



NATO Science for Peace and Security Series - A:
Chemistry and Biology

Detection of Bacteria, Viruses, Parasites and Fungi

Bioterrorism Prevention

Edited by
Mariapia Viola Magni



Springer



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Detection of Bacteria, Viruses, Parasites and Fungi

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Series A: Chemistry and Biology

Detection of Bacteria, Viruses, Parasites and Fungi

Bioterrorism Prevention

edited by

Mariapia Viola Magni

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Italy



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Introduction

This publication represents the result of the fruitful workshop organised with the aim to attract the attention on the possibility of bio terrorism attack, with the support of NATO funds. In the last years the attention was strongly concentrated on the terrorism view similar to “military type attacks:” bomb on the trains, kamikazes, airplanes etc. As consequence many devices studied are directed to prevent these attacks such as the control of the passengers before the flight.

For the people terrorism is therefore equivalent to bomb or similar and nobody think that there is also other possible and sophisticated means that can be used by the terrorist. In 1995 Sarin gas in the Tokio subway killed 12 people and affected 5,000 persons. In the USA anthrax was sent by mail to many federal offices. These events and other cases attract the attention on these possible terrorist attacks and the first recommendations for preventing these events were elaborated in the United State and in Europe.

The possible agents and the modality that can be used for the diffusion are analysed and food and water are considered the principal and more favourable way.

The story and the principal decision about this were reported in the first article of this collection which introduces the concept of bio-terrorism.

Then the attention was concentrated on the possible and more diffuse agents which may contaminate the food, the water and the environment and the possible methods which may be sufficient precise, rapid and easy to use on a large number of person and samples.

Dr. Coenye elaborated a fast method consisting in solid-phase cytometry which permits to analyse many samples and gives the possibility to visualise few bacteria or other kind of cells also if they are in small number. The analysis was made on air samples, on water and food samples.

As regards the water possible contamination of the source in relation to the particular structure of the soil was suggested by Vardanyan and Aginian.

Then the study was divided in four sectors of possible contaminant: bacteria especially the more diffuse, virus, parasites and pollutants.

Of each the possible way of diffusion, the consequent diseases and the more modern and safe methods used for diagnosis were presented and discussed in view of the results obtained.

All the authors have a long experience and in many cases they presented a complete revue of the subject.

Madic concentrated his analysis on a bacteria extremely diffuse and saprophyte of our intestine: *Escherichia coli*. Some species are pathogens and can produce a toxins similarly to *Shigella* which may cause diarrhoea till haemorrhagic colitis which may be complicated by haemolytic-uraemic syndrome. Domestic ruminants, especially cattle, are the major reservoir and the transmission to the man may happen through the consumption of undercooked ground beef, water, or dairy products contaminated by bovine faeces. The presence of toxins can be tested by studying the modifications of the shape of Vero cells cultured in presence of contaminated sample or through enzymatic tests. Actually the most safe methods are the molecular methods based on DNA; a commercial kit is produced for doing the diagnosis with security and in short time. It is in fact important to make this assay in all food preparation because, also if the manifestation may be mild as simple diarrhoea, they can interest through, for example, a lot of contaminated milk many person on the same time.

Other diffuse bacteria are the Enterococci. They are present in many foods, they are resistant to the heat temperature and in some of them like cheese give a special flavour typical of the product like happens for *Bryndza* cheese a special kind produced in Slovakia. In general they are not pathogen, but they can be transformed modifying their DNA and acquiring virulence capacity, so in this case it is important to make an exact diagnosis especially using molecular diagnostic tests.

Salmonella infections have increased recently as common gastroenteritis disease. The clinical manifestation are diarrhoea, fever, abdominal cramps, tenesmus, headache, myalgia and occasionally nausea and vomiting. The contamination of foods can affect many people: in USA in 1985 contaminated milk caused a disease in 170,000 person and in China in 1991 224,000 people are affected through a contaminated ice cream. The main reservoir of *Salmonella* are poultry, eggs and egg products. Serological test may be useful for the diagnosis.

Particular diffuse agent in the hospitals and community is the *Staphylococcus aureus* They can cause abscesses, bacteremia and infections of heart, nervous system, lung, bone and urinary tract.

It is therefore important to have available methods for the identification of various species.

The most precise tests are the genotypic including characterisation of chromosomal, plasmid or total genomic DNA. In this way it is possible to study the polymorphism and separate the various clones. An accurate analysis of the methods available is made in this paper.

Sometimes some gastroenteric diseases are attributed erroneously to some more frequent enteric bacteria whereas the responsible is *Bacillus cereus* which is destroyed by the high temperature, but its spores survive and can multiply at normal temperature. For these reasons, cooked food, if it is stored not adequately, can present a contamination by these bacteria. The foods which may be contaminated are a long list including beer, pastas, wine, boiler rice, cooked vegetables and other foods present in general in the supermarket. It is therefore important to apply some

control and to make precise tests for the presence of *Bacillus cereus*. These bacteria produce toxins which can cause not only intestine diseases, but also eye infections, skin infections, orthopaedic wounds, meningitis, bacteraemia and pulmonary infections. Also in this case molecular methods are more reliable, for example, PCR.

Finally possible contamination by *Listeria monocytogenes* of ham or crude meat was taken in consideration. Experiments made with ham contaminated artificially with various quantity of these bacteria had shown that the salt treatment reduces its presence and also some chemical treatment can limit the development of these bacteria. The experiments therefore showed that the ham is practically safe as regard a possible contamination from this bacterium which is common in pig skin.

The second section is dedicated to virus infection. Hepatitis B is taken in consideration for his consequence and since it is easy to diffuse not only through blood, but also through contaminated instruments, sexual contacts and to the infants from infected mother. The diagnosis is fundamental in order to prevent the diffusion, since the disease tends to be chronic and causes cirrhosis. Vaccination could prevent the illness.

Another emergent viral hepatitis is that causes by E virus, a single stranded RNA virus, which is endemic in many areas like North America and Asia. It can be transmitted via the faecal-oral route, water and also food. This virus causes acute hepatitis and it is important to distinguish it from other kind of viral hepatitis. The pathogenesis of hepatitis E is not well known and the diagnosis is actually mainly serological even if a molecular method represents that preferable to be developed.

Another emergent disease is due to Dengue viruses which may cause infections similar to a simple flu up to more severe syndromes like haemorrhagic fever or dengue shock syndrome. The genome of Dengue virus is formed by RNA of approximately 11 kb in length, composed by three structural genes and seven non structural protein. Since this virus does not give origin to a specific disease, it is necessary to make a precise diagnosis; at moment many methods exist, like immunological test and the authors in their article make a comparison between them.

In the third section parasites have been taken in consideration an infection which can be transmitted mainly from food either vegetable or animal including fishes. A complete review of the possible parasites contamination and especially of some emerging or re-emerging ones is presented in the paper of Dei Cas and others.

They first take in consideration the difference between the various geographical areas underlined the fact that these infections affect more than three millions of people in the area in which the people live with less than \$2 per day. These infections are also responsible of mental and physical underdevelopment of Africa children.

The most diffuse are *Cryptosporidium* and *Giardia intestinalis* which may be transmitted with the faeces of cat or domestic dog. Less diffuse but equally important for the pathological consequences are *Trypanosoma cruzi* and *Toxoplasma gondii* which are diffuse also in America and Europe.

The presence of some parasites, like *Diphyllibotrium*, in fishes favours the contamination especially when are used not cooked such sushi or marinated fishes infected with larval plerocercoids. The diffusion is mainly in the freshwaters and in Europe especially in Switzerland and in Italian, French Alpine lake region.

Echinococcus also may infect human through the ingestion of eggs eliminated by carnivores faeces which can contaminate water and vegetable. The form of infection may concern the liver or the pulmonary apparatus or may be polycystic in various organs depending on the species. *Taenia solium* is another re-emerging parasitic disease and pigs which are coprophage may facilitate the diffusion as intermediate hosts. Another emerging parasite infection is due to Anisakiasis which causes a digestive disease through the ingestion of crustaceous, fishes and even crocodiles. Finally it must remember that also *Trichinella* may infect human through the meat of pork, wild boar, horse, bear and reptile which are not controlled and their request as food is increasing.

The following two papers are dedicated to this kind of parasite *Trichinella* survives in the non cooked meat and infected the human through the intestine and then localises chronically in the muscle. A particular attention was devoted to the enzyme thymidylate synthase which appear expressed at high level by the *Trichinella spiralis* and *pseudospiralis*. The test was made by using monoclonal antibody and can be detected with immunofluorescent technique. In this way it was possible also to study the precise localization. The possible meaning of the presence of this enzyme which is in general related to cell duplication is discussed.

The same parasite was considered for its influence on the immune system, in fact it seems to have positive effect in allergic disease like asthma or in chronic inflammatory pathology like Crohn's disease. The mechanisms of this interactions are analysed and experimentally tested in order to see how the presence of such parasite can be utilised in the treatment of some pathologies. On the other hand the parasite can be considered as favouring agent of some tumour. The paper by Creusy and others deals with the experimental demonstration in immune depressed mice inoculated with *Cryptosporidium parvum* develop tumours in the gastro-enteric tissue. This observation agrees with other reports concerning parasites especially *Schistosoma haematobium* which seems responsible of urinary bladder carcinoma and *S. japonicum* and *S. mansoni* which are linked to hepatic and colorectal carcinoma. Since the way of diffusion is not well known a possible presence of parasites and the relative consequence may represent a risk.

Biological pollutant are due to fungi toxic metabolites which can contaminate foods are known as micotoxins. These products are not necessary for the fungi growth and they can be present in the environment as well as in foods. Their effect on the human health depend not only by its characteristic, but also by the immune system of each person. A biosensor system is developed in order to evaluate contaminants in very simple and quick way. The results are compared with standard methods such as HPLC and ELISA.

In conclusion, our analysis includes a large spectrum of diffuse contaminants which may spread mainly with foods and can be the causes of many pathologies some of them could be very severe. The development of quick and un-expensive new methods represent an help for diagnostic purposes since in many case it is necessary to analyse a large number of samples. The use of some of them to prevent bio-terrorist attack may interest a large number of people in the same time or may in some cases be responsible of chronic diseases with high mortality. It is easy to

contaminate foods with mycotoxins or with *Escherichia coli* producing toxins like *Shigella*. At present time, people is strongly worried about the new A flu, which may interest at the same time millions of people, the same can be obtained by the terrorist spreading on the environment any toxins or bacteria producing toxins using not special and rare agents, but simply utilizing that are more diffuse and easy to collect and cultivate or simply using already infected samples.

This publication intends to focus the attention on these possible attack and on the necessity to have at disposal methods easy to apply which permit in short time to make a diagnosis.

Mariapia Viola Magni

Part I

Bioterrorism Prevention

Bioterrorism: A Potential Weapon for Terrorist Attacks Through Food and Water Contamination: Evolution of Our Understanding of the Use of Chemical and Bacteriological Weapons

Vincenzo Costigliola and Franco Quagliata

Abstract Bioterrorism is possibly an even older phenomenon than the relatively recent “military-type” attempts, culminating in the 9/11 attacks to the New York Twin Towers, and which have skyrocketed into a worldwide series of true war like destructions. These situations range from those by individuals who usually give up their lives in the attempts, to the true military operations, such as those, which almost daily make countries such as Iraq, Afghanistan, the Philippines to mention only a few. There are many differences between the two types of terrorism, and they will be discussed in detail. One of the most obvious is primary target, which in the case of the use of explosive in many different forms has as the main target man, while bioterrorism is aimed at man in a much more indirect form, targeting man through hitting his food supplies, such as his animals, the waters he drinks and so on.

Keywords Chemical weapons • Bacteria • Toxins • Agroterrorism • Biosurveillance • Preparedness • Biological warfare • Biocontainment • Decontamination

Useful Abbreviations

AG	Australia Group
BEP	Biosecurity Engagement Program
BSL	Biosafety level
BTWC	Biological and Toxin Weapon Convention
BWC-IS	Biological Weapons Convention-Implementation Support Unit
CDC	Center for Disease Control
COM	European Community Commission
CSS	Health Security Committee
CTR	Cooperative Threat Reduction
CWC	Chemical Weapon Convention

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DG SANCO	Direzione Generale Salute e tutela dei Consumatori
DHS	Department of Homeland Security
DoD	Department of Defense
DoE	Department of Energy
ECID	European Center for Infective Diseases
EFTA	European Free Trade Association
EME	European Medicines Agency
ENP	European Neighborhood Policy
EPR	Epidemic and Pandemic Alert and Response
EU	European Union
FAO	Food and Agricultural Organization of the United Nations
FETP	Field Epidemiology Training Program
GEIS	Global Emerging Infections Surveillance and Response System
GAO	Government Accountability Office
GDD	Global Disease Detection
GHSAG	Global Health Security Action Group
FSC	Security Cooperation Forum
GHSI	Global Health Security Initiative
GHSAG	Global Health Security Action Group
GU	European Union Official Gazzetta
HHS	Health and Human Services
IDSR	Integrated Disease Surveillance and Response
IHR	International Health Regulations
MEC	European Common Market
NATO	North Atlantic Treaty Organisation
OIE	World Organization for Animal Health
OECD	Organization for Economic Cooperation and Development
OCSE	Organizzazione per la Cooperazione e lo Sviluppo Economico
OPAC	Organizzazione per la Proibizione delle Armi Chimiche
OPCW	Organization for the Prohibition of Chemical Weapons
PE	European Parliament
PESD	Foreign Politics and Common Defense
PSI	Proliferation Security Initiative
SEE	European Economic Space
TCE	European Community constitutional Treaty
TUE	European Union Treaty
UN	United Nations Organization
UN-ODA	United Nations Office for Disarmament Affairs
UNMOVIC	United Nations Monitoring Verification and Inspection Commission
UNODC	United Nations Office on Drugs and Crime
UNSCR	United Nations Security Council Resolution
USAID	United States Agency for International Development
WFCC	World Federation of Culture Collections
WCO	World Customs Organization
WHO	World Health Organization
WIP	World Intellectual Property Organization

Introductory Considerations

NATO has been directly involved in the Science Programme for Peace and Security (SPS) since 1956, when a report by three Foreign Ministers, Halvard Lange of Norway, Gaetano Martino of Italy, and Lester B. Pearson of Canada, emphasized the importance of political, economic and scientific consultations for Allied security.

When we speak of terrorism, especially since 9/11 we unconsciously think of airplanes, cars, trains, Kamikaze attacks and all other “military-type” activities. However, though much less “explosive” for real and for the attention, which they receive in the media, of paramount relevance are those events, which are lumped under the term “Bioterrorism” because are not delivered via any of the means indicated above.

A Bioterrorist Attack with viruses, bacteria or similar agents can affect people, animals or plants (“agroterrorism”). While agents such as anthrax affect only the targeted individuals, other, such as smallpox, can spread between individuals, reaching a much larger target.

Bioterrorism is even historically older: the Romans at times contaminated with dead and rotting animals the wells around enemy cities to poison their waters and destroy their forces. In the fourteenth century bubonic plague was used to create panic among the enemy and to weaken their forces with the illness.

An attack against water supplies, with much more sophisticated means than what was used in Roman times would have a devastating effect on the health of humans and animals: a reservoir, even relatively small lake, if contaminated with infectious agents could affect entire populations. In a river, the flow of the waters might dilute the contaminant, thus reducing the effect and limiting it in time. In the case of food the attack can occur at each of the stages of its production and preparation. From the harvest of crops, which can be sprinkled with the contaminants, to the infection of animal feed, to the introduction of toxins or infectious agents at the level of restaurants, institutional cafeterias, the food is a very accessible target: careless handling, obviously a non terrorist event, has caused at various times infections even of a considerable degree of gravity.

Bioterrorism effects can occur not only from a planned malicious activity, but at times, they are the unwanted and unanticipated result of accidental situations, for which, the ethical implications are clearly extremely different. An accidental explosion (Chernobyl) contaminated inland waters, caused short term burns and wounds, and permanent diseases such as leukemia: in a planned war situation (Hiroshima) the effects were very similar. It is therefore very difficult to decide if infections coming from infected water results from occasional contamination by a person contaminating them with biological material, or from a planned terrorist action, aimed at harming the population drinking the water.

In 1942 US President Roosevelt started a biological weapon program, which, at least officially was shut down by Nixon in 1969. We still do not know for sure if some (or all) the countries, which signed in 1972 the Treaty to ban germ warfare agents, really did so: agents were not specified, controls were non existent, ineffective and questionable (see Iraq’s first Gulf war).

The Communicable Disease Center created the Laboratory Response Network for Terrorism (LRNT) and a Rapid-Response Advanced Technology (RRAT) laboratory, both linked to the Environmental Protection Agency.

The Common Infectious Agents can be grouped in three categories.

1. Those posing a risk to National Security, such as anthrax, botulism, cyanide, plague, hemorrhagic fever virus, ricin
2. Those disseminating less easily, such as smallpox, tularemia, nerve agents, i.e. sarin
3. Those with a future potential of a major health impact

Anthrax not contagious, can occur spontaneously in several animal species. It was sent through the mail in the USA. It can be released in an attack, as in Sverdlovsk (USSR) in 1979. Up to 200 people died, but the KGB suppressed most of the information. Winds spread the anthrax about 30 miles away.

Smallpox provides the interesting example of the very effective vaccination program in Yugoslavia, where no cases had been seen since 1927. In 1972 a pilgrim came back to Kosovo and infected 11 friends: the disease was initially not recognized having been absent for 45 years. Yugoslavia closed its borders and vaccinated 19 million people in less than 2 weeks. Entire cities were quarantined.

Sarin was used in Japan, in 1994 in Matsumoto, where an attack was planned against Judges who were going to deliver on a dispute on a site for a “cult” group: it failed for banal logistic reasons. Again the same group planned an attack on the Tokyo subway, where 12 people died and 3,500 got sick.

Are there countries with active programs on Biological Weapons (BW)? Although the Treaty of 1972 would point in the direction of an absence of active research in the field, we know that such programs are active. Iraq is the most publicized culprit, though no hard evidence has been gathered. At least 15 other countries have developed them, among them: Iran, Israel, both Koreas, South Africa, China, India and Russia. The UK and the USA had large stocks, which they claim to have destroyed.

While the Soviets were developing their “biological arsenals” 1960–1980, USA and UK were so focused on the “nuclear threat” that they almost abandoned even the search for potential antidotes and vaccines.

The CDC identified Key Focus Areas, which go from Preparedness and Prevention to Response and Communication to counteract possible attacks. Recently electronic chips with live nerve cells have been identified as capable to detect many bacterial toxins. Fiber-optic tubes can detect specific pathogens such as anthrax, botulinum and ricin.

After the September 11 attack The Food and Drug Administration (FDA) has boosted its already very effective emergency response plans. It works closely with other Federal, State and Local safety authorities and regulatory agencies abroad to protect food and respond promptly to any evidence of threats to the food and water supply. The impressive amount of information, which the Agency can provide, makes it probably the best source of relevant and updated information. FDA can be contacted toll free at 1-800 SAFEFOOD, or on line at www.foodsafety.gov.

Definition

“Bioterrorist attack”: deliberate release of viruses, bacteria, or other agents used to cause illness or death in people, animals, or plants (agroterrorism).¹ They are typically found in nature, but it is possible to change them to increase their ability to cause disease, make them resistant to treatment, or to increase their ability to be spread into the environment either through the air, through water, or in food. Terrorists may use biological agents because they are difficult to detect and do not cause illness immediately. Some bioterrorism agents, like the smallpox virus, can be spread from person to person and some, like anthrax, cannot.

Bioterrorism is terrorism by intentional (occasionally accidental) release of biological agents (bacteria, viruses, or toxins; these may be in a naturally occurring status or undergone specific manipulations.

Classification

Bioterrorism – Example 1

Events leading to an effect, which we now define as “bioterrorism” result from a wide variety of situations: some of these are devoid of any foul doing by the individual, and completely involuntary.

At times an action, in itself completely normal and acceptable, can produce an unanticipated effect resulting in an accident: a father cleans a hunting gun and because of a phone call turns around, hits the table and the 5 years old child who plays there is wounded.

Other times an omission can lead to the same effect, again without any fault by the individual: the same child asks the father to help him getting up as it fell: the father helps him and in bending down knocks the gun off the table and the shot ensuing wounds the child.

In other cases the events are planned to produce damage as the first and only goal: a thief enters the house and to fence off the father shoots the child.

The effect in all three cases can be very similar if not identical: only ethically they are profoundly different

Bioterrorism – Example 2

Various Types

Planned or casual: their severity, and the impact, which they might have on the person or people being targeted can vary considerably.

An HIV-positive narcotic addict might contaminate with a syringe someone who refuses to give him/her money.

An HIV-infected individual might crush into a healthy person and with his/her blood might contaminate the person not wanting to do it.

Again, very similar objective results, but completely different subjective motives.

Bioterrorism – Example 3

An accidental explosion in a nuclear plant, i.e. Chernobyl, can contaminate inland waters for years and induce short term, burns and wounds, and permanent diseases, leukemia, etc. in the population.

An atomic explosion in a war situation, i.e. Hiroshima, produced similar effects.

Also in this case, we have the same type of effect for a casual event and for a specifically planned military operation.

History

Early Bioterrorism

Though not the first example of a covert operation, biological terrorism was known during the Roman civilization, where dead and rotting animals were thrown into wells to poison enemy water supplies.² It continued on into the fourteenth century where the bubonic plague was used to infiltrate enemy cities, both by instilling the fear of infection in residences, in the hope that they would evacuate, and also to destroy defending forces that would not yield to the attack.³ Disease as a weapon in this stage of history exhibited a lack of control of the aggressors over their own biological weapons. Primitive medical technology provided limited means of protection for the aggressor and a battle's surrounding geographical regions. After a battle won, the inability to contain enemies who escaped death led to widespread epidemics affecting not only the enemy forces, but also surrounding regions' inhabitants. The use of these biological weapons, and the apparent lack of medical advancement necessary to defend surrounding regions from them, widespread epidemics such as the bubonic plague quickly moved across all of Western Europe, destroying a large portion of its population. The affected victims became weapons themselves. This was noted in the Middle Ages, but medical advancements had not progressed far enough to prevent the consequences of a weapons use.³

More Recent Bioterrorism

With increased sophistication, countries were developing weapons that delivered much higher effectiveness and less chance of infecting the wrong party. An important

step in this direction was the first use of anthrax⁴. Though initially limited by the need of large doses, it became the weapon of choice because it is easily transferred, has a high mortality rate, and can be easily obtained. Moreover, variants of the anthrax bacterium can be found all around the world making it the biological weapon of choice in the early nineteenth century. Another property of anthrax that favored its use is its poor ability to spread much past the initial target population (see also Section [Modern Bioterrorism Events](#) below).

By the time World War I began, anthrax was directed at animal populations. This was ineffective. And at that point, mustard gas became the poisonous biological weapon of choice. Its horrifying effects lead to the treaty of the Geneva Protocol of 1925. The treaty was created to prevent the use of asphyxiating gas as a method of biological warfare⁵. While this was a significant advancement toward the prevention of biological weapon use, the treaty said nothing about weapon development. Secretly, biological weapon development programs existed in many nations. The lack of their use is probably the result of the programs' immaturity and the programs immaturity and not the unwillingness to use them.

American biological weapon development began in 1942. President Franklin D. Roosevelt placed George W. Merck in charge of the effort to create a development program. These programs continued until 1969, when by executive order President Richard Nixon shut down all programs related to American offensive use of biological weapons (<http://fas.org/nuke/guide/usa/cbw/bw.html>).

North Korea accused the US of their use in Vietnam, but most likely those accusations were anti US propaganda.

Current Bioterrorism

As the 1970s passed, global efforts to prevent the development of biological weapons and their use were widespread. In 1972 – International Treaty to ban germ warfare agents: which agents were not specified and controls non existent, ineffective or questionable (see Iraq's first Gulf war). This led to the prohibition of development, production and stockpiling biological weapons was developed. In the 1980s Iraq made substantial efforts to develop and stockpile large amounts of biological weapons. By the end of the 1980s Iraq had several sites dedicated to the research and development of biological warfare. They began to test their findings in the late 1980s. 1989 – Biological Weapons Anti-Terrorism Act. These actions lead to the first Gulf war in which Iraq's biological weapons were dismantled and destroyed.

Since that time, efforts to use biological warfare has been more apparent in small radical organizations attempting to create fear in the eyes of large groups. Some efforts have been partially effective in creating fear, because of the lack of visibility associated with modern biological weapon use by small organizations. In 1995 a small terrorist group, then called Aum Shinrikyo now called Aleph, launched a Sarin gas attack on the Tokyo subway system. The attack killed twelve people and affected more than 5000⁶ (see below). The response of Japanese emergency services successfully prevented an outcome with much higher mortality rates.

In the United States a more recent biological terrorism attack occurred in 2001 when letters laced with infectious anthrax were delivered to news media offices and the US Congress. The letters killed five people⁷. While many believed this attack to be in relation to Iraq's development of biological weapons, the anthrax strand used seemed to indicated a domestic source.

Types of Biological Agents

Note: Some examples of their effects will be indicated under the agents involved

The CDC has defined and categorized bioterrorism agents according to priority as follows:

Category A Agents

These are anthrax, smallpox, plague, botulism, tularemia, viral hemorrhagic fevers and ricin. They can disseminate and present a high risk on public health. Most of them require Level 4 Biosafety laboratories.

Anthrax

Anthrax infections, which are not contagious, can occur spontaneously in several animal species. They have a bi-phasic course: a flu-like initial, mild phase and a more serious relapse for which there is no treatment. There is a vaccine, which requires series of many injections.

It has been delivered through the mail in the USA: also to the offices of several United States Senators 2001. This led to the vaccination of federal employees.

It can be released in a terrorist attack, as in Sverdlovsk in Russia in 1979. Eighty to two hundred people died, but most of the information was taken away by the KGB. The winds spread the anthrax about 30 miles away⁸.

Smallpox

Is highly contagious and it affects only humans; spreads through the atmosphere and has a 20–40% mortality rate.

The first use of smallpox as a biological weapon dates back to the American Revolution, when Lord Jeffrey Amherst, the British commander of the Army fighting against a contingent of French soldiers, which together with the Delaware Indians were helping the Continental Army, decided to contaminate the tents of the

Indians by donating them blankets and handkerchiefs, infected with smallpox. This caused an epidemic, which decimated the Delaware tribe, spread to the Shawnee, the Mingo and to the Cherokees in Tennessee, weakening the anti British alliance.⁹

Mandatory worldwide vaccinations were eliminated in the 1970s. USA and USSR laboratories kept the agent in their laboratories and, after the end of the Soviet Union cultures were available in other countries. The vaccination of the pre 1970 under the WHO program does not maintain the immunity as in other infections, since it lasts only for 3–5 years. It is more dangerous as a biological weapon than other infections because both the infected individuals and their pox can transmit the disease.¹⁰

In Yugoslavia, because of a very effective vaccination program, no cases were seen since 1927. In 1972 a pilgrim returned to Kosovo and infected 11 of his friends: the private physicians and the staff of two hospitals did not recognize the disease, absent for 45 years: several hospital wards were contaminated and Yugoslavia closed the borders and vaccinated 19 million people in 10–12 days. Entire groups and even whole cities were quarantined.¹¹

Botulinum Toxin

Caused by bacterium *Clostridium botulinum*. Deadly: botulism causes death by respiratory failure and paralysis.

Plague

It is caused by the *Yersinia pestis* bacterium. Rodents are the normal host of plague, and the disease is transmitted to humans by flea bites and occasionally by aerosol in the form of pneumonic plague. The disease has a history of use in biological warfare dating back many centuries (see Roman times, above), and is considered a threat due to its ease of culture and ability to remain in circulation in rodents for long periods.

Viral Hemorrhagic Fever

This includes the *Filoviridae* (Marburg and Ebola genera), and the *Arenaviridae* (i.e. Lassa or Machupo). Ebola has fatality rates ranging from 50–90%. No cure currently exists, although vaccines are in development. The United States and the erstwhile Soviet Union both investigated the use of Ebola for biological warfare, and the Aum Shinrikyo group⁶ possessed cultures of the virus. Ebola kills its victims through multiple organ failure and hypovolemic shock. The Marburg virus was first discovered in Marburg, Germany. Fatality rates ranged from 25–100%, no specific treatment is available and a vaccine is only at the research stage. The Arenaviruses are less virulent, but more widespread, especially in central Africa and South America.

Tularemia

Tularemia, or rabbit fever, is severely debilitating, but has a very low fatality rate. It is caused by the *Francisella tularensis* bacterium.

Ricin

It is the third most toxic substance known after plutonium and botulism: it is a protein toxin that is extracted from the castor bean (*Ricinus communis*). The USA Centers for Disease Control (CDC) considers 500 µg¹² to be the lethal dose of ricin in humans if exposure is from injection or inhalation.¹³ Ricin is poisonous if inhaled, injected, or ingested, acting by the inhibition of protein synthesis. While there is no known antidote, the US military has developed a vaccine.¹⁴

Category B Agents

They have a low mortality rate and disseminate with moderate ease.

Brucellosis

This is an infectious disease caused by the *Brucella* species. Are transmitted primarily among animals, and cause disease in many different vertebrates: sheep, goats, cattle, deer, elk, pigs, dogs, and several other animals. Quite frequent in shepherds at time of delivery, brucellosis can cause a range of symptoms that are similar to the flu and may include fever, sweats, headaches, back pains, and physical weakness. Severe infections of the central nervous systems or lining of the heart may occur. Brucellosis can also cause long-lasting or chronic symptoms that include recurrent fevers, joint pain, and fatigue

Clostridium perfringens produces Epsilon toxin

Salmonella species, *E. coli* O157:H7, *Shigella*, Staph can pose food safety threats

Glanders (*Burkholderia mallei*)

Melioidosis (*Burkholderia pseudomallei*)

Psittacosis (*Chlamydia psittaci*)

Q fever (*Coxiella burnetii*)

Staphylococcal enterotoxin B

Typhus (*Rickettsia prowazekii*)

Viral encephalitis (alphaviruses, e.g.: Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis)

Mad cow disease bovine spongiform encephalopathy (in man Creutzfeldt-Jakob disease)

Vibrio cholerae, *Cryptosporidium parvum* can pose water supply threats

Avian Influenza virus: infecting humans as H5N1 virus

Category C Agents

These are pathogens that might be engineered for mass dissemination since are easy to produce and have potential for high morbidity or mortality (examples: nipah virus, hantavirus and multi-drug resistant Tuberculosis [MTB]). Sarin gas is a chemical weapon of mass destruction, discovered in 1939 by German scientists, and which can be used as a nerve agent. In 1994 in Matsumoto (north of Tokyo) a private group planned an attack on Judges who were to deliver on a dispute against a “cult”: it failed for a banal reason: the person in charge of delivering the truck overslept. A few months later the same group released sarin in the Tokyo subway killing 12 people, and making sick close to 5,000. Their action was aimed at preventing police raids against cult facilities (see Section [Current Bioterrorism](#) above).

What follows are some general considerations regarding some of the agents, which we have encountered, and that we will lump under the term the “Villains”.

1. Food- and water-borne illnesses may be caused by toxins created by growing bacteria; toxins produced by the harmful algal species; or contamination of food and/or water with certain bacteria, viruses or parasites. Many cases of food poisoning happen when someone eats food that has harmful bacteria in it. The bacteria or the toxins produced by them can then make the person sick. Bacteria also can get into the water supply and make someone sick.
2. Food- or water-borne illnesses are not spread from casual contact with another person. A person can come into contact with food- or water-borne bacteria by eating or drinking something that has bacteria in it.
3. Food- or water-borne bacteria as weapons: The use of these bacteria is less likely than using bacteria that can be spread through the air because it is hard to expose a lot of people at the same time. However, all of the above bacteria may be used to contaminate public water supplies or food at large events. Standard treatment of public water supplies would most likely kill the bacteria before people were able to drink it.

Please note: Just because you come into contact with a food- or water-borne bacteria does not mean you will get sick from it.

4. Most infections with food- and water-borne bacteria cause diarrhea, nausea, vomiting, fever and stomach cramps.
5. Individual features
 - **Salmonella:** bloody diarrhea, fever, headache, lack of energy
 - **Shigella:** blood or mucus in the stool
 - **E.coli:** bloody diarrhea. Some people (2–7%) may develop a syndrome (hemolytic uremic syndromes – HUS) that results in the destruction of red blood cells and kidney problems. HUS is more common in children than adults. About 3–5% of those who develop HUS may die
 - **Parvum:** watery diarrhea, crampy stomach pain
 - **Cholera:** About 5% of people who come into contact with Cholera may develop severe diarrhea, vomiting and leg cramps

6. Are there vaccines for food- and water-borne illnesses?
7. Where can one get more information about food- and water-borne illnesses?

Illinois Department of Public Health <http://www.idph.state.il.us>

Government Entities Counteracting Bioterrorism

A. USA

The CDC created the Laboratory Response Network for Terrorism (LRNT) and a Rapid-Response Advanced Technology (RRAT) laboratory, both linked to the EPA. Amongst their functions an important one is to help distinguishing between (a) Naturally occurring infectious disease and (b) Maliciously spread infections.

The CDC designs and implements strategies to integrate countries' existing disease surveillance response systems providing laboratory confirmation and additional data to public health activities. It works with WHO's regional office for Africa in 46 countries, providing direct technical assistance to eight of them.

Key Focus Areas for CDC are currently:

- Preparedness and Prevention
- Detection and Surveillance
- Diagnosis and Characterization of biological and chemical agents
- Response
- Communication

Contact CDC: 800-CDC-INFO (800-232-4636); 888 232-6348 (TTY); also: cdcinfo@cdc.gov

The Food and Drug Administration (FDA) is the US government agency whose primary mandate is to protect public health, both in groups and in individuals; but also the health of animals and plants. Controlling food and pharmacological agents, which represent a potential hazard to those target population is the single largest protective shield ever put together by any national and international entity.

- It has increased surveillance of food imports and food production.
- It started an educational program realizing that consumers are the final judges and the potential victims of the food they buy. One essential step for their protection is to check whether the food package or can is intact before opening it. If it has been damaged it should not be used or even handled.
- It has issued recommendations on what to do in suspicious situations: e.g. what to do if there is suspicion that a food product has been contaminated or tampered with?
- Should the consumer assume drugs (such as antibiotics) as a protective measure?
- It has provided a toll free line to learn about food safety at +1 888 SAFEFOOD, and has advertised the online information and support at www.foodsafety.gov.

The FDA since the September 11 attack, FDA has increased its emergency response capability by realigning resources for possible use to counter terrorism, and by reassessing and strengthening its emergency response plans. The agency also continues to work closely with other federal, state, and local food safety authorities and with regulatory agencies abroad to maximize coordination of efforts to protect food and to respond rapidly to evidence of threats to the food supply.

The events of 9/11 convinced the US Congress to pass the Public Health Security and Bioterrorism Act, a milestone, signed by the President on June 12, 2002. It consisted of five Titles (I–V) and two Subtitles (A and B), covering all potential harmful agents and situations.

On November 7, 2008 FDA issued the Final Rule of the Act on Public Health Security and Bioterrorism Preparedness and Response act of 2002.

In October 2008 it was drafted the Compliance Policy Guide – Guidance for FDA and CBO Staff (Prior Notice of Imported Food); and also the Compliance Information Registration.

The National Security Act of 1947 created the Office of Director of Central Intelligence (DCI). George Tenet (in the late nineties was Deputy Director of CIA) in February 2004 reported to Congress that “Bin Laden considered the acquisition of weapons of mass destruction a religious obligation.” He added that “more than two dozen other terrorist groups are pursuing CBRN (chemical, biological, radiological, and nuclear materials.”

B. E.U.

Many of the initial rules set by the Community were recommendations in the form of Soft Law measures, therefore non binding for the member nations.

In October 2007 the European Parliament and Council deliberated on the institution of a second health program for the period January 1, 2008 through December 31, 2013. This occurred in accordance with the TCE art. 152, which stated that “... the Community’s action aims at the improvement of public health...and at eliminating sources of danger for human health.” It was also instituted the white book “A common commitment for health: Strategic approach of the EU for the period 2008–2013.” This book “should have” contributed at achieving a higher level of protection from bio-chemical threats. A MAJOR CAVEAT: sanitary discrepancies between the 27 Member Nations, post January 1, 2007. The plan was supposed to have four major objectives, broad coordination between the member States, quick detection and identification of pathogens, storage of medicines and vaccines, and guidelines for the EU (See: G/FS – 2001 GG).

The very clear cut principle of precaution, art. 174 TCE, fights with the complexity of the issues, so broad as to arrive at political issues so detailed as to get to the level of the health of the single individual.

A table of the objectives starting between May and September 2002 and to be completed between July and October of the same year is very clearly outlined, and a very positive report has been published: however, it is not clearly defined which are the involved institutions that are supposed to implement the obligations in the member nations. (Ibidem)

Various public agencies and private corporations have collaborated in attempting at discovering new detection means against BW.

In July 2005 (modified in October 2008) USDA, FDA, DHS, and FBI joined private industry in a plan to protect food and agricultural supplies from agroterrorism.¹

USDA +1 202 720-4623; FDA +1 301 827-6244; DHS +1 202 282- 8010; FBI (Press Office) +1 202 324-3691

Modern Bioterrorism Events

1915–1916 Livestock Sabotage by Germany

Dr. Anton Dilger, a German-American physician, worked for Germany in the USA (Chevy Chase and Baltimore) in 1915 and 1916 with cultures of anthrax and glanders with the intention of biological sabotage, infecting live stock, mainly horses, in the USA on behalf of the German government.¹⁵ Other German agents are known to have undertaken similar sabotage efforts during WWI in Norway, Spain, Romania and Argentina.

1984 Rajneeshee Salmonella Attack

In 1984, followers of the Bhagwan Shree Rajneesh attempted to control a local election by incapacitating the local population. This was done by infecting salad bars in eleven restaurants, produce in grocery stores, doorknobs, and other public domains with *Salmonella typhimurium* bacteria in the city of The Dalles, Oregon, at the time of the elections. The attack infected 751 people with severe food poisoning. However, there were no fatalities. This incident was the first known bioterrorist attack in the United States in the twentieth century.¹⁶

1994 Serin Incidents

In 1994 in Matsumoto (north of Tokyo) a private group planned an attack on Judges who were to deliver on a dispute against a “cult”: it failed for a banal reason: the person in charge of delivering the truck overslept. A few months later the same

group attacked in the Tokyo subway killing 12 people, and making sick 5,000. Their action was aimed at preventing police raids against cult facilities (see Section [Current Bioterrorism](#) above).

2001 Anthrax Attack

In September and October 2001, several cases of anthrax broke out in the United States in the 2001 anthrax attacks, caused deliberately. This was a well-publicized act of bioterrorism, which was perpetrated through anthrax-laced mail. It motivated efforts to define biodefense and biosecurity, where more limited definitions of biosafety had focused on unintentional or accidental impacts of agricultural and medical technologies.

Planning for and Reacting to a Bioterrorist Attack

Planning may involve the development of biological identification systems.

Until recently in the United States, most biological defense strategies have been geared to protecting soldiers on the battlefield rather than ordinary people in cities. Financial cutbacks have limited the tracking of disease outbreaks. Some outbreaks, such as food poisoning due to *E. coli* or *Salmonella*, could be of either natural or deliberate origin.

Preparedness and Response to a Biological Attack

Biological agents are relatively easy to obtain by terrorists and are becoming more threatening in the USA, and laboratories are working on advanced detection systems to provide early warning, to identify contaminated areas and populations at risk, and to facilitate prompt treatment. Methods for predicting the use of biological agents in urban areas as well as assessing the area for the hazards associated with a biological attack are being established in major cities. In addition, forensic technologies are working on identifying biological agents, their geographical origins and/or their initial application. Additional efforts soon to come include decontamination technologies to restore facilities without causing additional environmental concerns.¹⁷

Biosurveillance Strategies

In 1999, the University of Pittsburgh's Center for Biomedical Informatics deployed the first automated **bioterrorism** detection system, called RODS (Real-Time Outbreak Disease Surveillance). RODS is designed to draw collect data from

many data sources and use them to perform signal detection, that is, to detect the a possible bioterrorism event at the earliest possible moment. RODS, and other systems like it, collect data from sources including clinic data, laboratory data, and data from over-the-counter drug sales. In 2000, Michael Wagner, the codirector of the RODS laboratory, and Ron Aryel, a subcontractor, conceived of the idea of obtaining live data feeds from “non-traditional” (non-health-care) data sources. The RODS laboratory’s first efforts eventually led to the establishment of the National Retail Data Monitor, a system which collects data from 20,000 retail locations nation-wide.

On February 5, 2002, George W. Bush visited the RODS laboratory and used it as a model for a \$300 million spending proposal to equip all 50 states with biosurveillance systems. In a speech delivered at the nearby Masonic temple, Bush compared the RODS system to a modern “DEW” line (referring to the Cold War ballistic missile early warning system).

The principles and practices of biosurveillance, a new interdisciplinary science, were defined and described in the *Handbook of Biosurveillance*, edited by Michael Wagner, Andrew Moore and Ron Aryel, and published in 2006. Biosurveillance is the science of real-time disease outbreak detection. Its principles apply to both natural and man-made epidemics (bioterrorism).

Data which potentially could assist in early detection of a bioterrorism event include many categories of information. Health-related data such as that from hospital computer systems, clinical laboratories, electronic health record systems, medical examiner record-keeping systems, 911 call center computers, and veterinary medical record systems could be of help; researchers are also considering the utility of data generated by ranching and feedlot operations, food processors, drinking water systems, school attendance recording, and physiologic monitors, among others. Intuitively, one would expect systems which collect more than one type of data to be more useful than systems which collect only one type of information (such as single-purpose laboratory or 911 call-center based systems), and be less prone to false alarms, and this appears to be the case.

In Europe, disease surveillance is beginning to be organized on the continent-wide scale needed to track a biological emergency. The system not only monitors infected persons, but attempts to discern the origin of the outbreak.

Researchers are experimenting with devices to detect the existence of a threat:

Tiny electronic chips that would contain living nerve cells to warn of the presence of bacterial toxins (identification of broad range toxins).

Fiber-optic tubes lined with antibodies coupled to light-emitting molecules (identification of specific pathogens, such as anthrax, botulinum, ricin).

New research shows that ultraviolet avalanche photodiodes offer the high gain, reliability and robustness needed to detect anthrax and other bioterrorism agents in the air. The fabrication methods and device characteristics were described at the fiftieth Electronic Materials Conference in Santa Barbara on June 25, 2008. Details of the photodiodes were also published in the February 14, 2008 issue of the journal *Electronics Letters* and the November 2007 issue of the journal *IEEE Photonics Technology Letters*.

Limitations of Bioterrorism

Bioterrorism is inherently limited as a warfare tactic because of the uncontrollable nature of the agent involved. A biological weapon is useful to a terrorist group mainly as a method of creating mass panic and disruption to a society. However, technologists such as Bill Joy (co-founder of Sun Microsystems) have warned of the potential power which genetic engineering might place in the hands of future bio-terrorists,¹⁸ a bacterial agent might be engineered for genetic or geographical selectivity. Such a scenario formed the plot of the science fiction novel *The White Plague* and the action novel *Area 7*.

Other Forms of Bioterrorism

The use of agents that do not cause harm to humans but disrupt the economy have been discussed.^[2, 3, 15] A highly relevant pathogen in this context is the foot-and-mouth disease (FMD) virus, which is capable of causing widespread economic damage and public concern (as witnessed in the 2001 and 2007 FMD outbreaks in the UK), whilst having almost no capacity to infect humans.

The genomic revolution requires scientists to follow a recognised Code of Conduct. The ‘dual-use’ technology dilemma implicates issues further; good scientific inventions can be reapplied along a sinister vector.

See Also

- Defense Advanced Research Projects Agency (DARPA)
- Defense Threat Reduction Agency (DTRA)
- Biological warfare (BW)
- Biological Weapons Convention (BWC)
- Biocontainment
- Decontamination
- Global Health Security Initiative (GHSI)
- National Science Advisory Board for Biosecurity
- Center for Biosecurity
- Australia Group (AG)

Further Reading

- Milanovich F (1998 June). Reducing the threat of biological weapons. *Sci Technol Rev*, pp 4–9. Retrieved from <http://www.llnl.gov/str/Milan.html>
- Christopher GW, Cieslak TJ, Pavlin JA, Eitzen EM Jr. (1998) Biological weapons (Adapted from *Biological Warfare: A Historical Perspective*). Fort Detrick, MD:

Operational Medicine Division, United States Army Medical Research Institute of Infectious Diseases

- Iraq's Biological Weapon Program (n.d.). Retrieved 19 December 2005. <http://www.iraqwatch.org/profiles/biological.html>
- Paquette L (2002) Bioterrorism and health and medical services administration. New York, Dekker
- Wagner M, Moore A, Aryel R (2006) Handbook of Biosurveillance. Academic Press, San Diego

Additional Useful Links

- EU Health Portal Information on EU activities related to bioterrorism
- Green Goo – Life In The Era Of Humane Genocide by Nick Szabo
- Bioterrorism: Plague as a biological weapon
- The CDC's Emergency Preparedness & Response (Bioterrorism) Website
- Fighting Fear of a Bioterrorism Event With Information Technology: Real-World Examples and Opportunities
- USAMRIID's Medical Management of Biological Casualties Handbook
- The Sunshine Project: Protection or Proliferation? Map and Publications on the US Biodefense Program
- Bioterrorism News from Genome News Network (GNN)
- NOVA: Bioterror

Shortcomings

The US Department of State on July 24, 2006 noted: "Existing international biological weapons non proliferation policies are not adequate to address the evolving nature to address the evolving nature of the biological weapons threat": the international community (World Health Organization and World Organization for Animal Health) though they have published biosafety guidelines, have failed to embrace obligatory biosecurity standards.^{19, 20} However, the Organization for Economic Cooperation and Development has issued biosecurity guidelines to implement its global network of Biological Resource.²¹

Very little effort by the EU or other scientifically advanced allies to coordinate medical capabilities to fight infectious diseases.²² Most of the European efforts have focused on advocating procedures and controlling the dissemination of sensitive dual-use research, educate researchers in the ethics, more that in the practice of implementing mechanisms for the direct oversight of such sensitive dual-use research.

Concluding Considerations

Bioterrorism

What You Need to Know About It

Learn about Bioterrorism 1

(“Safe at Home: A Family Survival Guide”)

This is the only such guide available today. The book’s linchpin is a straightforward, q&a-style set of guidelines for everything from choosing a filtration mask and putting together a disaster supply kit to preparing children for emergencies without giving them nightmares.

The rest of the book, also in q&a format, provides basic information on the most likely bioterrorism agents, such as anthrax, smallpox, plague and botulism.

It clearly and knowledgeably explains the symptoms, incubation period and available treatments for each agent, providing specific details, like the definition of “weaponized” anthrax and the government plan for containing a smallpox outbreak. Sidebars describe how the organisms have been used as weapons in the past.

The book also includes a chapter on chemical weapons and one on the food and water supply. Though his tone is generally optimistic, it is candid about weaknesses in the public health system, such as the dearth of vaccine research on children or the FDA’s inability to meet its food inspection goals.

It is concerned, above all, about the lack of rapid communication among doctors and health agencies (citing that “one of five public health offices do not have e mail”), and concludes with his proposals to increase funding for state and local public health organizations and other suggestions for government action.

This reassuring, thorough resource undoubtedly will prove a comfort for many readers and, in the case of a bioterrorist attack, has the potential to save countless lives. Color photo insert of organisms and, to aid in diagnosis, of skin rashes (comparing, for instance, smallpox to chickenpox).

Additional Information

1. Public health, guard food and water
2. Proposed after the anthrax attacks last fall, the law authorizes the spending of billions of dollars to improve state, local, and hospital preparedness, expand the National Pharmaceutical Stockpile, increase inspections of imported foods, assess the security of drinking-water systems, and upgrade facilities at the Center for Disease Control and Prevention (CDC).
3. “Biological weapons are potentially the most dangerous weapons in the world”, Bush said before signing the bill. “Last fall’s anthrax attacks were an incredible

tragedy to a lot of people in America, and it sent a warning that we needed. We must be better prepared to prevent, identify and respond. And this bill I'm signing today will help a lot in this regard."

4. Congress sent the Public Health Security and Bioterrorism Preparedness and Response Act of 2002 to the president 3 weeks ago. The measure is a compromise between somewhat different versions that were passed by the Senate and House last December. The original bills were sponsored by Bill Frist, R-Tenn., and Edward Kennedy, D-Mass., in the Senate and by Billy Tauzin, R-La., and John Dingell, D-Mich., in the House.
5. At present, commercial food testing laboratories can neither identify nor rule out bioterrorism agents in food products. Requests for such testing should be directed instead to state public health laboratories.

(For a contact list, see the links provided by the Association of Public Health Laboratories.) These laboratories are part of the national Laboratory Response Network (LRN), a multilevel system designed to link state and local laboratories with advanced-capacity biosafety facilities and to provide surge capacity in the event of a bioterrorism incident.

6. A different type of history is that of the high morbidity event related with the recent assumption by Chinese children of powdered milk enriched with melanine. Close to 100,000 children have been victims of the product, while approximately 11,000 are still hospitalized. Though not a *bonafide* terrorist attack, its impact is so strong that it is worth mentioning (Asia news.it 10.01.2008).
7. Efforts to prevent and respond to potential deliberate contamination or disruption in the food system encompass a broad range of public and private sector activities. Some of these efforts grew out of traditional food safety responsibilities, while others were developed in response to the potential threats of large-scale, coordinated attacks. The latter includes improvements in security, threat assessment, disease surveillance, laboratory detection, communications, and coordination among federal, state, and local agencies responding to emergencies involving the production and distribution of food nationwide.

Important Issues

One is the answer to the question: where are today the Biological Weapons?

Iraq is the most publicized of the countries with BW. However, at least 15 other countries have developed them: amongst them: Iran, Israel, both Koreas, South Africa, China, India, and Russia. Britain and the USA had substantial stocks, which they claim to have destroyed.

Another one is what has been defined Nuclear Blindness versus Biological Awareness, at the time of the Cold War.

While the Soviets were developing their "biological arsenals" in the 1960–1980s, the USA and Britain were so intensely concerned with the "nuclear threat" to the point of neglecting BW even arriving at abandoning the search for potential antidotes and vaccines.

Next Steps

It seems of paramount relevance to take advantage of the information gathered so far to plan and implement educational programs to inform physicians and all other health providing organization (national and international) of the issues, which would warrant a safer and more effective delivery of medical care.

References (Only Those Not Listed in the Text)

1. Civil liability for agroterrorism: in House Bill 98 (2008)
2. Block SM (2001, Jan–Feb) The growing threat of biological weapons. *American Scientist* 89:1. Accessed 15 Dec 2005
3. Eitzen E, Takafuji E (1997) Historical overview of biological warfare. In Office of the Surgeon General, Department of the Army (ed) *Textbook of Military Medicine: Medical Aspects of Chemical and Biological Warfare* 4
4. Blanc HW (1890) Anthrax: the disease of the Egyptian plagues. *New Orleans Med Surg J* 18:1–25
5. Duchovic RJ, Vilensky JA (2007). Mustard gas: Its pre-world war I history. *J Chem Educ* 84:944. <http://jchemed.chem.wisc.edu/Journal/Issues/2007/Jun/abs944.html>
6. Tu AT (2000) Overview of sarin terrorist attacks in Japan. *ACS Symp Ser* 745:304–317
7. Bohn K (2008, 6 August) U.S. officials declare researcher is anthrax killer. CNN. <http://www.cnn.com/2008/CRIME/08/06/anthrax.case/index.html>. Accessed on 2008-08-078
8. Guillemin J (1999) *ANTHRAX, the investigation of a Deadly Outbreak*. University of California Press, California. ISBN 0-520-22917-7, names of victims, pp 275–277
9. Weslager CA (1972) *The Delaware Indians: A History*. Rutgers University Press, New Brunswick
10. Koch R (1843–1910) Anthrax discoverer. http://german.about.com/library/blr/f_koch.htm. Accessed on 13 Aug 2008
11. Contemporary challenges of the immune system: Antrax Infection in Yugoslavia, 1972 (2009, 31 March) http://www.cbs.dtu.dk/courses/27685.imm/presentations/Ole/Ch2_Challenges.ppt
12. Ricin: biotoxin. Emergency response safety and health database. National Institute for Occupational Safety and Health (2009, April 20)
13. Wedin GP, Neal JS, Everson GW, Krenzelok EP (1986) Castor bean poisoning. *Am J Emerg Med* 4(3):259–261
14. http://www.dcmilitary.com/dcmilitary_archives/stories/090105/36813-1.shtml
15. Woods Lt. Col. Jon B (ed) (April 2005) *USAMRIID's Medical Management of Biological Casualties Handbook*, (6th edn). U.S. Army Medical Institute of Infectious Diseases, Fort Detrick, Maryland, p 67
16. *Salmonella Serotype Typhimurium Outbreak Associated with Commercially Processed Egg Salad, Oregon, 2003*. CDC, *MMWR* Dec 10, 2004. 53(48):1132–1134
17. *Public Health Security and Bioterrorism Preparedness and Response. Protection of Food Supply. Title III – Protecting Safety and Security of Food and Drug Supply, Subtitle A – Protection of Food Supply AT*. <http://thomas.loc.gov>., Bill number H.R. 3448
18. Joy B (2000) *On Newstands Now. Why the future doesn't need us*
19. <http://www.who.int/topics/biosafety/en/>
20. http://www.oie.int/eng/edito/en_edito_jun03.htm
21. http://www.oecd.org/document/50/0,3343,en_2649_34537_1911986_1_1_1_1,00.html
22. http://eur-lex.europa.eu/LexUriServ/site/en/com/2007/com2007_0399en01.pdf

Detection and Quantification of Bacteria and Fungi Using Solid-Phase Cytometry

Lies ME Vanhee, Eva D'Haese, Ils Cools, Hans J Nelis, and Tom Coenye

Abstract Solid-phase cytometry (SPC) was developed to meet the demand for fast and sensitive microbial detection and quantification methods. By combining the principles of epifluorescence microscopy and flow cytometry, this technique allows accurate, fast and automated detection of single microbial cells. SPC analysis is a five-step procedure, including membrane filtration, fluorescent labelling of the retained cells, scanning of the membrane filter, data analysis by a computer and microscopic validation. The aim of this review is to present the basic principles of SPC, its advantages and disadvantages and to discuss the existing applications as well as some perspectives for future research.

Keywords Solid-phase cytometry • rapid detection • quantification

Introduction

Each year microbiologists analyse millions of clinical, water, food and beverage samples to determine total plate counts (total number of culturable cells) and to demonstrate the presence or absence of specific undesirable microorganisms.

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However, traditional culture-based techniques are often labour-intensive and may take days to yield a result. Additionally, they often underestimate the number of microorganisms. First of all, only culturable microorganisms are detected, while the non-culturable ones, which can comprise a significant percentage of the total, escape detection. Secondly, because of differences in growth requirements, no single condition will allow growth of every microorganism. Finally, fast-growing microorganisms may overgrow slower ones.

In industrial, environmental and clinical settings, real-time microbial monitoring is often required for rapid decision making. Analytical microbiology has undergone great changes with the introduction of modern instrument-based technologies, each with a different performance and application range. The detection limit, accuracy, speed of analysis and type of sample to be analysed are important criteria for the selection of an instrumental technique.

Fluorescence-based microbial detection systems, including epifluorescence microscopy (EFM), flow cytometry (FC) and solid-phase cytometry (SPC), lend themselves to rapid, in-situ analysis of individual microorganisms, without the need for multiplication (Lemarchand et al. 2001; Lisle et al. 2004). This review will focus on the basic principles of SPC, its advantages, disadvantages and applications, and outline some future perspectives.

Basic Principles of SPC

In SPC, the main principles of EFM and FC, being fluorescent labelling of cells and laser detection, are combined (Lemarchand et al. 2001). The different steps in a SPC protocol are presented in Fig. 1.

First, samples are filtered over a black membrane filter (e.g. polyester or polycarbonate) with an appropriate pore size (i.e. 0.4 μm for bacteria and 0.8–2 μm for eukaryotic cells). These screen filters are used because of their low background fluorescence and high contrast, which facilitates validation using the epifluorescence microscope (see below) (Brailsford 1996). Secondly, the retained cells are fluorescently stained using one or more physiological or taxonomic probes (see Section [Fluorescent Stains for SPC](#)).

Next, the fluorescence emitted by the labelled cells is detected using a solid-phase cytometer (ChemScan C or RDI), which consists of an argon laser for fluorophore excitation and two to three photomultiplier tubes (PMTs) for signal detection. A laser beam from the argon laser (488 nm) is guided via a two-axis scanning device and a spot, 7 μm in diameter, is focused on the membrane surface. The beam scans the surface at a speed of 1 m/s in the X-direction. Along one scan line, PMTs with wavelength windows set for the green (500–530 nm) and amber (540–585 nm) regions collect the fluorescent light emitted at 0.5 μm intervals (samples). As each scan line is being offset in the Y direction by 3 μm from the previous one, the two-directional scanning is thus fully overlapping, ensuring that every point of the membrane is scanned at least twice by the laser beam. An entire

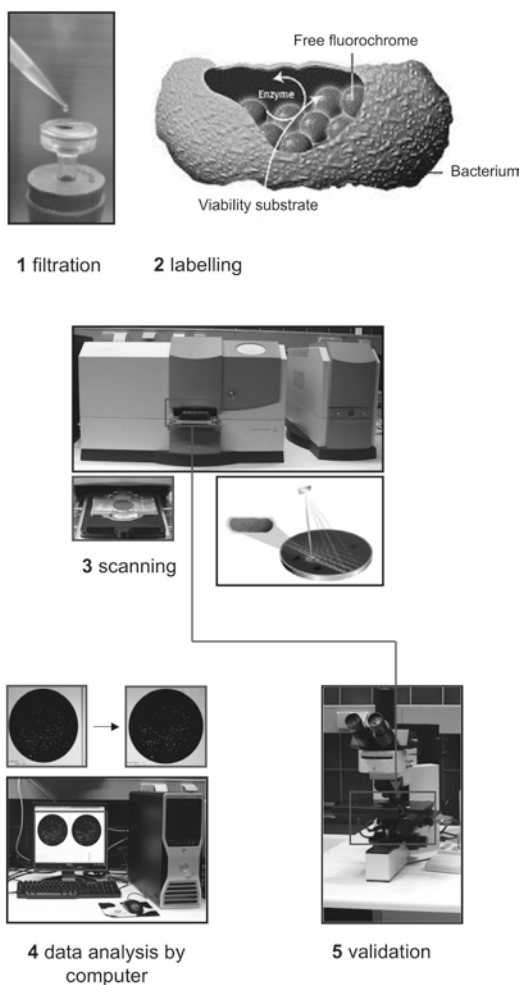


Fig. 1 Schematic overview of solid-phase cytometry. Steps include: membrane filtration, fluorescent labelling, scanning, data analysis by a computer and microscopic validation

membrane surface of 25 mm diameter can thus be scanned in 3 min. To this end, the membrane filter is placed in a stainless steel holder, which is automatically brought in focus and is cooled by Peltier elements.

Subsequently, the produced signals are processed by a computer to differentiate valid signals (labelled microorganisms) from fluorescent particles by evaluating data for several software parameters such as the size of the fluorescent spot, the specific intensity, the color ratio and the signal pattern (Fig. 2) (Brailsford 1997a, b; Guyomard 1997; Mignon-Godefroy et al. 1997; Rolland et al. 1999; Wallner et al. 1997). Upper and lower constraints can be placed on the number of scan lines and samples (reflecting the size) as well as on the fluorescence intensity admissible for a positive event.

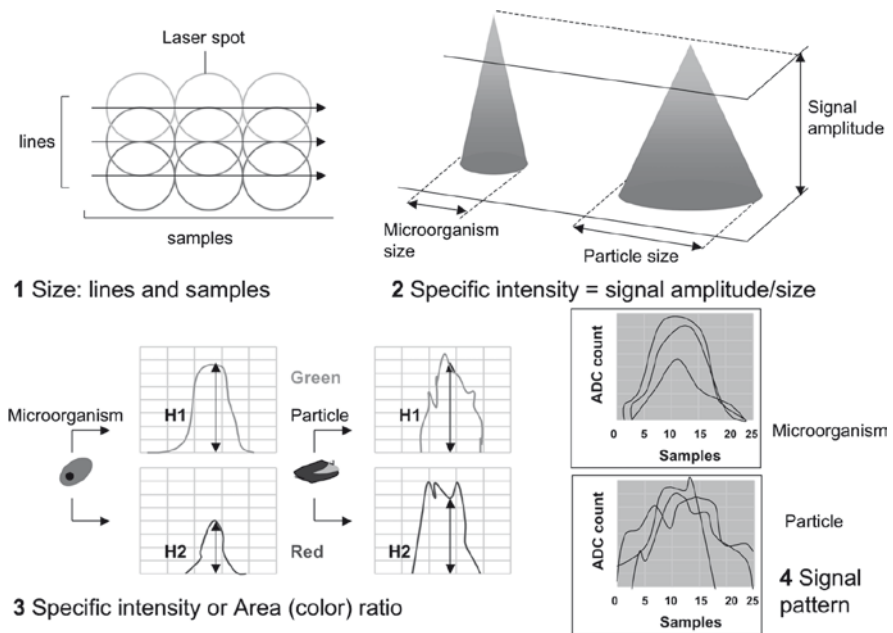


Fig. 2 Overview of the software parameters used by the computer to discriminate among microorganisms and autofluorescent particles. The four main discriminants are size, specific intensity, color ratio and signal pattern of the detected fluorescent spot

Additionally, the integration of the fluorescence by the computer further allows the calculation of a red to green ratio for each event. Finally, the degree of fitting of the fluorescence curves with the Gaussian interpolation is a reflection of the shape of the detected fluorescent spot, and can also be used as a parameter for discrimination. At the end of the analysis procedure, results are displayed as green spots on a membrane filter image in a primary and, after software elimination of background spots, in a secondary scan map (Mignon-Godefroy et al. 1997).

Finally, to further analyse their properties, the retained spots are visually inspected using an epifluorescence microscope equipped with a computer-driven moving stage. To that end, the sample holder is transferred to the motorized stage in exactly the same orientation as in the ChemScan. Highlighting of a green spot in the secondary scan map directs the microscope to the respective position on the membrane filter, allowing rapid and accurate visual discrimination between labelled cells and fluorescent particles ('validation').

Fluorescent Stains for SPC

Fluorescent stains used in EFM, FC and SPC include physiological and taxonomic probes (Joux and Lebaron 2000). An overview of their target sites is given in Fig. 3.

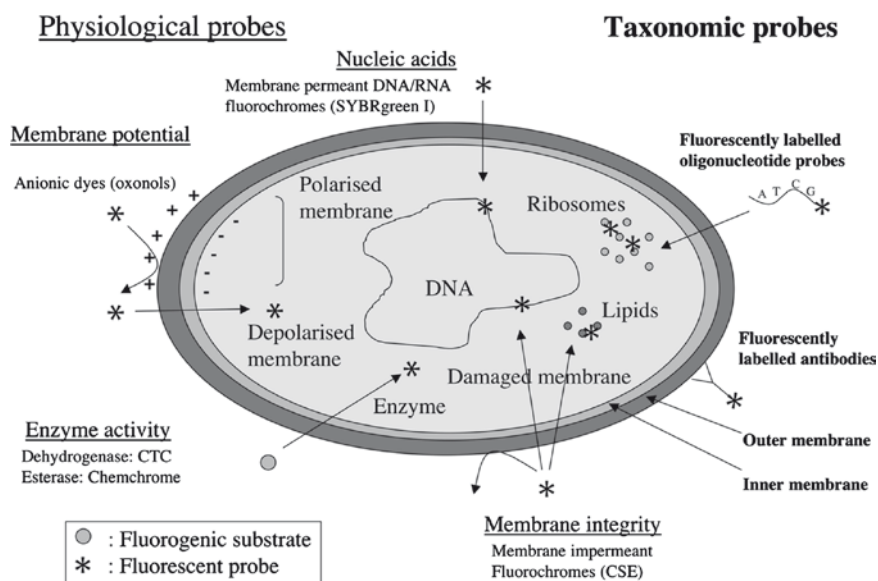


Fig. 3 Different cellular target sites for physiological and taxonomic fluorescent dyes (Based on Joux and Lebaron 2000)

Physiological probes directly bind to particular molecules in the cell or are markers of metabolic activities (such as enzyme activity and membrane potential). These probes are non-specific, i.e. their action is independent of the type and the identity of the cell. Depending on whether a stain is membrane permeant or impermeant it will label both viable and dead cells or dead cells only, respectively. ChemChrome V6 (formerly ChemChrome B or V3) contains carboxyfluorescein diacetate (Fig. 4), a nonfluorescent compound that is taken up by metabolically active cells and cleaved by intracellular esterases to yield an intensely green fluorescent product (carboxyfluorescein). When used in conjunction with an appropriate labelling buffer (ChemSol B12), the fluorescent carboxyfluorescein is only retained in cells with an intact cytoplasmatic membrane (Catala et al. 1999; Parthuisot et al. 2000). As the dye will rapidly leak from dead cells because of their damaged membranes, ChemChrome V6 functions as an activity and cell integrity probe that measures both enzymatic activity and cell-membrane integrity.

Little has been reported on the use of other fluorescent dyes for SPC. Broadaway et al. (2003) described the use of SYBR Green I in combination with ChemScan detection. Van Poucke and Nelis (2000a, b) reported the specific SPC detection of a target bacterium (*Escherichia coli*) using a physiological probe, i.e. fluorescein- β -D-digluconide, a substrate for the marker enzyme β -glucuronidase. Although some non-target bacteria occurring in water also contain this enzyme, specificity for *Escherichia coli* was derived from a quantitative difference in

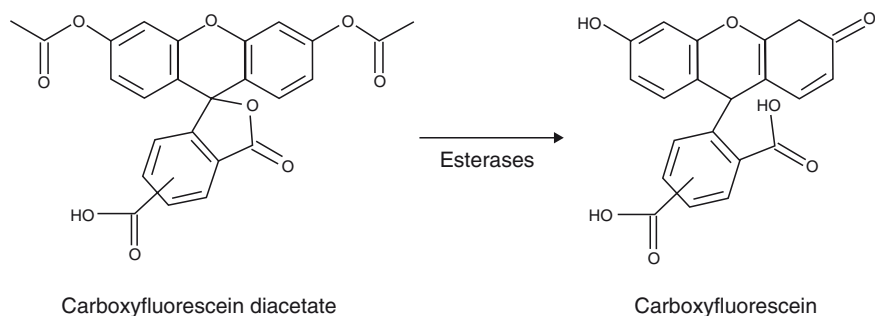


Fig. 4 Cleavage of ChemChrome V6 by esterases to yield the green fluorescent carboxyfluorescein

β -glucuronidase activity resulting from the use of a proprietary cocktail of inducers and stabilizers.

Taxonomic probes, including antibodies and nucleic acid or peptide nucleic acid (PNA) probes selectively stain particular target cells by association with antigens or DNA/RNA. The corresponding approaches are designated as immunofluorescence (IF) and fluorescence in situ hybridisation (FISH), respectively. The major limitation of these labelling procedures is the low fluorescence intensity, which results in poorly labelled cells that escape detection.

Signal intensity can be increased by double antibody labelling, a procedure in which both the primary and secondary antibody are tagged with fluorescein isothiocyanate (FITC). As these two fluorescent signals are additive, the signal intensity will be markedly increased (Aurell et al. 2004).

In tyramide signal amplification (TSA), the target cells are labelled with an antibody or a nucleic acid probe, followed by secondary detection with a horseradish peroxidase (HRP) labelled antibody. HRP activates multiple copies of fluorescently labelled tyramide derivatives, yielding fluorescent tyramide radicals that are deposited in the vicinity of the HRP-target interaction site (Fig. 5).

The implementation of a direct viable count (DVC) approach may also lead to an increase of the fluorescence intensity. This procedure is based on the activation of the cellular metabolism in the presence of a nutritive source and a DNA gyrase inhibitor (e. g. nalidixic acid), which stops cell division, increases the intracellular rRNA content and causes elongation of sensitive cells. This leads to a higher fluorescence intensity as there are more rRNA targets available for subsequent FISH labelling (Baudart et al. 2002, 2005).

Finally, a double labelling with antibodies and a viability substrate can be performed. De Vos and Nelis (2003, 2006) combined ChemChrome V6 with tetramethyl rhodamin isothiocyanate (TRITC) labelled antibodies for the detection of *Aspergillus fumigatus*. In these approaches, the ChemChrome reagent, yielding green fluorescence, ensures the primary detection by the ChemScan, whereas the TRITC label results in red fluorescence, to be observed microscopically.

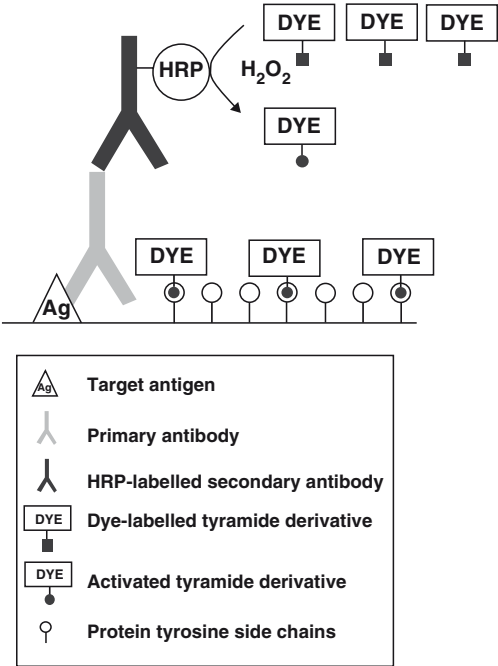


Fig. 5 Schematic presentation of tyramide signal amplification (TSA)

Applications of SPC

The fact that pathogenic microorganisms can be present in low numbers often hampers their detection. Conventional culturing may take several days and underestimate the microbial load. Therefore, more rapid and sensitive microbial detection methods would be useful for many applications to complement or replace these traditional culture methods. As SPC is a fast and sensitive tool to detect low numbers of microorganisms, it has been used for the detection and quantification of several important species.

Applications of SPC (summarized in Table 1) can be divided into three main categories. The first one concerns SPC methods (using physiological probes for fluorescent labelling) used to determine a total (viable) microbial count for quality assessment of water and air samples (Section [Determination of the Total \(Viable\) Count of Water and Air Samples](#)). A second group (Section [Specific Detection of Target Organisms](#)) consists of methods for the detection of specific target organisms in water, air, food and clinical samples. This type of SPC analysis requires taxonomic probes for selective labelling, sometimes in conjunction with a physiological probe to assess the viability of the cells. In the third category of applications (Section [Studies on the Physiological State of Microorganisms](#)), SPC is used as a tool for studies on the physiological state of microorganisms, e.g. the formation of

Table 1 SPC detection of different types of cells, the matrix in which detection was performed, as well as the fluorescent label used

Application	Matrix	Fluorescent label	Reference
Total counts	Water	SYBR Green I	Broadaway et al. 2003
Total viable counts	Natural waters	ChemChrome B, ChemChrome V6	Parthuisot et al. 2000
	Pharmaceutical water	ChemChrome V3	Guyomard 1997
	Pharmaceutical water	ChemChrome V3	Brailsford 1996
	Pharmaceutical water	ChemChrome V3	Jones et al. 1999
	Pharmaceutical water	ChemChrome (not specified)	Wallner et al. 1997
	Pharmaceutical water	ChemChrome (not specified)	Wallner et al. 1999
	Potable water	ChemChrome B	Reynolds et al. 1997
	Potable water	ChemChrome B	Reynolds and Fricker 1999
	Tap water, seawater, purified water	ChemChrome V6	Catala et al. 1999
	Water	ChemChrome V3	Lisle et al. 2004
	Air	ChemChrome V6	Vanhee et al., 2008, Vanhee et al., 2009a
Total viable fungal count	Hospital waters	ChemChrome V6 + TRITC-concanavalin A	De Vos and Nelis 2006
Specific target organisms			
Water samples			
<i>Enterobacteriaceae</i>	Freshwater, drinking water	Nucleic acid probe + DVC + TSA	Baudart et al., 2002, 2005
<i>Escherichia coli</i>	Drinking water	Nucleic acid probe + TSA	Lepeuple et al. 2003
<i>Escherichia coli</i>	Tap water	PNA probe + TSA	Prescott and Fricker 1999
<i>Escherichia coli</i>	Tap water, well water, surface water	Fluorescein- β -D-glucuronide	Van Poucke and Nelis 2000a, b
<i>Escherichia coli</i> O157:H7	Tap water, seawater	FITC-Ab	Lemarchand et al. 2001
<i>Escherichia coli</i> O157:H7	Water	FITC-Ab + CTC	Pyle et al. 1999
<i>Legionella pneumophila</i>	Hot water systems	FITC-Ab (prim. & sec.)	Aurell et al. 2004
<i>Cryptosporidium parvum</i>	Raw and potable water	FITC-Ab	de Roubin et al. 2002; Reynolds et al. 1999; Rushton et al. 2000
<i>Naegleria fowleri</i>	Surface waters	Biotin-Ab + streptavidin-RPE-Cy5 HRP-Ab + TSA	Pougnaud et al. 2002

(continued)

Table 1 (continued)

Application	Matrix	Fluorescent label	Reference
<i>Prymnesium parvum</i>	Seawater	Ab + FITC-Ab	West et al. 2006
<i>Prymnesium parvum</i>	Seawater	Nucleic acid probe + TSA	Töbe et al. 2006
Air samples			
<i>Aspergillus fumigatus</i>	Air	Ab + TSA	Vanhee et al. 2009b
Food samples			
<i>Escherichia coli</i> O157:H7	Meat	FITC-Ab + CTC	Pyle et al. 1999
<i>Mycobacterium paratuberculosis</i>	Milk	ChemChrome V6	D'Haese et al. 2005
Clinical samples			
<i>Aspergillus fumigatus</i>	BAL, sputum	ChemChrome V6 + TRITC-Ab	De Vos and Nelis 2003, 2006
<i>Cryptococcus neoformans</i>	CSF, serum	FITC-Ab, ChemChrome V3	Bauters et al. 2003
<i>Human Papilloma Virus</i>	Solid biopsy material	Biotin-nucleic acid probe + FITC-avidin	Butor et al. 1997
Physiological state applications			
<i>Campylobacter jejuni</i>	Pure cultures	ChemChrome V6	Cools et al. 2005
Total viable count	Spiramycin	ChemChrome V6	Ramond et al. 2000
Colistin	Milk	ChemChrome V6	D'Haese and Nelis 2000
Total viable count	Pharmaceutical oils	ChemChrome V6	De Prijck et al. 2008
Other applications			
Anaerobic bacteria and spores	Pure cultures	ChemChrome V6	Vermis et al. 2002
Fetal cells	Maternal blood	FITC-Nucleic acid probe + FastRed-Ab	Serradell et al. 2000
Somatic cells	Milk	ChemChrome V6	D'Haese et al. 2001
Filament count	<i>Candida albicans</i> cultures	ChemChrome V6	Nailis et al. 2009

TRITC: tetramethyl rhodamin isothiocyanate

DVC: direct viable count

TSA: tyramide signal amplification

PNA: peptide nucleic acid

FITC: fluorescein isothiocyanate

Ab: antibody

CTC: cyanoditotyl tetrazolium chloride

RPE-Cy5: R-phycoerythrin conjugated with Cy5

HRP: horseradish peroxidase

BAL: bronchoalveolar lavage fluid

CSF: cerebrospinal fluid

viable but non-culturable (VBNC) forms. Finally, SPC has proven its usefulness in various less common research applications (Section [Other Applications](#)).

Determination of the Total (Viable) Count in Water and Air Samples

Analysis of Water Samples

Multiple studies have applied SPC for the determination of the total viable count (TVC) of mainly pharmaceutical and potable waters. For this purpose, viability stains such as ChemChrome B (Parthuisot et al. 2000; Reynolds et al. 1997; Reynolds and Fricker 1999a), ChemChrome V3 (Brailsford 1996; Guyomard 1997; Jones et al. 1999; Lisle et al. 2004) and ChemChrome V6 (Catala et al. 1999; Parthuisot et al. 2000) were used. ChemChrome V6 resulted in superior labelling when compared to the now obsolete ChemChrome B or V3 (Parthuisot et al. 2000). Although SPC counts were often higher than plate counts using R2A agar, a very good correlation was found between the results obtained with both methods (Brailsford 1996; Jones et al. 1999; Wallner et al. 1997, 1999). Only one study (Broadaway et al. 2003), however, determined the total count (viable and non viable cells) by using the nucleic acid dye SYBR Green I in addition to the TVC obtained with ChemChrome V6.

De Vos and Nelis (2006b) applied a double labelling for the selective detection of fungi in hospital waters (dialysis fluid and rinse water for endoscopic equipment), combining ChemChrome V6 for viability assessment and TRITC-concanavalin A for selective labelling of fungal cells.

Analysis of Air Samples

Recently, we have developed a novel approach for the rapid enumeration of airborne bacteria and fungi based on SPC. Air samples are collected by impaction on a water soluble polymer that is subsequently dissolved. For labelling of the airborne microorganisms, the viability stain ChemChrome V6 was used (Vanhee et al. 2008, 2009a).

Specific Detection of Target Organisms

Analysis of Water Samples

For the specific detection of target organisms taxonomic probes have to be used. In spite of the practical problems with the fluorescence intensity when these taxonomic probes are used (see Section [Fluorescent Stains for SPC](#)), several target

microorganisms have been detected in water samples by means of SPC, though with variable success.

The enumeration of *Enterobacteriaceae* and *Escherichia coli* in water using SPC has been the subject of several studies. These microorganisms serve as indicators of faecal contamination as part of the monitoring of the quality of raw and partially purified waters. They are also used to demonstrate the compliance of a final product with legal standards. For the selective detection of viable *Enterobacteriaceae*, Baudart et al. (2002, 2005) used a nucleic acid probe targeting the 16S rRNA. In order to enumerate the viable cells and to increase the fluorescence intensity, FISH was combined with a DVC procedure and TSA. Using this approach, as little as one fluorescent target cell could be demonstrated in the presence of 10^7 – 10^8 non-fluorescent other cells.

Several studies have focused on the specific detection of *Escherichia coli* with SPC, using different labelling procedures. While Lepeuple et al. (2003) used a 16S rRNA directed nucleic acid probe, Prescott and Fricker (1999) used a PNA probe. In both methods TSA was included to increase the fluorescence intensity. Van Poucke and Nelis (2000a, b) on the other hand used an enzyme substrate (fluorescein- β -D-diglucuronide) for the demonstration of β -glucuronidase activity in *Escherichia coli*. The quantitative difference in enzyme activity between target and non-target bacterial cells allowed for the specific detection of *Escherichia coli*.

Real-time monitoring of the quality of water is also important to prevent outbreaks caused by pathogenic microorganisms. Therefore, rapid and sensitive SPC detection methods have also been developed for selected pathogens including *Escherichia coli* O157:H7, *Legionella pneumophila*, *Cryptosporidium parvum*, *Naegleria fowleri* and *Prymnesium parvum* (Table 1). Lemarchand et al. (2001) used FITC-labelled antibodies without amplification to detect *Escherichia coli* O157:H7 in water. Pyle et al. (1999), on the other hand, used an antibody both for capturing (immunomagnetic separation, IMS) and labelling (FITC conjugated). Additionally, an incubation step with cyanoditotyl tetrazolium chloride (CTC) to determine cellular respiratory activity was incorporated. The resulting red fluorescence was observed microscopically during the validation of the scan results.

Aurell et al. (2004) were able to demonstrate *Legionella pneumophila* cells in water using a double antibody technique with specific monoclonal antibodies conjugated to FITC. A very simple amplification technique, using a secondary, FITC conjugated, antibody was used to increase the fluorescence intensity.

Cryptosporidium oocysts were selectively extracted (antibodies coupled to magnetic beads) from water concentrates using IMS. These oocysts were subsequently visualized using FITC conjugated monoclonal antibodies. However, dissociation from the immunomagnetic beads before labelling proved to be necessary (de Roubin et al. 2002; Reynolds et al. 1999; Rushton et al. 2000).

Naegleria fowleri (Pougnard et al. 2002) has been detected by SPC in surface water. A monoclonal antibody was conjugated with biotin or HRP and revealed by streptavidin conjugated to RPE-Cy5 (R-phycoerythrin conjugated with the cyanine dye Cy5) or FITC-conjugated tyramide, respectively. The RPE-Cy5 protocol was the most efficient and allowed the detection of both trophozoite and cyst forms in water.

Finally, two studies used SPC for the rapid quantification of the toxic alga *Prymnesium parvum* in seawater. West et al. (2006) used a specific monoclonal antibody and a FITC-conjugated secondary antibody, while Töbe et al. (2006) detected the algal cells with a new oligonucleotide probe conjugated to HRP and TSA.

Analysis of Air Samples

A novel approach for the quantification of *Aspergillus fumigatus*, based on SPC and immunofluorescent labelling, has recently been developed. Air samples were collected by impaction as described previously (Section Analysis of Air Samples). This was followed by labelling with a monoclonal anti-*Aspergillus* antibody and TSA to detect the cells. Additionally, a growth step at 47°C was included to improve the specificity (Vanhee et al. 2009b).

Analysis of Food Samples

Given the unfilterability of foods, reports on the application of SPC in this field are scarce (D'Haese and Nelis 2002). However, the few experimental methods that were developed may serve as a proof of concept.

Pyle et al. (1999) used SPC for the rapid detection of *Escherichia coli* O157:H7 in meat. IMS allowed the isolation of target cells from the food matrix and was followed by a double labelling, using green fluorescent FITC-conjugated antibodies and the red fluorescent viability substrate CTC.

D'Haese et al. (2005) studied the potential of SPC for the rapid enumeration of the extremely slow-growing *Mycobacterium paratuberculosis* in milk. However, as the viability stain ChemChrome V6 was used for labelling, no specific detection of the bacteria was obtained and only spiked samples were analysed.

Analysis of Clinical Samples

SPC has not yet found its way to clinical microbiology, although several studies have demonstrated its potential applicability. De Vos and Nelis (2003, 2006) describe the specific detection of *Aspergillus fumigatus* in bronchoalveolar lavage fluid (BAL) and sputum by means of a double labelling using ChemChrome V6 and TRITC-conjugated antibodies.

Bauters et al. (2003) demonstrated *Cryptococcus neoformans* in cerebrospinal fluid (CSF) and serum. Their 30-min procedure was based on the non-specific labelling with ChemChrome V3 in combination with a second analysis using immunofluorescence. To that end, cells were labelled with a specific primary antibody against a capsular polysaccharide and a secondary antibody conjugated with FITC.

Butor et al. (1997) were able to detect and map Human Papilloma Virus (HPV) infected cells labelled by means of FISH in cervical condyloma biopsies using a cDNA probe conjugated with biotin and subsequent detection with FITC conjugated avidin.

Studies on the Physiological State of Microorganisms

Cools et al. (2005) used SPC with ChemChrome V6 labelling to demonstrate the existence of a viable but non-culturable (VBNC) form of *Campylobacter jejuni* in water. For freshly cultured viable cells, an excellent correspondence was noticed between culture and SPC. Therefore, if discrepancies between the two results occur in older cultures, they can be attributed to the transition of culturable *Campylobacter jejuni* cells into the VBNC form.

Addition of pure cultures of four microorganisms to the antibiotic spiramycin (Ramond et al. 2000) did not compromise their recovery (irrespective of their susceptibility) using a SPC procedure with ChemChrome V6 labelling. In contrast, on a conventional plate, only spiramycin resistant strains were completely recovered.

Another study tested to what extent antibiotics affect the membrane integrity of *Escherichia coli* and hence inhibit the ChemChrome V6 labelling. Inhibition of the fluorescent staining was only observed for membrane permeabilizing antibiotics, even at concentrations below the MIC but not for antibiotics with other mechanisms of action e. g. β -lactams. As an application, colistin could be determined in milk by measuring the decrease in the number of labelled *Escherichia coli* cells relative to the initial number that had been added to the milk (D'Haese and Nelis 2000).

For the evaluation of the survival of different strains of bacteria in pharmaceutical oils, SPC was recently compared to the plate method. In agreement with previous studies, differences in recovery between the two methods were indicative of the formation of VBNC cells (De Prijk et al. 2008).

Other Applications

The applicability of SPC has also been evaluated in several other areas. Vermis et al. (2002) were able to label (ChemChrome V6) and enumerate vegetative cells and spores of eight strains of anaerobic bacteria under aerobic conditions. For vegetative cells a labelling time of 3 h (as compared to the 30 min labelling needed for aerobic bacteria) was necessary, whereas spores required an anaerobic activation of 3 h followed by a 1 h labelling.

A preliminary study showed that fetal cells can be detected in maternal blood using SPC and an immuno-FISH labelling protocol (Serradell et al. 2000). This could be promising for the prenatal diagnosis of chromosomal abnormalities.

SPC was also used for the rapid enumeration of somatic cells in milk (D'Haese et al. 2001). However, comparison with the routinely used fluoro-opto-electronic method revealed a poor comparability. Furthermore, problems of milk filterability and the interference of fluorescent particles hamper this application of SPC.

Finally, SPC has also been used to determine the fraction of *Candida albicans* filaments in a culture using labelling with ChemChrome V6 and a microscopic discrimination between yeast cells and filaments (Nailis et al. 2009).

Advantages and Disadvantages of SPC

One of the prominent advantages of SPC is its speed. As this method does not rely on culturing the microorganisms, quantitative results can be obtained within a few hours. Additionally, the membrane filter is scanned by the laser in only 3 min. Therefore, SPC can be used for real-time monitoring to provide an early warning and a rapid implementation of corrective measures.

The use of viability stains results in the quantification of not only the culturable cells but all viable cells. As recently stated by Newby (2007), the detection of VBNC microorganisms is crucial as pathogenic organisms may retain pathogenicity during the VBNC state. In general, SPC yields higher counts than plate methods because the VBNC microorganisms are also enumerated. Consequently, current warning and action limits based on plate counts need to be redefined.

SPC has a theoretical detection limit of one cell per filtered volume, but can also be used to determine the microbial load of highly contaminated samples because of its high dynamic range with an upper limit of approximately 10,000 cells per membrane filter. Unlike EFM and FC, SPC has the potential of detecting rare events, i.e. of finding low numbers of target cells among an excess of non-target cells (Mignon-Godefroy et al. 1997; Lemarchand et al. 2001; Lisle et al. 2004).

Microscopic inspection after the scan, to determine if a recorded fluorescent event represents a microorganism or a particle, is possible. Furthermore, SPC counts all microorganisms on the membrane filter, so that errors associated with counting microorganisms in a limited number of microscope fields and subsequent extrapolation of the counts to the total number of cells are minimized (Lemarchand et al. 2001).

SPC also has its limitations. A first one is the limited availability of compatible stains. Since the instrument is equipped with a single Ar laser source, the staining is restricted to the range of probes and dyes excitable at approximately 488 nm and emitting light in the range of 500–530 nm.

The most fundamental limitation of SPC is the requirement of filterable samples. Only clear, aqueous solutions can be used unless samples are more or less extensively pre-treated. For example, such pre-treatment proved necessary to obtain a modest improvement in the ability to filter BAL and sputum samples (De Vos et al. 2006). Alternatively, microorganisms can be isolated from complex matrices by e.g. IMS (de Roubin et al. 2002; Pyle et al. 1999; Reynolds et al. 1999; Rushton et al. 2000).

Furthermore, the possibility to culture and identify the microorganisms on the membrane filter would present a significant improvement.

In some samples, the occurrence of fluorescent particles may lead to an aborted scan or to a cumbersome validation when numerous spots are present in the secondary scan map. By adding a counterstaining step (Catala et al. 1999) and/or using a filter with a larger pore size, this problem can sometimes be overcome. Additionally, the fluorescence intensity of cells labelled using FISH or immunofluorescence is often low, making signal amplification necessary (see Section [Fluorescent Stains for SPC](#)).

The final disadvantage of SPC is its high cost and low throughput. A ChemScan is an expensive piece of laboratory equipment (approximately €170,000) and the cost for a traditional analysis with ChemChrome V6 is also relatively high (approximately €7.5 per sample).

Conclusions and Perspectives

The different applications of SPC clearly illustrate its usefulness in microbiology. However, some modifications could broaden the applicability of this method. The modification of SPC to a high throughput system (e. g. microtiter plate based) would enhance its use in industrial settings.

The introduction of other dyes for fluorescent labelling might provide information about other physiological cellular states and the development of new labelling procedures using taxonomic probes might enable the detection of other cells. Microorganisms genetically modified to express the green fluorescent protein are also likely to be detectable with SPC, although there are at present no reports in this regard.

Due to the use of a single light source, the choice of probes is rather limited. When two probes are combined, the technique becomes cumbersome and loses its advantage of speed. Therefore, extension of the instrument with a second or even a third light source would open the possibility for a whole range of new applications. For example, if a viability probe and a probe for the detection of a specific species could be combined, detailed studies on the physiological behaviour of bacteria in a complex community would be facilitated.

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References

- Aurell H, Catala P, Farge P, Wallet F, Le Brun M, Helbig JH, Jarraud S, Lebaron P (2004) Rapid detection and enumeration of *Legionella pneumophila* in hot water systems by solid-phase cytometry. *Appl Environ Microbiol* 70:1651–1657
- Baudart J, Coallier J, Laurent P, Prévost M (2002) Rapid and sensitive enumeration of viable diluted cells of members of the family *Enterobacteriaceae* in freshwater and drinking water. *Appl Environ Microbiol* 68:5057–5063
- Baudart J, Olaizola A, Coallier J, Gauthier V, Laurent P (2005) Assessment of a new technique combining a viability test, whole-cell hybridization and laser-scanning cytometry for the direct counting of viable *Enterobacteriaceae* cells in drinking water. *FEMS Microbiol Lett* 243:405–409
- Bauters TGM, Swinne D, Stove V, Nelis HJ (2003) Detection of single cells of *Cryptococcus neoformans* in clinical samples by solid-phase cytometry. *J Clin Microbiol* 41:1736–1737
- Brailsford M (1996) Real-time microbial analysis of pharmaceutical water. *Microbiol Eur* 4:18–20
- Brailsford M (1997a) Making the switch to real-time microbiological process control. *Manuf Chemist* (March):35–36
- Brailsford M (1997b) Profiting from a lack of growth. *Pharm Manuf Rev* (October):19–20

- Broadaway SC, Barton SA, Pyle BH (2003) Rapid staining and enumeration of small numbers of total bacteria in water by solid-phase laser cytometry. *Appl Environ Microbiol* 69:4272–4273
- Butor C, Duquenne O, Mignon-Godefroy K, Mougin C, Guillet JG (1997) Solid phase cytometry allows rapid in situ quantification of human Papilloma Virus infection in biopsy material. *Cytometry* 29:292–297
- Catala P, Parthuisot N, Bernard L, Baudart J, Lemarchand K, Lebaron P (1999) Effectiveness of CSE to counterstain particles and dead bacterial cells with permeabilised membranes: application to viability assessment in waters. *FEMS Microb Lett* 178:219–226
- Cools I, D'Haese E, Uyttendaele M, Storms E, Nelis HJ, Debevere J (2005) Solid phase cytometry as a tool to detect viable but non-culturable cells of *Campylobacter jejuni*. *J Microbiol Meth* 63:107–114
- De Prijck K, Peeters E, Nelis HJ (2008) Comparison of solid-phase cytometry and the plate count method for the evaluation of the survival of bacteria in pharmaceutical oils. *Lett Appl Microbiol* 47:571–573
- de Roubin M-R, Pharamond J-S, Zanelli F, Poty F, Houdart S, Laurent F, Drocourt J-L, Van Poucke S (2002) Application of laser scanning cytometry followed by epifluorescent and differential interference contrast microscopy for the detection and enumeration of *Cryptosporidium* and *Giardia* in raw and potable waters. *J Appl Microbiol* 93:599–607
- De Vos MM, Nelis HJ (2003) Detection of *Aspergillus fumigatus* hyphae by solid phase cytometry. *J Microbiol Meth* 55:557–564
- De Vos MM, Sanders NN, Nelis HJ (2006) Detection of *Aspergillus fumigatus* hyphae in respiratory secretions by membrane filtration, fluorescent labelling and laser scanning. *J Microbiol Meth* 64:420–423
- De Vos MM, Nelis HJ (2006) An improved method for the selective detection of fungi in hospital water by solid phase cytometry. *J Microbiol Meth* 67:557–565
- D'Haese E, Nelis HJ (2000) Effect of antibiotics on viability staining of *Escherichia coli* in solid phase cytometry. *J Appl Microbiol* 89:778–784
- D'Haese E, Nelis HJ, Reybroeck W (2001) Determination of somatic cells in milk by solid phase cytometry. *J Dairy Res* 68:9–14
- D'Haese E, Nelis HJ (2002) Rapid detection of single cell bacteria as a novel approach in food microbiology. *J AOAC Internat* 85:979–983
- D'Haese E, Dumon I, Werbrouck H, Dejonghe V, Herman L (2005) Improved detection of *Mycobacterium paratuberculosis* in milk. *J Dairy Res* 72:125–128
- Guyomard S (1997) Validation of a scanning laser system for microbiological quality control (QC) analysis. *Pharm Technol Eur* (September):50–54
- Jones DL, Brailsford MA, Drocourt JL (1999) Solid-phase, laser-scanning cytometry: a new two-hour method for the enumeration of microorganisms in pharmaceutical water. *Pharmacoepial Forum* 25:7626–7645
- Joux F, Lebaron P (2000) Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. *Microb Infect* 2:1523–1535
- Lemarchand K, Parthuisot N, Catala P, Lebaron P (2001) Comparative assessment of epifluorescence microscopy, flow cytometry and solid phase cytometry in the enumeration of specific bacteria in water. *Aquat Microb Ecol* 25:301–309
- Lepeuple AS, Delabre K, Giloupe S, Intertaglia L, de Roubin MR (2003) Laser scanning detection of FISH-labelled *Escherichia coli* from water samples. *Water Sci Technol* 47:123–129
- Lisle JT, Hamilton MA, Willse AR, McFeters GA (2004) Comparison of fluorescence microscopy and solid-phase cytometry methods for counting bacteria in water. *Appl Environ Microbiol* 70:5343–5348
- Mignon-Godefroy K, Guillet JG, Butor C (1997) Solid phase cytometry for detection of rare events. *Cytometry* 27:336–344
- Nailis H, Vandenbroucke R, Tilleman K, Deforce D, Nelis H, Coenye T (2009) Monitoring *ALS1* and *ALS3* gene expression during in vitro *Candida albicans* biofilm formation under continuous flow conditions. *Mycopathologia* 167:9–17

- Newby P (2007) The significance and detection of VBNC microorganisms. *Eur Pharm Rev* 3:87–93
- Parthuisot N, Catala P, Lemarchand K, Baudart J, Lebaron P (2000) Evaluation of Chemchrome V6 for bacterial viability assessment in waters. *J Appl Microbiol* 89:370–380
- Pougnard C, Catala P, Drocourt J-L, Legastelois S, Pernin P, Pringuez E, Lebaron P (2002) Rapid detection and enumeration of *Naegleria fowleri* in surface waters by solid-phase cytometry. *Appl Environ Microbiol* 68:3102–3107
- Prescott AM, Fricker CR (1999) Use of PNA oligonucleotides for the *in situ* detection of *E. coli* in water. *Mol Cell Probes* 13:261–268
- Pyle BH, Broadaway SC, McFeters GA (1999) Sensitive detection of *Escherichia coli* O157:H7 in food and water by immunomagnetic separation and solid-phase laser cytometry. *Appl Environ Microbiol* 65:1966–1972
- Ramond B, Rolland X, Planchez C, Cornet P, Antoni C, Drocourt J-L (2000) Enumeration of total viable microorganisms in an antibiotic raw material using ChemScan solid phase cytometer. *PDA J Pharm Sci Technol* 54:320–330
- Reynolds DT, Fricker EJ, Purdy D, Fricker CR (1997) Development of a rapid method for the enumeration of bacteria in potable water. *Water Sci Tech* 35:433–436
- Reynolds DT, Fricker CR (1999) Application of laser scanning for the rapid and automated detection of bacteria in water samples. *J Appl Microbiol* 86:785–795
- Reynolds DT, Slade RB, Sykes NJ, Jonas A, Fricker CR (1999) Detection of *Cryptosporidium* oocysts in water: techniques for generating precise recovery data. *J Appl Microbiol* 87:804–813
- Rolland X, Cornet P, Drocourt JL (1999) The ChemScan RDI: a new in-process control tool combining speed with sensitivity in microbial detection. In: Connor TH, Weier H-U, Fox F (eds) *Biotechnology International II*. Universal Medical Press, San Francisco, CA, pp 309–314
- Rushton P, Place BM, Lightfoot NF (2000) An evaluation of a laser scanning device for the detection of *Cryptosporidium parvum* in treated water samples. *Lett Appl Microbiol* 30:303–307
- Serradell L, Dupont J-M, Ferré F, Rabineau D, Cornet P (2000) Fetal cell detection in maternal blood using the solid phase cytometer ChemScan RDI: preliminary studies. In: Hahn S, Holzgreve W (eds) *Fetal cells and fetal DNA in maternal blood*. New developments for a new millennium. 11th Fetal cell workshop. Karger, Basel, pp 82–90
- Töbe K, Eller G, Medlin LK (2006) Automated detection and enumeration for toxic algae by solid-phase cytometry and the introduction of a new probe for *Prymnesium parvum* (Haptophyta: *Prymnesiophyceae*). *J Plankt Res* 28:643–657
- Vanhee LME, Nelis HJ, Coenye T (2008) Enumeration of airborne bacteria and fungi using solid phase cytometry. *J Microbiol Meth* 72:12–19
- Vanhee LME, Nelis HJ, Coenye T (2009a) Detection and quantification of viable, airborne bacteria and fungi using solid-phase cytometry. *Nat Protoc* 4:224–231
- Vanhee LME, Nelis HJ, Coenye T (2009b) Rapid detection and quantification of *Aspergillus fumigatus* in air using solid-phase cytometry. *Environ Sci Technol* 43:3233–3239
- Van Poucke SO, Nelis HJ (2000a) A 210-min solid phase cytometry test for the enumeration of *Escherichia coli* in drinking water. *J Appl Microbiol* 89:390–396
- Van Poucke SO, Nelis HJ (2000b) Rapid detection of fluorescent and chemiluminescent total coliforms and *Escherichia coli* on membrane filters. *J Microbiol Meth* 42:233–244
- Vermis K, Vandamme PAR, Nelis HJ (2002) Enumeration of viable anaerobic bacteria by solid phase cytometry under aerobic conditions. *J Microbiol Meth* 50:123–130
- Wallner G, Tilmann D, Haberer K, Cornet P, Drocourt JL (1997) The ChemScan system: a new method for rapid microbiological testing of water. *Eur J Parent Sci* 2:123–126
- Wallner G, Tilmann D, Haberer K (1999) Evaluation of the ChemScan system for rapid microbiological analysis of pharmaceutical water. *PDA J Pharm Sci Technol* 53:70–74
- West NJ, Bacchieri R, Hansen G, Tomas C, Lebaron P, Moreau H (2006) Rapid quantification of the toxic alga *Prymnesium parvum* in natural samples by use of a specific monoclonal antibody and solid-phase cytometry. *Appl Environ Microbiol* 72:860–868

Vulnerability Assessment and Mapping of Akounq Groundwater Body, Armenia

Vahram Vardanyan and Artashes Aginian

Abstract Assessment of intrinsic and bacteriological vulnerability of water-bearing bodies was made, as well as 3D hydrostratigraphic model of study area was constructed.

Qualitative evaluation of water table aquifers vulnerability we have conducted considering the depths to water-table from land surface and thickness of poorly permeable soils in aeration zone. According to this method following types of soils have been distinguished in the cross-section of aeration zone:

1. Loamy sand or fractured matrix rocks
2. Loams or relatively confining matrix rocks
3. Clay or confining matrix rocks

Quantitative assessment of water-table aquifers vulnerability is being carried out when there is risk of bacteriological infection. This method is based on groundwater downward time-of-travel during which percolated bacteria or polluted water may reach to water table.

Quantitative assessment of confined aquifer vulnerability that is first from land surface is carried out considering two parameters: total thickness of overlain soils with slow infiltration rates and interrelation between water-table level and established level of water of confined aquifer.

Keywords Aquifer • vulnerability • intrinsic • permeability

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Introduction

Tremendous economic activities negatively impact on hydrogeological conditions, mainly on aquifers that result in contamination and depletion of storage.

Contamination hazard of fresh water aquifers many times exceeds the danger of the storage depletion. Hence, conservation of ground water as of sustainable source for drinking water supply is of main importance.

Ground water is the only source for economic and drinking water supply in the Republic of Armenia. Ground water recharge areas are mountain ranges that naturally discharge both at washed versants and intermountain basins.

Industrial manufacturing and agriculture including gardening, vegetable growing and growing of other industrial crops are developed at intermountain basins and those foothills. Most of settlements have no discharge network. Hence, ground water is under contamination risk and vulnerability evaluation of fresh ground water bodies of primarily importance (Bochever 1979). Ground water contamination is a change of water quality under the impact of human activities comparing with the quality of water in natural conditions (background level) that leads partially or entirely to uselessness.

The analysis of ground water vulnerability is made by lithologic cross-sections of 15 wells.

Characteristics of the Study Area

The study area is a volcanic plateau, extending over 10 km². Agriculture, mainly farming and vegetable-growing is developed here.

Plateau is enclosed by quaternary volcanic ridges from south-west sawed through narrow gorge of Akunk River. Akunk lacustrine-alluvial valley is a fragment of old river valley and has an oval form (length 4 km, width 2 km) with altitudes 1,425–1,443 masl (PNIIS 1970). Water-bearing rocks are represented by discontinuously water-bearing quaternary lacustrine-alluvial sediments, discontinuously water-bearing quaternary and pliocene fractured lava streams, lenses of locally water-bearing interformational sedimentary rocks (Fig. 1). The 3-d stratigraphic model was constructed (Ross et al. 2004). The till, loams of pliocene provide confining of the aquifer units, which act as regional aquitard. The capacity of this source is about 2,500 l/s and feeds aqueduct of capital Yerevan for drinking purposes. Aquifer of confined-unconfined nature. Ground water naturally discharges at land surface with capacity 2,000 l/s. Another part of water is pumped out of 15 wells. The level of water in wells is about 45 m.

Method

Two types of ground water contamination depending on the quantity of contaminating products are being distinguished:

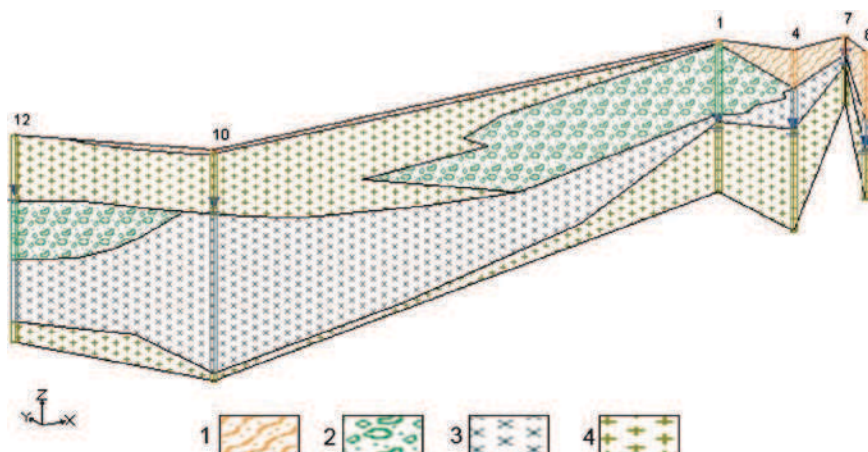


Fig. 1 Hydrostratigraphic section through wells NN 1, 4, 7, 8, 10, 12. (1) Loam with gravel, pebble, basalt detritus, waterless. (2) Basalt detritus, pebble filled with loam. (3) Monolithe basalt. (4) Fractured basalt

1. Concentration of contaminating products in water greater than in natural conditions, continues to grow, but less than maximum permissible concentration (MPC)
2. Concentration of contaminating products in water greater than MPC (Goldberg 1988)

The first type is evaluated as a start of contamination, and the second type as already to be contaminated. It is important to reveal the tendency of water impairment at initial stages to prevent the further contamination of ground water. Percolation or seepage of industrial and domestic liquids, pesticides, as well as natural substandard waters into operating aquifers may result in ground water contamination. Abandoned wells might also result in ground water contamination.

In the territory of the Republic of Armenia we have investigated aquifers with various degrees of contamination caused by above described possible contamination sources and seepage pathways.

At present qualitative and quantitative evaluation of water table aquifers and compiling of respective maps are of highly practical importance (Goldberg 1988; Grozdova 1979).

Vulnerability of water-table aquifers we have evaluated by depths to water table from land surface and by thickness of poorly permeable soils in aeration zone. According to this method in the cross-section of aeration zone following types of soils with low infiltration rates have been distinguished:

- $K = 0.1\text{--}0.01$ m/day (loamy sand or fractured matrix rocks)
- $K = 0.01\text{--}0.001$ m/day (loams or relatively confining matrix rocks)
- $K < 0.001$ m/day (clay or confining matrix rocks), where K is permeability/conductance

Table 1 Numerical scores assigned to water level below land surface (b.l.s.) and to soils according their thickness and lithology

Water-table level b.l.s. (m)						Total thickness (m) and lithology of poorly permeable layer (a, b, c)											
						<2			2–4			4–6			6–8		
<5	10–20	20–30	30–40	>40		(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
1	2	3	4	5		1	1	2	2	3	4	3	4	6	4	6	8
Total thickness (m) and lithology of poorly permeable layer (a, b, c)																	
8–10 m			10–12 m			12–14 m			14–16 m			16–18 m			18–20 m		
(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
5	7	10	6	9	12	7	10	14	8	12	16	9	13	18	10	15	20
															12	18	21

Table 2 Classification of water-table aquifer by vulnerability degree

Category	Total numerical scores	Description
I	<5	Highly vulnerable
II	5–10	Vulnerable
III	10–15	Slowly protected
IV	15–20	Protected
V	20–25	Well protected
VI	>25	Highly protected

Table 1 presents numerical scores assigned to depth to water table and to poorly permeable soils in aeration zone based on their thickness.

As can be seen from the table high scores are assigned to poorly permeable soils in contrast to water level scores. Category of water-table aquifer vulnerability is being derived by summing of numerical scores of Table 1. Water-table aquifers vulnerability is assessed by six categories where first category is assessed as highly vulnerable and sixth category as highly protected, see Table 2:

Quantitative assessment of water-table aquifers vulnerability is being carried out when there is risk of bacteriological infection. This method is based on groundwater downward time-of-travel (TOT) during which percolated bacteria or polluted water may reach to water table. Percolation time is defined by formula

$$t_0 = \frac{M_0 * \pi_0}{K_0} \tag{1}$$

where

t_0 – percolation time, days

M_0 – thickness of aeration zone, m

π_0 – porosity of soils in aeration zone, m

K_0 – permeability (conductance) of soils in aeration zone, m

Table 3 Evaluation of water-table aquifer vulnerability by bacteriological contamination

Category	Time of travel (days)	Vulnerability
I	<10	Highly vulnerable
II	10–50	Vulnerable
III	50–100	Slowly protected
IV	100–200	Protected
V	200–400	Well protected
VI	>400	Highly protected

Period of vital functions of bacteria under slow contamination is taken 200 days, under mass infection (natural or man-caused disaster) or continuous contaminant spill conditions it is taken 400 days. Six categories of ground water vulnerability to bacteriological contamination have been distinguished (Table 3):

Quantitative assessment of confined aquifer vulnerability that is first from land surface is carried on the base of two parameters: total thickness of overlain soils that have slow infiltration rates (m_0) and by levels of water table (H_1) and established level of water in confined aquifer (H_2).

Three categories of confined aquifer vulnerability have been singled out.

I – protected ($m_0 > 10$ m, $H_2 > H_1$)

II – relatively protected (m_0 5–10 m, $H_2 > H_1$ or $m_0 > 10$ m; $H_2 \leq H_1$)

III – vulnerable ($m_0 \leq 5$ m; $H_2 \leq H_1$).

Results

Because ground water body is of confined-unconfined nature, the higher risk of contamination is assessed in locations where unconfined conditions.

Only three wells out of 15 has encountered water-table horizons (#1, 3, 11), and vulnerability assessment of unconfined conditions has been made by numerical scores and TOT (Tables 4 and 5).

Vulnerability assessment from bacteriological contamination is given in Table 5. The water-table aquifer is evaluated as slowly protected (TOT = 50–100 days) and protected (100–200 days).

Intrinsic vulnerability of confined aquifer is given in Table 6. Because of considerable thickness of overlain strata confined aquifer in the territory of boreholes is evaluated as protected and corresponds to I category of vulnerability.

Yerevan water supply aqueduct feed only groundwater bodies related to fractured basalts, and analyses of wells cross-sections indicate that water body in the location of wells #3, 11 are not overlaid by impermeable strata. And it is strongly recommended to limit economic activities in these locations.

Table 4 Water-table aquifer intrinsic vulnerability assessment by numerical scores

Borehole number	Thickness of aeration zone (m)				Ground water level, m	Evaluation of numerical scores			On water table level	Aquifer vulnerability		
	Total	Soils with low infiltration rates by indexes				On indexes of soils with low infiltration rates (see Table.1: a, b, c)				Total	On categories	Vulnerability
		(a)	(b)	(c)		(a)	(b)	(c)				
1	10	8	2	-	10	4	3		2	9	II	Vulnerable
3	39	15			39	8			4	12	III	Slowly protected
11	43	6			43	4			5	9	II	Vulnerable

Table 5 Water-table aquifer natural vulnerability assessment from bacteriological contamination

Borehole number	Thickness of low permeable soils of aeration zone m_0 (m)	Permeability K_0 (m/days)	Porosity n_0	Vertical percolation time t_0 (days)	Aquifer vulnerability	
					On categories	Evaluation
1	10	0.02	0.3	150	IV	Protected
3	15	0.03	0.3	150	IV	Protected
11	6	0.02	0.3	90	III	Slowly protected

Table 6 Confined aquifer natural vulnerability assessment

Borehole number	Overlaid thickness m_0 (m)	Water table level H_1 (m)	Established level of water of confined aquifer H_2 (m)	Parameters	Vulnerability category	Evaluation
1	44	10	43.5	$m_0 > 10$ m $H_2 < H_1$	II	Relatively protected
2	18	–	8.6	$m_0 > 10$ m	I	Protected
4	161	–	45.78	$m_0 > 10$ m	I	Protected
5	51.5	–	49.44	$m_0 > 10$ m	I	Protected
7	16	–	13.38	$m_0 > 10$ m	I	Protected
8	53	–	51.9	$m_0 > 10$ m	I	Protected
10	35	–	31.5	$m_0 > 10$ m	I	Protected
12	35.6	–	33.26	$m_0 > 10$ m	I	Protected
13	18.7	–	11.4	$m_0 > 10$ m	I	Protected

References

- Bochever FM (1979) Groundwater conservation from contamination. Nedra Press, Moscow, pp 156–174
- Goldberg VM (1988) Methodical recommendations for revealing and assessment of ground water contamination. VSEGINGEO Press, Moscow
- Grozдова OI (1979) Mapping and regional predictions of man-caused modifications of underground hydrosphere, hydrogeology and engineering geology. VIEMS Press, Moscow
- Ross M et al. (2004) 3D geologic framework models for regional hydrogeology and land use management. Hydrogeol J, vol 1, pp 7–14
- PNIS M (1970) On results of ground water prospecting in location of Katnaghbur springs for Yerevan water supply, vol 2, pp 13–14, vol 1, p 161 (Report of Geological Survey)

Part II

Bacteria Diagnostic Tests

Methods for Detection of Shiga-Toxin Producing *Escherichia coli* (STEC)

Jordan Madic

Abstract Shiga-toxin producing *Escherichia coli* (STEC) also referred to as Verocytotoxic *E. coli* (VTEC) are currently considered as important emerging food-borne bacterial pathogens of public health concern. STEC strains are causes of sporadic and epidemic human cases of hemorrhagic colitis (HC), which can lead to severe and life threatening hemolytic and uremic syndrom (HUS), especially in young infants. The major part of cases are associated with ingestion of food or water contaminated with STEC strains belonging to the serotype O157:H7. However, more than 200 different serotypes were found in association with HC or SHU in humans. The presence of STEC in low amounts in contaminated matrix and the absence of a unique marker shared by all the pathogenic STEC make their detection very laborious. A wide panel of detection methods are now available, ranging from conventional methods based on phenotypic and biochemical properties, immunological methods, to most recent DNA-based techniques. In this review, different diagnostic procedures are described, including commercially available kits for food industry and human medicine. Recent strategies for the detection of the major serotypes associated with human disease are also discussed, and their drawbacks and advantages are detailed.

Keywords Shiga-toxin producing *Escherichia coli* (STEC) • detection • biochemical methods • immunological methods • DNA-based methods

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Introduction

Escherichia coli is a Gram negative, catalase positive, oxydase negative, nitrate reductase positive, indol positive and facultative anaerobic bacillus. This micro-organism is a natural host of the gut microflora of Human and various warm-blooded animal species. It can comprise up to 1% of the gastrointestinal population of mammals. *E. coli* are serotyped on the basis of their O (somatic) and H (flagellar) surface antigen profile. More than 170 O antigens have been found, each defining a serogroup (Nataro and Kaper 1998). *E. coli* is used as an indicator of environmental fecal contamination of water supplies (Callaway et al. 2009). Some strains of *E. coli* are responsible for infantile gastro-enteritis and diarrhea. The different pathotypes of *E. coli* include intestinal pathotypes such as enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffusely adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EaggEC or AAEC), enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) (Nataro and Kaper 1998).

In 1982 in the USA, two outbreaks of severe colitis were associated with eating of undercooked meat contaminated by *E. coli* O157:H7 serotype (Riley et al. 1983). Strains of this serotype were found to produce a toxin similar to that of *Shigella dysenteriae*: the shiga-toxin also called verotoxin due to its cytotoxic activity on Vero cells (Karmali et al. 1983).

E. coli strains producing this toxin are called Shiga toxin (Stx)-producing *E. coli* (STEC). Enterohaemorrhagic *E. coli* (EHEC) constitute a subset of STEC strains associated with severe clinical symptoms. These symptoms range from mild diarrhoea to haemorrhagic colitis (HC) and may be complicated by haemolytic-uraemic syndrome (HUS), the main cause of acute renal failure in children (Paton and Paton, 1998b). Since 1982, numerous cases of outbreaks and individual cases were reported all over the world (Deisingh and Thompson 2004) and STEC are now considered as an important emergent food borne pathogen.

Domestic ruminants, especially cattle, are a major reservoir of STEC whose transmission to humans occurs through consumption of undercooked ground beef, water, or dairy products contaminated by bovine feces (Besser et al. 1999) or via direct contact from infected persons or animals.

E. coli O157:H7 is the principal *E. coli* serotype implicated in human pathology and the most commonly identified STEC serotype in clinical cases worldwide. However, increasing numbers of human STEC infections are being caused by other serotypes. In Europe, over the period 2002–2006, non-O157:H7 serotypes accounted for 34% of all human STEC infections where serotypes were established (Anonymous 2007). Although a large range of O:H serotypes have been implicated in human STEC infections, the vast majority of sporadic cases and outbreaks of HC and HUS are caused by a limited number of serotypes including O157:H7, O26:H11, O103:H2, O111:H8, O145:H28 and their non-motile derivatives. In the classification of STEC strains by Karmali et al. (2003), these five serotypes have been categorized in seropathotypes (A and B) (showing the

highest incidence in human infections and association with serious and epidemic cases).

Shiga toxins, the main virulence factors contributing to pathogenicity, consist of two major types termed Stx1 and Stx2, each comprising several variants of both types. Shiga toxins are encoded by lysogenic bacteriophages and target globotriosyl ceramide (Gb₃) receptors at the surface of endothelial cells of various organs, including kidneys, intestine and brain (Lingwood et al. 1987). An exception is Stx2_e (the pig edema disease toxin) which targets the globotetraosylceramide (Gb₄) receptor present in pig (DeGrandis et al. 1989). In addition to Stx, typical EHEC strains carry in their chromosome the locus of enterocyte effacement (LEE), a pathogenicity island responsible for the production of attachment and effacement (A/E) lesions (Nataro and Kaper 1998). This locus contains the *eae* gene encoding an outer membrane adhesin, the intimin, which mediates close contact between EHEC and intestinal epithelial cells. This locus is also found in enteropathogenic *E. coli* (EPEC), a predominant cause of infant diarrhoea in developing countries (Yu and Kaper 1992). Considerable heterogeneity has been identified among the DNA sequences of the *eae* genes, especially in their 3' end. To date, at least 18 types and nine subtypes have been described in the literature. The gamma subtype is commonly found in EHEC strains of serotypes O157:H7 and O145:H-, whereas beta, epsilon and theta subtypes are commonly detected in EHEC strains of serotypes O26:H11, O103:H2 and O111:H-, respectively (Ito et al. 2007).

Because of the low infectious dose of STEC, laboratory diagnosis of STEC in foodstuffs is becoming of great importance. It's essential to dispose of rapid and reliable methods to detect STEC presence in a suspected sample and to eventually get back to the source of contamination. STEC detection methods are mainly applied in hospital for stool samples analysis from patients suffering from HC or HUS and in food industry. The latter is very demanding in methods to guarantee the safety of their products. This paper will review the principal methods for STEC detection and will discuss their advantages and their drawbacks.

Matrices and Enrichment Protocols

Isolation of STEC from complex matrices is challenging because animal feces, environmental and food samples may contain very low numbers of STEC. In addition, STEC cells may be in an injured or stressed physiological state as a result of exposure to food processing adverse conditions (e.g. low pH, low temperature, high NaCl concentration, etc...) or the presence of antibiotics in clinical specimens. Complex matrices may also contain very high levels of background microflora and natural inhibitors which interfere with detection of the pathogen. Given the low infectious dose of STEC and the sensitivity of the available detection and isolation methods, an enrichment step is always

performed in order to increase the cell numbers of the pathogen to a detectable level and to allow recovery of injured cells. The efficiency of this step rely on three parameters: (i) the temperature, (ii) the length of incubation and (iii) the the broth composition. While growth of STEC is usually carried out at 37°C, some authors also incubate the broth at 41°C (Vimont et al. 2006). The length of incubation is also variable, and may vary depending on the protocol and the matrice enriched, ranging from 6 to 24 h. Possé et al. (2008a) performed an enrichment method including a pre-enrichment phase at 42°C and an enrichment at 37°C. The two most employed enrichment media for *E. coli* O157:H7 and other STEC serogroups are modified trypton soy broth (mTSB) and *E. coli* broth (EC). These media may be supplemented with various selective agents such as bile salts, potassium tellurite and antibiotics among which novobiocin is the most commonly used (Vimont et al. 2006). However, the use of antibiotics may interfere with STEC growth and concentration of these additive has to be carefully chosen (Foster et al. 2003; Vimont et al. 2006).

The International Standard Organization (ISO) reference method for isolation of *E. coli* O157 (ISO 16654) from food recommends an enrichment in mTSB supplemented with novobiocin at 41.5°C for a total period of 18–24 h. In contrast, there is no standard method for enrichment of non-O157 *E. coli*, though a number of enrichment protocols have been reported for the isolation of a wide range of STEC or the recovery of some serogroups (Table 1).

Table 1 Enrichment media used for STEC detection

Enrichment media	Group/Serogroup	References
Non-selective enrichment media		
Trypton Soy Broth (TSB)	STEC	
Modified TSB	STEC	
Buffered Pepton Water	STEC	
<i>E. coli</i> Broth	STEC	
Selective enrichment media		
TSB + Vancomycin + Potassium Tellurite	O111	Catarama et al. 2003
TSB + Vancomycin + Cefixime + Potassium Tellurite	O26	
TSB + Novobiocin + vancomycin + Rifampicine + Bile salts + Potassium Tellurite	O26, O103, O111, O145, O157	Possé et al., 2008b
Modified TSB + Novobiocin	O157	ISO 16654
Modified TSB + Acriflavin	O157	AFNOR 12/08-07/00
Modified TSB + Cefixime + Cefsulodin + Vancomycin	O157, O26	Hepburn et al. 2002
Modified <i>E. coli</i> Broth + Novobiocin	O157, STEC	Perelle et al. 2007
Buffered Peptone Water + Vancomycin + Cefsulodin + Cefixime	O157	Foster et al. 2003
Buffered Pepton Water + Vancomycin	O26, O111	Drysdale et al. 2004

Phenotypical Methods

Detection of Shiga Toxin Production

Shiga toxins were initially discovered by Konowalchuk et al. (1977), who first observed their irreversible cytopathic effect on Vero cells monolayer and other cell lines in culture. Since Vero cells have a high concentration of Gb₃ and Gb₄ (target receptor for Stx_{2e}), they can be used to detect all Stx variants (Paton and Paton 1998b). But other cell lines can be employed to assess Stx cytotoxicity, such as Hela cells (O'Brien et al. 1982). This cell line however lacks Gb₄ and is therefore less sensitive to Stx_{2e} (Paton and Paton 1998b). The Vero cells assay (VCA) is the reference method for the detection of Stxs and is considered as the “gold standard” to which other methods should be compared for validation. However, several constraints have limited its routine use in diagnostic laboratory settings among which cost and labour-intensive maintenance of tissue cultures and time needed to obtain the results are significant. The Vero cells assay is performed by addition of STEC cell-free supernatants to tissue culture monolayers of Vero cells. In the presence of Stxs, Vero cells take a round shape and disconnect from one another. The degree of cytotoxicity can be estimated at 24 and 48 h with the help of a microscope. This method allows food and fecal sample enrichments as well as cell culture supernatants to be tested for the presence of Shiga toxins and can also differentiate between the toxin types 1 or 2 (Scotland et al. 1985). Several studies have shown that the VCA is highly sensitive (Rahn et al. 1996). However, since STEC strains may be present at very low levels and since some strains may display various degree of Stx production, low or moderate Stxs-producers could be sorely detectable by VCA. To increase the sensitivity of this method, Karmali et al. (1985) treated fecal extracts with polymyxin B to release cell-associated Stx and could reliably detect STEC at a low concentration.

However, the presence of cytotoxicity in the extract could be due to other toxins or bacterial products, it is necessary to perform a neutralization assay with specific antisera to confirm that the cytopathic effect is due to the production of Shiga toxins. Moreover, in absence of neutralization assay, VCA lacks specificity (Rahn et al. 1996). Therefore, the Vero Cells assay have been largely supplanted by immunologically-based and DNA-based method for the detection of STEC, but is still used for confirmation of Shiga toxin production from pure cultures.

Detection of Specific Serotypes

Phenotypic Properties of STEC O157

E. coli O157:H7 has the same phenotypic properties as the majority of *E. coli* species with the exception of sorbitol fermentation and β -D-glucuronidase activity. Unlike 95% of *E. coli* strains, O157:H7 strains do not ferment sorbitol in 2

days and are β -D-glucuronidase negative (Farmer and Davis, 1985, Thompson et al. 1990). Nevertheless, several studies have reported sorbitol-fermenting (SOR+) *E. coli* O157:H7 and O157:H- (Karch and Bielaszewska 2001; Feng et al. 2007; Rosser et al. 2008). Moreover, phenotypically atypical STEC O157 producing β -D-glucuronidase (GUD+) have been isolated from human outbreaks and sporadic cases in Germany, USA and in Japan (Nagano et al. 2004). The German isolates were also able to ferment sorbitol within 24 h. Phenotypic and molecular characterization of the German SOR+ GUD+ STEC O157:H- strains revealed that they represented a distinct clone within the *E. coli* O157 complex (Karch et al. 1993). The prevalence of those phenotypically atypical STEC O157 is still unknown.

Detection of STEC O157

The particular biochemical properties of most *E. coli* O157 led to the development of a specific agar for the routine detection and isolation. MacConkey Agar is a selective medium that inhibits the growth of Gram-positive bacteria due to the presence of crystal violet and bile salts. By using the lactose present in this agar, lactose-fermenting bacteria such as enterobacteriaceae appear as pink colonies. To enhance the selectivity of MacConkey Agar for *E. coli* O157:H7, March and Ratnam (1986) have added sorbitol instead of lactose to this medium. They detected *E. coli* O157:H7 in stool samples from patients with bloody diarrhea with a sensitivity of 100% and a specificity of 85%. Okrend et al. (1990) have improved the selectivity of SMAC medium by the addition of 0.1 g l⁻¹ 5 bromo-4 chloro-indoxy- β -D-glucuronide (BCIG). This approach improved the isolation of STEC O157 in artificially inoculated bovine ground beef. BCIG allows the differentiation between β -D-glucuronidase positive colonies from those which are β -D-glucuronidase negative. The enzyme β -glucuronidase splits the bond between the β -D-glucuronide and the chromophore 5-bromo-4-chloro-3-indolyle, which is released and colours the cells. Sorbitol-negative and β -D-glucuronidase negative *E. coli* O157 colonies appears white while sorbitol-negative and β -D-glucuronidase positive colonies appears blue or green.

Organisms such as *E. coli* from other serogroups and *Proteus* may not ferment sorbitol and thus may be confused with *E. coli* O157 on SMAC Medium (Chapman et al. 1991). To further improve the selectivity of this medium, Chapman et al. (1991) added rhamnose and cefixime. Rhamnose is not fermented by STEC O157, unlike most sorbitol non-fermenting *E. coli* of other serogroups and cefixime, a third generation cephalosporine, is more effective against *Proteus spp.* than *E. coli*. The major drawback of this method is that rhamnose is an expensive supplement. An alternative modification has been assessed by Zadik et al. (1993) who added potassium tellurite and cefixime in SMAC medium. Minimum inhibitory concentration were higher for STEC O157 than for other strains of *E. coli* and for *Aeromonas spp.* Thus, SMAC medium containing potassium tellurite and cefixime inhibited the growth of 67% of non-STEC O157 *E. coli* and all or most strains of

other sorbitol-non-fermenting bacterial species tested. This agar was tested on cattle rectal swabs and has increased the rate of STEC O157 isolation. Cefixime Tellurite Sorbitol MacConkey Agar (CT-SMAC) is now the media of choice in the ISO standard protocol (ISO 16654) for *E. coli* O157 although this medium has been reported to fail to isolate two sorbitol-fermenting STEC O157:H- on HUS patients (Karch et al. 1996).

Fluorogenic revelation for the detection of STEC O157 has also been tested (Thompson et al. 1990). The substrate 4-méthylumbelliferyl β -D-glucuronide (MUG) is cleaved by β -D-glucuronidase and provides a fluorescent end product, methylumbelliferone. When Thompson et al. (1990) evaluated it on bacterial strains isolated from human cases, very few organisms other than *E. coli* and 92.4% of *E. coli* strains were found MUG-positive whereas 88.3% of *E. coli* O157 were MUG-negative. The remaining 11.7% MUG-positive *E. coli* O157 did not produce Shiga toxins.

Beside Mac Conkey Agar and derivatives, several new agar (Table 2), based on the phenotypic properties of *E. coli* O157:H7, have also been developed to facilitate the isolation of STEC of this serotype e.g. by reducing the growth of other sorbitol-fermenting and non-sorbitol-fermenting organisms. Among these, Rainbow agar O157 (Bettelheim 1998a), CHROMagar O157 (Bettelheim 1998b) and ChromID O157:H7 (Bettelheim 2005) can be cited.

However, the absence of detection of phenotypically atypical STEC O157 with media based on sorbitol-fermenting profile and β -D-glucuronidase activity still remains a problem.

Table 2 Selective solid media for the detection of STEC serotypes

Selective Agar	Serogroup(s)
Sorbitol McConkey Agar (SMAC)	O157
SMAC + Potassium tellurite + cefixime (CT-SMAC)	O157
5-Bromo-4-Chloro-3-Indolyl- β -D-glucuronide Agar (BCIG)	O157
SMAC-BCIG	O157
SMAC-BCIG + Potassium tellurite + cefixime (SMAC-BCIG-CT)	O157
4-Methylumbelliferyl- β -D-glucuronide (MUG)	O157
Lactose Monensin Glucuronate Agar (LMG)	O157
Enterohemolysin Agar	STEC
Enterohemolysin Agar + Vancomycin + cefixime + Cefsulodin	STEC
Phenol Red Sorbitol Agar + MUG (PRS-MUG)	O157
FluorocultT® <i>E.coli</i> O157 Agar	O157
CHROMagar O157	O157
Biosynth Culture Medium® O157	O157
O157-ID Agar	O157
Rainbow Agar® O157	O157, O26, O111, O48
Rhamnose McConkey Agar (RMAC)	O26
RMA + Cefixime	O157
RMAC + Potassium tellurite + cefixime (CT-RMAC)	O26

Detection of Non-O157 STEC

Although most of the commercially available agars for STEC detection mainly focus on the identification of *E. coli* O157 serotype, some attempts have been made to develop new agar for the selective detection and isolation of other major pathogenic STEC serogroups such as O26, O103, O111 and O145. Table 2 lists most of them.

Non-O157 STEC strains display a heterogeneous range of phenotypic properties making it difficult to find a common medium that selectively and differentially will recover these pathogens. These strains, as well as sorbitol-fermenting O157 strains, are not detected on SMAC or CT-SMAC medium. Hiramatsu et al. (2002) have demonstrated the inability of the majority of STEC O26 strains to utilize rhamnose and to have a high tolerance for potassium tellurite. Accordingly, they have developed a rhamnose-MacConkey agar (RMAC) and a cefixime-tellurite rhamnose-MacConkey agar for the selective isolation of STEC O26. All of the STEC O26 strains generated colorless colonies on both media while potassium tellurite and cefixime effectively inhibited the growth of bacteria other than STEC O26. Both the RMAC and the CT-RMAC has better specificity for the routine isolation for STEC O26 in laboratory but CT-RMAC works better for the isolation from stool samples.

Recent researches led by Possé et al. (2008a) aimed to develop differential media based on 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) as a chromogenic compound to signal β -D-galactosidase activity. This chromogenic marker and a mixture of selected carbohydrates were combined with a pH indicator, which allow a colour-based identification of STEC serotypes fermentation profile, and several inhibitory components. Two sets of differential selective media were developed: the first set contained sucrose, sorbose and X-gal and allows phenotypical differentiation of O26, O103, O111 and O145 STEC serotypes. STEC O26 strains were found to be sucrose and sorbose fermenters while STEC O103 and STEC O111 strains only fermented sucrose. STEC O145 strains did not ferment any of these carbohydrates. The second set contained sorbitol and X-gal and targeted sorbitol-positive and sorbitol-negative O157 STEC serotypes. Suspected colonies obtained on each set of differential media should be plated onto confirmation media which are also based on the fermentation profile of the targeted STEC serotypes. Confirmation media contain, in addition to the pH indicator phenol red, a combination of carbohydrates among the following: dulcitol, rhamnose, arabinose and raffinose or rhamnose alone according to the strains that has to be confirmed. Dairy products, meat and cattle faeces were artificially contaminated using clinical STEC strains to evaluate both sets of differential and confirmation media (Possé et al., 2008b). The procedure resulted in confirmed isolates for 100% of the raw milk samples, 86.8% of the cheeses made from raw milk, 67.2% in ground meat and 28.9% in cattle faeces. Further tests of specificity in ground beef are currently performed through an international inter-laboratory study assay.

An alternative method to isolate non-O157 STEC strains is based on enterohemolysin agar. Beutin et al. (1989) have found an association between enterohemolysin and Stx production in 89% of *E. coli* strains belonging to nine different

serotypes. Enterohemolysin activity can be detected on blood agar containing washed sheep erythrocytes supplemented with calcium after overnight incubation. Isolated colonies showing an enterohemolysin activity must be next confirmed to be *stx*-positive since some enterohemolysin-producing non STEC strains can also be detected. However, sorbitol-fermenting O157 and non-O157 STEC strains that do not produce enterohemolysin will not be detected on enterohemolysin agar (Bielaszewska et al. 1998).

Immunological Methods

Immunological detection has become a widely used approach to detect STEC in various matrices, including mostly foodstuffs. Systems using this approach are generally applied to enriched broth after an overnight incubation and use antibodies against Stx toxins or the O-antigens, allowing in the latter case a serogroup-based detection. Immunological methods are generally reliable and allow a sensitive and specific detection. Most assays are easy to implement in laboratories and do not require expensive elements. Various commercialized kits have been developed.

Detection of E. coli O157 and O157:H7

As *E. coli* O157:H7 is the most implicated STEC serotype in human cases, numerous immuno-based methods for the detection of this specific serotype have been documented. They include conventional Enzyme Linked Immuno Sorbent Assays (ELISA) in microplates, one-step immuno-detection systems, fully automated systems and various non enzymatic immunological based systems like Rapid Plate Latex Agglutination (RPLA), Immuno-Magnetic Separation (IMS) or immuno-chromatography (Table 3).

Many systems for the detection of *E. coli* O157:H7 using Enzyme Linked Immunosorbent Assay (ELISA) have been reported. They generally involve the capture of a STEC-specific antigen between an immobilized antibody and a capture antibody. The detection is performed either by the capture antibody which is linked to an enzyme, or by a secondary enzyme-linked antibody targeting the capture antibody. Then, by adding the enzymatic substrate, a visible signal proportional to the amount of antigen is produced. The sensitivity of these tests is generally 10^5 *E. coli* O157 per mL of enriched broth (AFSSA 2003).

A fully automated system derived from ELISA method has been evaluated by Vernozy-Rozand et al. (1998), tested by the Association of Agricultural Chemists (AOAC) and validated by the French national organization for standardization (AFNOR). This system, the VIDAS *E. coli* O157 (BioMerieux, France) is an Enzyme Linked Fluorescent Assay (ELFA) using two ready-to-use components :

Table 3 Commercialized immunological methods for the detection and isolation of *E. coli* O157:H7 in food. (Vernozy-Rozand et al., 2002)

TEST	Target antigen	Revelation format	Format	Validation
EHEK-Tek (Organon Teknika)	O157 and H7	Colorimetric ELISA	Microplate	Health Canada Method MRLP-91
Tecra <i>E. coli</i> O157 VIA (Bioenterprises Pty Ltd)	O157	Colorimetric ELISA	Microplate	
Assurance EHEC EIA (BioControl Systems Inc.)	O157 and H7	Colorimetric ELISA	Microplate	AOAC Official Method 996.10
PATH-STIK <i>E. coli</i> O157 (Celsis-Lumac)	O157	Colorimetric Immunochromatographic	Dip-stick	
VIP EHEC (BioControl Systems Inc.)	O157 and H7	Colorimetric Immunoprecipitation	1 step	AOAC Official Method 996.09
Transia card <i>E. coli</i> O157 (Diffchamb)	O157	Colorimetric Immunochromatographic	1 step	
REVEAL <i>E. coli</i> O157:H7 (Neogen)	O157 and H7	Colorimetric Immunoassay	1 step	
ImmunoCard Stat! <i>E. coli</i> O157:H7 (Meridian Diagnostics)	O157 and H7	Colorimetric Immunochromatographic	1 step	AOAC Performance Tested
Biocard EHEC (ANI Biotech OY)	O157	Colorimetric Immunochromatographic	1 step	
Singlepath <i>E. coli</i> O157:H7 (Merck Ltd)	O157 and H7	Colorimetric Immunochromatographic	1 step	
VIDAS <i>E. coli</i> O157 (BioMérieux)	O157	Fluorescent ELFA	Automated	AFNOR 12/08-07/00
EiaFoss <i>E. coli</i> O157 (Foss Electric A/S)	O157	Fluorescent Immunoassay	Automated	
Dynabeads anti <i>E. coli</i> O157 (Dynal Ltd)	O157	–	Magnetic beads	AFNOR DYN 16/2-0696. ISO EN 16654
<i>E. coli</i> O157 Immunocapture system (Tecra Ltd)	O157	–	Dip-stick	
VIDAS Immunoconcentration <i>E. coli</i> O157 (ECO) (BioMérieux)	O157	–	Automated	AFNOR 12/08-07/00

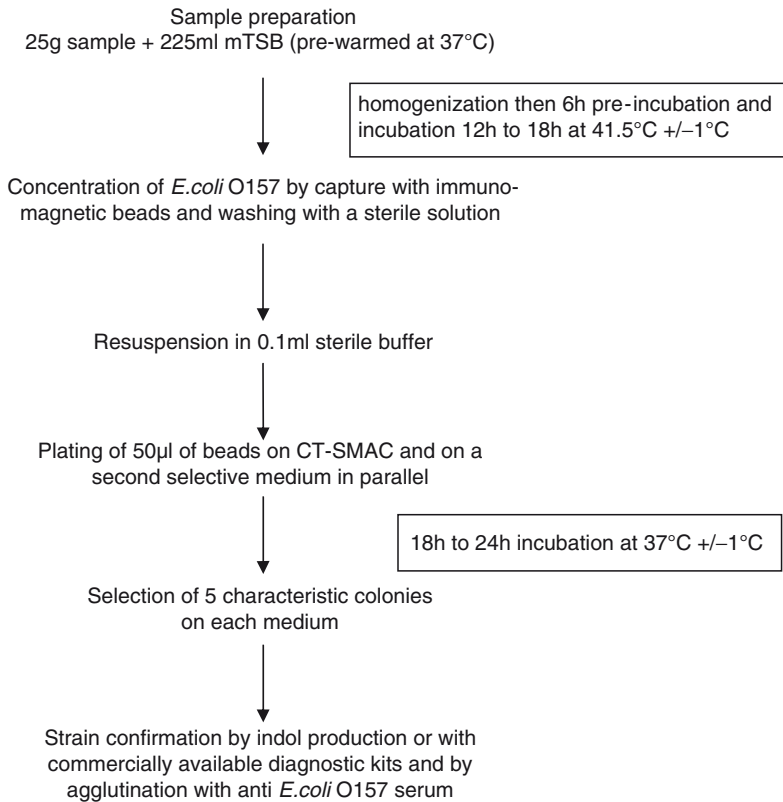


Fig. 1 IMS protocol for the recovery of *E. coli* O157:H7 from food (ISO EN-16654) (AFSSA, 2003)

(i) disposable tips called Solid Phase Receptacle (SPR) internally coated with anti-O157 antibodies and (ii) a strip containing the reagents (washing and revealing solutions) required for the reaction. A 0.5 mL aliquot of enriched broth is placed in the strip and successively aspirated in and out the SPR. Finally, the presence of target antigen is revealed through a fluorogenic reaction in presence of alkaline phosphatase enzyme. The immuno-capture and recovery of *E. coli* O157 strains can also be performed by a variation of the VIDAS system, the VIDAS Immuno-concentration *E. coli* O157 (ECO) (BioMerieux, France).

New techniques based on immuno-chromatography uses colloidal gold-labeled monoclonal antibodies to detect *E. coli* O157:H7. These antibodies form complexes with *E. coli* O157:H7 as the test sample migrates over a membrane. Positive reactions show a colored band that is compared to a reference scale provided by the manufacturer. This technique enables a quick detection and was employed in the “ImmunoCard Stat! *E. coli* O157:H7” assay (Meridian Diagnostics). Mackenzie et al. (2000) evaluated this commercial kit on stool samples. All the 14 culture samples positive for *E. coli* O157:H7 were detected in contrast to the 263 culture-negative specimens that were not. The authors therefore concluded that the test was highly sensitive and specific.

The international reference method to detect and recover *E. coli* O157 (ISO EN 16654, Fig. 1) includes a step of Immuno-Magnetic Separation (IMS), which requires the use of magnetic beads coated with polyclonal antibodies against O157 antigen (Dynabeads anti *E. coli* O157, Dynal Ltd, Norway). The cell bead complexes are recovered from the medium by the application of a magnetic field causing the complexes to be concentrated in the tube, and enabling the bulk medium to be discarded. The concentrated cells are next plated onto CT-SMAC for strain recovery and confirmation. Chapman et al. (1994) found that IMS was 100-fold more sensitive for detection of *E. coli* O157:H7 than direct culture on isolation media.

It should be noted however that all the commercial kits described above enable the detection of the O157 antigen (associated in EHEC strains with the H7 flagellar antigen). A positive result only means that a strain carrying an O157 antigen is present in the sample. However, in some cases, *stx*-negative O157 strains or non-O157 strains are detected with these methods as cross reactions have been observed with the O157 polyclonal antiserum (e.g. with *Salmonella* group N, *Yersinia enterocolitica* serogroupe O9, *Brucella abortus*, *Brucella melitensis* and *Pseudomonas maltophilia* 555; AFSSA 2003). Isolation of the strains and confirmation for *E. coli*, serotype and presence of *stx* and other virulence factors are thus needed to conclude.

Detection of STEC Strains

Immunological detection of STEC strains are mainly based on Shiga-toxin production detection, in general after an enrichment step. Several ELISA have been described for the detection of Shiga-toxins. Some of them use monoclonal antibodies against Stx, while others are based on Shiga-toxins binding to glycolipids containing a terminal α -D-Gal-(1-4)-D-Gal, purified Gb₃, Lyso-Gb₃ and hydatid cyst fluid. In both assays, bound toxin is then detected using monoclonal or polyclonal antiserum against Shiga-toxin, revealed by alkaline phosphatase labeled antiserum and enzyme substrate. In general these methods have not proved to be as sensitive as the Vero cell assay. In addition, as the toxins shows variations in their antigenicity and binding properties, detection of all Stx is not guaranteed (Smith and Scotland 1993). Commercial ELISA kits include the “Premier EHEC” targeting Shiga-toxins (Meridian Diagnostics Inc., USA) which was evaluated in stools by Kehl et al. (1997) and by Mackenzie et al. (1998) along with the “Premier *E. coli* O157” kit. This test is performed in microplate directly from stools samples or after an enrichment protocol. It was found to have a sensitivity and a specificity of 100% and 99.7%, respectively, whereas the sorbitol macConkey method had a sensitivity and a specificity of 60% and 100%, respectively. This test was found valuable for practical routine detection of STEC in several laboratories. The “Premier EHEC” was also compared by Willford et al. (2009) with two other commercially available ELISA kits, the Ridascreen Verotoxin Enzyme Immunoassay (rBiopharm, Germany) and The ProSpecT Shiga Toxin *E. coli* Microplate Assay (Remel Inc.). Both assays are performed in microplate from enriched sample. The specificities of the three tests were comparable, and the sensitivities of two of them were within the same order of magnitude. However, the ProSpecT Shiga Toxin *E. coli* Microplate Assay was approximately tenfold less sensitive. All

three tests were unable to detect the Stx2_d and Stx2_e variants thus indicating that some STEC strains may not be detected by Stx ELISA.

Reverse Passive Agglutination Assays (RPLA) use antibodies-coated beads which interact with Stx and produce a diffuse layer at the base of the reaction-wells. RPLA assays using antibodies against Shiga-toxin have been developed and commercialized. Karmali et al. (1999) have compared the VEROTOX-F assay (Denka Seiken, Japan) with the Vero cells assay and found a good sensitivity (0.7 ng ml⁻¹ for Stx1 and 0.6 ng ml⁻¹ for Stx2). This assay was able to detect Stx1, Stx2 and Stx2_e variants. The ability of the VTEC RPLA (Unipath Limited, UK) to detect and identify Stx1 and Stx2 extracted with polymyxin from a supernatant of pure culture of STEC was evaluated by Beutin et al. (1996). Human pathogenic STEC strains were reliably isolated and characterized for their Stx type within 72–96 h. Another kit, the STEC-Screen “Seiken” RPLA was also evaluated and compared with the Vero cell assay by Beutin et al. (2002). This test was accurate, rapid and easy to perform, thus being suitable for the routine screening of clinical stool specimens for STEC.

Immunochromatography-based methods using monoclonal antibodies to detect Shiga-toxins have been developed. These methods enable a quick detection, within 10 min for the Duopath verotoxin test (Merck, Germany) (Park et al. 2003). This test was compared to the Premier EHEC test, used as a gold standard, and demonstrated a sensitivity of 100%, showing no cross reactivity with other enteric organisms.

For the detection and the recovery of specific strains belonging to major STEC serogroups, IMS can be performed using magnetic beads coated with antibodies specific for O26, O111, O103 and O145 serogroups (commercially available; Dynal Ltd, Norway). However, strains recovered with this method must be confirmed for the presence of *stx* gene to be considered as STEC strains. This methods have not been ISO certified like the IMS for *E. coli* O157:H7, but have been employed to help recover STEC strains from food samples (Auvray et al. 2007) or animal faeces (Jenkins et al. 2003).

DNA-Based Methods

Besides phenotypical and immunological tests used in food industry, which may require important delays to perform an analysis, DNA-based methods represent a rapid alternative to these techniques. In addition to reducing the time needed to carry out the analysis, DNA-based methods may offer a substantial amount of advantages over phenotypical and immunological assays. In opposition to phenotypical assays, based on biochemical characteristics, they are less affected by the type of medium used, the emergence of mutants and the presence of bacteria having similar phenotypes (Bouvet and Vernozy-Rozand 2000). They may be also less affected by specificity compared to immunological techniques where antibodies used may cross react with other bacteria leading to false positives. However, careful design of DNA-based assays must be achieved in order to give advantages on “traditional” techniques.

Detection of EHEC-associated markers is performed either directly from the whole genome (DNA-hybridization) or indirectly after amplification by Polymerase Chain Reaction (PCR) of specific gene fragments.

DNA Hybridization and Oligonucleotide Probes

DNA hybridization assays rely on the properties of single strand DNA to hybridize with complementary sequences. They also use the property of DNA to bind to nitrocellulose membranes (Bouvet and Vernozy-Rozand 2000). The availability of cloned *stx1* and *stx2* genes allow the development of DNA probes for the detection of STEC. Initially, probes labeled with ^{32}P or ^{35}S were used to screen a large number of *E. coli* fecal isolates by colony hybridization. These techniques were specific and sensitive and could differentiate between *stx1* and *stx2* if stringent conditions were used.

Karch and Meyer (1989) designed four synthetic oligonucleotide probes representing four different regions of the *stx1* gene and one oligonucleotide derived from the *stx2* gene of an *E. coli* O157:H7 strain. Using colony hybridization assay, these probes detected 50 Stx-producing isolates and did not hybridize with 416 nontoxigenic *E. coli* strains. Stx1-producing *E. coli* and *E. coli* O157:H7 strains producing both Stx1 and Stx2 were detected with all four probes, suggesting a high homology between the *stx1* region selected and the probes. Other probes were directed against less highly conserved regions: synthetic oligonucleotide probes homologous to the nucleotide sequence coding for the A subunit of Stx1 and the B subunit of Stx2 have been designed by Brown et al. (1989). By modifying the hybridization and the washing conditions, probes were able to differentiate between *stx1*, *stx2*, and *stx2e* genes.

Feng et al. (1993) developed a PF-27 oligonucleotide probe specific for the β -glucuronidase encoding gene *uidA* for the detection of *E. coli* O157:H7. This gene shows a specific mutation in position 92 corresponding to the replacement of a thymine by a guanine in all the *E. coli* O157:H7 lacking β -glucuronidase activity. The PF-27 probe was tested on 253 bacterial isolates and only O157:H7 strains were detected with this method. Radioactive probes have disadvantages such as long exposure time, short half life of probes and safety problems associated with handling and disposal of radioisotopes (Paton and Paton, 1998b). These issues were resolved without any loss of specificity and sensitivity by using non radioactive probes labeled with digoxigenin or biotin and visualized by chromogenic or chemiluminescent enzymatic reactions) (Thomas et al. 1991). Although hybridization with DNA or oligonucleotide probes is not a particularly sensitive means of screening broth cultures or fecal extracts for the presence of STEC, it is a powerful tool for distinguishing isolated colonies containing *stx* genes from commensal organisms (Paton and Paton, 1998b).

PCR Based Methods

PCR Techniques and Detection Chemistries

In recent years, PCR-based assays have become very important as techniques for the detection of bacteria. Crude lysates or DNA extracts obtained from single colonies, as well as mixed broth cultures, colony sweeps, or even direct extracts of feces

or foods can be used as DNA templates for PCR (Paton and Paton, 1998b). In conventional PCR, the amplification products are usually detected by ethidium bromide staining after separation by agarose gel electrophoresis. However, several variations of standard PCR have appeared and assisted, among other things, in producing more rapid detection methods. Of these, multiplex PCR and real-time PCR are proving to be the most popular. Multiplex PCR allows several targets to be co-amplified simultaneously in the same reaction by combining several primer pairs (Deisingh and Thompson 2004) and real-time PCR uses fluorescence to monitor at each cycle the number of products or amplicons generated during the amplification. Monitoring of amplification can be achieved by several means such as the use of SYBR Green, a double-stranded DNA binding dye that detects PCR products as they accumulate during PCR cycles. SYBR Green dye chemistry will detect all double-stranded DNA, including non-specific reaction products. However, amplicons can be distinguished following analysis of the dissociation curve, whereby the melting temperature (T_m) of the PCR product is determined by the reduction in relative fluorescence as all double stranded DNA are denatured to their single-stranded form (Yoshitomi et al. 2006). The presence of two or more peaks indicates that more than one amplicon was produced and amplification was not specific. A more specific detection can be obtained by the use of specific fluorogenic labeled-probes which are designed to hybridize with the DNA fragment located between the forward and reverse PCR primers. To perform real-time multiplex PCR, these probes are labeled with dyes emitting at different wavelengths detected in separate specific channels by the real-time PCR instrument. Several types of fluorogenic hybridization probes have been described for the specific detection of PCR amplified products. Hybridization probes refer to a pair of oligonucleotides labeled with distinct fluorophores. The probe closer to the forward primer is labeled with a donor dye and the other one with an acceptor dye. When the two probes anneal with the template before the elongation step, the donor dye absorbs the energy from the light source of the instrument and transfers it to the dye from the other probe by fluorescence resonance energy transfer (FRET). The later emit a signal which is detected and recorded by the real-time PCR instrument (Dorak, 2006). Hydrolysis probes, or TaqMan probes, use the 5' nuclease activity of Taq DNA polymerase. They are labeled with two fluorophores, termed the reporter and the quencher at the 5' and at the 3' end respectively. While the probe is intact, the proximity of the quencher dye absorbs the fluorescence emitted by the reporter dye by FRET through space. If the target sequence is present, the probe anneals downstream from one of the primer binding site and is cleaved by the 5' nuclease activity of Taq DNA polymerase as this primer is extended. The cleavage separates the reporter dye from the quencher dye, liberating the reporter's energy and allowing detection of its fluorescence signal. Thus, the number of cleaved probes and the quantity of fluorescence accumulated is proportional to the amount of PCR products generated at each cycle. The use of dual-labeled probes is common to another system designed by Tyagi and Kramer (1996) and called molecular beacons. These are single-stranded oligonucleotides that form stem-loop ("hair-pin") structures in solution, keeping the reporter and quencher dyes in close contact,

and thus preventing fluorescence. The signal is restored when the probe hybridizes to its target nucleic acid between the primer binding sites and adopts a linear conformation, moving away the reporter dye from the quencher and generating fluorescence. Many other detection chemistries have been developed, and some of them use only labeled-primers that quench the fluorescent signal when the dye is in proximity to the double stranded stem formed by the primer in solution (hairpin primers) (Dorak 2006). The development of real-time PCR has greatly increased the specificity and speed of PCR-based detection methods. That is why conventional PCR which requires time-consuming detection of amplicons by gel electrophoresis is now gradually replaced by real-time PCR assays.

Target Genes

Although many genes such as colonization and virulence factors have been targeted using both conventional and real-time PCR, the majority of PCR tests for EHEC detection include one or both of the Shiga-toxin producing genes, *stx1* and *stx2* (Bouvet and Vernozy-Rozand 2000; Yoshitomi et al. 2006). Various oligonucleotide primers for the amplification by conventional PCR of *stx* genes have been designed. While *stx1* has a relatively homogeneous nucleotide sequence, several variants of *stx2* have been described. There is a large number of primer pairs available which detect most *stx2* variants in a single assay and others which discriminate between the variants. The first conventional PCR system was developed by Karch and Meyer (1989) who used a single pair of degenerated primers binding to a conserved region of *stx* genes to amplify both *stx1* and *stx2*. Amplicons are visualized on agarose gel after ethidium bromide staining and distinction between *stx1* and *stx2* is performed by southern blot with two hybridization probes complementary to a portion of the amplified sequences. Brian et al. (1992) designed two pairs of oligonucleotide primers to amplify *stx1* and *stx2* in a single reaction. Amplification product derived from 100 to 1000 bacteria was visible on agarose gel with ethidium bromide staining. After hybridization by Southern blot with *stx1* and *stx2* specific probes labeled with ^{32}P , amplification products obtained from DNA extracted from one to ten bacteria could be visible. In order to confirm the identity of amplified products, analysis with restriction fragment length polymorphism was also employed to differentiate *stx2* variants (Lin et al. 1993; Russmann et al. 1994; Read et al. (1992) have designed a pair of oligonucleotide primers targeting conserved sequences found in *stx1*, *stx2* and *stx2e* genes. A total of 223 STEC isolated from various food and feces and belonging to 50 different serotypes, 72 non-STEC and 76 other bacteria strains were tested. All STEC strains were detected and none of the other strains were positive with this PCR assay with the exception of *shigella dysenteriae* type 1 which is known to produce a toxin similar to Stx (O'Brien et al. 1982). The sensitivity of this method made it possible to detect one page of DNA, corresponding to 17 CFU. Chen et al. (1998) employed the same primer pair to perform a double step assay comprising (i) an asymmetric PCR overproducing one strand of the target and (ii) a semi nested PCR using a double stranded primer

labeled with a donor and an acceptor dye. During the second step, the fluorescent primer was dissociated and incorporated in the amplification product. The decrease of the fluorescent signal, proportional to the amount of primer incorporated, was monitored. In this study, the authors could detect 112 CFU of STEC par ml (equivalent to 8.6 CFU per PCR) of post-spiked ground meat.

The *rfbE* gene of the O157 antigen cluster was targeted by a primer pair in an assay described by Desmarchelier et al. (1998). The PCR detected less than 1 CFU of *E. coli* O157 per ml in raw milk following enrichment.

Various STEC-associated genes were used as targets for STEC detection such as *fliC*-H21 associated with STEC O91:H21 (Auvray et al. 2008), *eae* and *tir* variants, *katP*, *espP*, *espD*, *ehxA*, *etpD* and *saa* (Nielsen et al., 2003), which were detected by 5'nuclease real-time PCR assays. Primer pairs specific for the *fliC*-H7 gene were combined with other primers targeting the *stx1* and *stx2* genes and the *E. coli* O157:H7 *eae* gene in a multiplex conventional PCR assay by Gannon et al. (1997). Hu et al. (1999) have described a multiplex PCR containing five sets of primers which amplified specific regions of *eae* specific of O157, *stx1*, *stx2*, *fliC*-O157 and *rfbE*-O157 genes. This assay could successfully distinguish 49 *E. coli* O157:H7 from 33 non-O157:H7 and could detect all bovine feces samples seeded with six or more bacteria. Campbell et al. (2001) have tested this multiplex PCR in soil and water samples. They could detect 1 CFU ml⁻¹ of drinking water and 2 CFU g⁻¹ of soil in one day. Cebula et al. (1995) designed a conventional multiplex PCR targeting the *stx1* and *stx2* genes and a unique conserved single-nucleotide polymorphism (SNP) in the *uidA* gen of *E. coli* O157:H7. Jinneman et al. (2003) selected the same targets to design a multiplex 5'-nuclease real-time PCR assay but detected the *uidA* gene of *E. coli* O157:H7 with an hydrolysis probe labeled with a minor groove binding (MGB) molecule at its 3' ends. The MGB molecule, by increasing the affinity and lowering the T_m requirements of the probe was primarily used for SNP and allelic discrimination assays (Dorak 2006). Yoshitomi et al. (2003) designed a multiplex real-time PCR assay using SYBR Green dye which enabled the simultaneous amplification of *stx1*, *stx2* genes and the *uidA* gene of O157:H7 using three specific primer pairs. A double mismatch introduced into the 3' end of the forward primer reduces amplification of the non-O157:H7 *uidA* gene. As only a single mismatch to the O157:H7/H-*uidA* sequence occurs, amplification of this serotype is preferentially obtained. A real-time PCR assay using SYBR Green (O'Hanlon et al. 2004) was also developed targeting the somatic antigen-associated genes *per* and *wzy* of serogroups O157 and O111, respectively, and the flagellar-associated *fliA* and *fliC* genes of O26. Primer pairs and probes specific for the *rfbE* gene of O157, the *wzx* gene of O26, the *wbdI* gene of O111 serogroup O-antigen gene cluster as well as the *ihpI* gene of O145 serogroup and the *eae* gene of O103 were designed (Perelle et al. 2004) and combined in a multiplex real-time 5'nuclease PCR assay to screen a collection of food raw materials of bovine origin (Perelle et al. 2007).

Finally, reverse transcriptase (RT) PCR was employed for the detection of viable *E. coli* O157:H7. RT is a DNA polymerase which is capable of synthesizing single-stranded DNA from RNA in the 5'–3' direction. Yaron and Matthews (2002) have studied several genes (*rfbE*, *fliC*, *stx1*, *stx2*, *mobA*, *eae*, *hly* and 16S rRNA) as

indicator for viability of *E. coli* O157:H7 cells under typical growth conditions before and after thermal treatment. They found that *rfbE*, *stx1*, *hly* and 16S RNA amplicons were detected during all growth phase. But only the RT-PCR signal of 16S rRNA and *rfbE* was stable. After heating at 60°C for 20 min, all the mRNA species were degraded, except the rRNA. The authors suggested that the *rfbE* gene is the most appropriate target for the detection of viable *E. coli* O157:H7. However,

Table 4 Detection methods by PCR of STEC-associated genes

Target genes	Detection method	Reference
<i>stx1</i> , <i>stx2</i> , <i>eae</i>	SYBR Green real-time multiplex PCR	Chassagne et al. 2009
<i>fliC</i> -H21	Taqman real-time-PCR	Auvray et al. 2008
O157:H7 single nucleotides polymorphisms	SYBR Green real-time PCR, HairPin primers	Riordan et al. 2008
<i>stx</i> , <i>eae</i> , <i>wzx</i> -O26, <i>wzx</i> -O103, <i>wzx</i> -O111, <i>wzx</i> -O121, <i>wzx</i> -O145, <i>wzx</i> -O157	Conventionnal multiplex PCR	Monday et al. 2007
<i>stx1</i> , <i>stx2</i> , <i>uidA</i> O157	SYBR Green real-time multiplex PCR	Yoshitomi et al. 2006
O157, <i>stx</i> (Bellin et al. 2001)	SYBR Green real-time PCR	Heijnen and Medema 2006
Subtilase, <i>stx1</i> , <i>stx2</i> (Paton and Paton, 1998a)	Conventionnal multiplex PCR	Paton and Paton, 2005
<i>stx1</i> , <i>stx2</i> (Sharma et al.1999)	Taqman real-time-PCR	Sekse et al. 2005
<i>stx1</i> , <i>stx2</i> , <i>wzx</i> -O103, <i>wzy</i> -O103	multiplex PCR	Fratamico et al. 2005
<i>wzx</i> -O26, <i>eae</i> -O103, <i>wbdI</i> -O111, <i>ihp1</i> O145, <i>rfbE</i> -O157	Taqman real-time multiplex PCR	Perelle et al. 2004
<i>perO157</i> , <i>wzy</i> O111, <i>fliA</i> -O26, <i>fliC</i> -O26	SYBR Green real-time PCR	O'Hanlon et al. 2004
<i>eae</i> and <i>tir</i> variants, <i>katP</i> , <i>espP</i> , <i>espD</i> , <i>ehxA</i> , <i>etpD</i> , <i>saa</i>	Taqman real-time-PCR	Nielsen et al., 2003
<i>stx1</i> , <i>stx2</i> (Bellin et al. 2001)	FRET real-time-PCR	Pulz et al. 2003
<i>stx1</i> , <i>eae</i>	Taqman real-time multiplex PCR	Ibekwe and Grieve 2003
<i>stx1</i> , <i>stx2</i> (Pollard et al. 1990), <i>eae</i> O157:H7	Taqman real-time multiplex PCR	Ibekwe et al. 2002
<i>stx2</i>	Conventionnal PCR	Hamabata et al. 2002
<i>rfbE</i> , <i>fliC</i> , <i>stx1</i> , <i>stx2</i> , <i>mobA</i> , <i>eae</i> , <i>hly</i> , 16S rRNA	RT-PCR	Yaron and Matthews 2002
<i>stx1</i> , <i>stx2</i>	FRET multiplex real-time-PCR	Reischl et al. 2002
<i>eae</i> , <i>hly</i>	FRET multiplex real-time-PCR	

(continued)

Table 4 (continued)

Target genes	Detection method	Reference
<i>stx1, stx2</i>	Molecular beacons real-time-PCR	Belanger et al. 2002
<i>stx1, stx2</i>	SYBR Green real-time PCR	Jothikumar and Griffiths 2002
<i>stx1, stx2, eae, ehxA</i>	Conventionnal multiplex PCR	Paton and Paton 2002
<i>stx1, stx2, uidAO157</i>	Taqman real-time multiplex PCR	Jinneman et al. 2003
<i>stx1, stx2</i>	FRET multiplex real-time-PCR	Bellin et al. 2001
<i>stx1, stx2, eae</i> O157:H7	Taqman real-time multiplex PCR	Sharma et al. 1999
<i>eae</i> _{O157} , <i>stx1, stx2, fliC</i> -O157 and <i>rfbEO157</i>	Conventionnal multiplex PCR	Hu et al. 1999
<i>rfb-O157</i>	Conventionnal PCR	Desmarchelier et al. 1998
<i>stx1, stx2, stx2e</i> (Read et al. 1992)	Fluorescent PCR	Chen et al. 1998
<i>stx1, stx2, eae, hly, rfb</i> -O111, <i>rfb</i> -O157	Conventionnal multiplex PCR	Paton and Paton, 1998a
<i>fliC</i> -H7, <i>stx1, stx2, eae</i> O157	Conventionnal multiplex PCR	Gannon et al. 1997
<i>stx1, stx2, eae</i>	Conventionnal multiplex PCR	China et al. 1996
<i>stx1, stx2, uidAO157</i>	Conventionnal multiplex PCR	Cebula et al. 1995
<i>stx</i>	Conventionnal PCR	Paton et al. 1993
<i>stx1, stx2, stx2e</i>	Conventionnal PCR	Read et al. 1992
<i>stx1, stx2</i>	Conventionnal multiplex PCR	Brian et al. 1992
<i>stx1, stx2</i>	Conventionnal PCR	Pollard et al. 1990
<i>stx1, stx2</i>	Conventionnal PCR	Karch and Meyer 1989

further research are needed to lower the detection limit of this method as 10^7 CFU per PCR reaction are needed to achieve a positive detection without enrichment.

Several other conventional and real-time PCR systems targeting STEC-associated gene have been developed, Table 4 provides a non exhaustive list of these methods that can be found in the literature.

Limits of PCR Based Methods

Optimization of the sensitivity of PCR methods is of importance as the amount of STEC in fecal samples or foodstuffs may be very low. However, all of the

PCR assays performed on DNA extracted from complex matrix such as food, feces or environmental samples have to overcome the problems related with inhibitors of Taq polymerase. In the study performed by Brian et al. (1992), the sensitivity of the PCR was 100-fold lower when DNA was extracted from feces seeded with known number of STEC compared to pure broth cultures. Several authors recommended to isolate the bacteria or extract their DNA before performing the PCR to avoid the presence of inhibitors (Bouvet and Vernozy-Rozand 2000). Addition of bovine serum albumin, proteinase inhibitors and tween have also been tested (Chen et al. 1998). It has to be noticed that broth enrichment used to increase the number of target sequences also dilutes the inhibitors from the sample. Some protocols have been developed to perform PCR detection directly from food. Begum and Jackson (1995) revealed that fat content of ground meat was a major inhibitory component. They managed to reduce the amount of inhibitors by diluting the samples 1000-fold. Using this technique, it was possible to detect as few as 30 STEC ml⁻¹ of ground beef homogenate by PCR. Chen et al. (1998) used an alternative method to remove inhibitors. Biotin-labeled DNA probes specific for *stx1* and *stx2* genes were used to capture the target DNA from STEC. After isolation by streptavidin-coated magnetic beads, the DNA target was directly amplified by conventional PCR. With this technique, ground beef samples artificially contaminated by 10⁰ STEC were tested positive after a 15-h enrichment phase. In order to control the effect of PCR inhibitors when performing PCR detection with complex matrix, it is highly recommended to use an internal inhibition control (IAC). This approach allow the feasibility of the amplification to be assessed and avoid false-negative results. Thus, Fach et al. (2001) used an internal control which is co-amplified with the *stx* genes and detected by a sandwich hybridization assay using microwells coated with streptavidin. This ELISA-PCR used two sets comprising an immobilized biotin-labeled capture probe and a digoxigenin-labeled detection probe specific for *stx* genes and for the internal control. Revelation was carried out with a peroxidase-labeled anti-digoxigenin antibody and a chromogenic substrate. The limit of detection of this method in dairy products was 10² CFU. ml⁻¹. Several real-time PCR assays based on *stx1* and *stx2*-specific probes have been proposed for STEC detection, however, most have them are not suitable as diagnostic tools as they lack an IAC and could lead to false-negative results. The use of an IAC in real-time PCR assays for the specific detection of *stx* has been reported (Belanger et al. 2002; Stefan et al. 2007). In both cases, amplification of the IAC was performed using a different set of primers than for *stx* which may not accurately reflects the target amplification. Auvray et al. (2009) have developed an internally controlled real-time PCR assay where a competitive IAC is co-amplified with all *stx1* and *stx2* variants, except *stx2_p*, by using a single primer pair, and three specific probes to allow targets distinction. After enrichment, this real-time PCR assay was able to detect STEC in minced beef inoculated with as low as 10 CFU per 25 g.

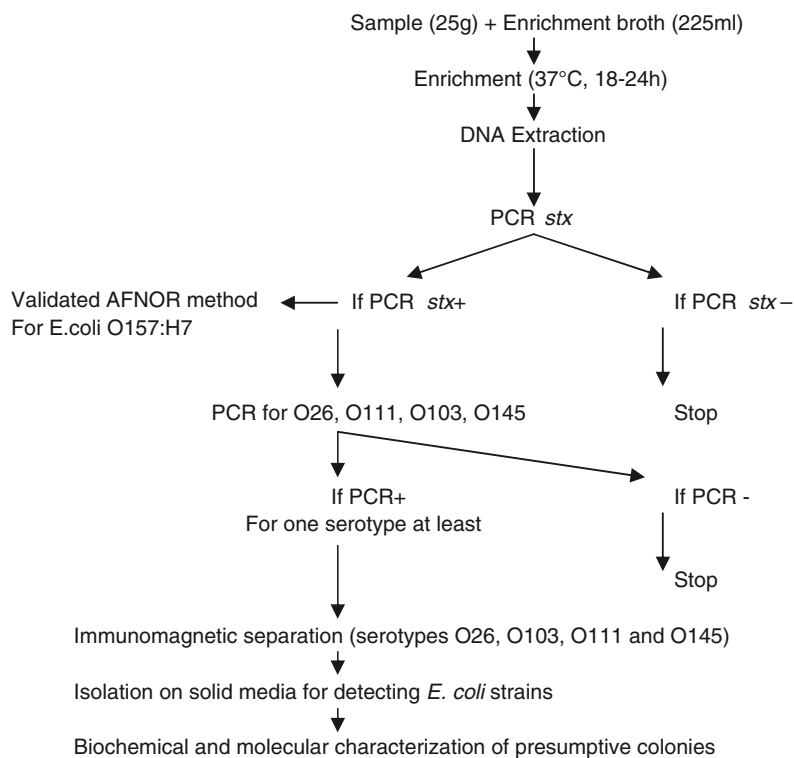


Fig. 2 AFSSA screening strategy to detect the five major STEC serogroup involved in human cases (EFSA 2007)

PCR-Based Strategy to Detect STEC

A PCR-based strategy (Fig. 2) has been recommended by the French Food Safety Agency (AFSSA) for the detection of STEC belonging to the five main pathogenic serotypes. The method used is based on the definition by AFSSA of these serotypes : *E. coli* strains has to harbor *stx1* and/or *stx2* genes, the *eae* gene and belong to one of the following serotypes : O157, O26, O111, O103 and O145. Screening is based on two PCR steps : the first one allows the detection of the *stx* genes and the second one the genes specific for the five major STEC serotypes associated with human diseases : *rfbE*-O157, *wzx*-O26, *wzx*-O103, *wbdI*-O111 and *ihp1*-O145. The samples positives for *stx* genes and one or more of these O antigen associated genes are submitted to an IMS step specific for the respective O antigen detected by PCR. As several DNA targets detected by PCR within an enriched broth can originate from

distinct bacteria, the isolation of a strain carrying all the genetic markers described above is an essential condition to assess whether the sample contains a pathogenic STEC strain. A modified version of this strategy including an initial screening step for *eae* together with *stx* has been retained by a CEN expert committee (European Normalisation Committee, CEN,TC275/WG6) for evaluation in order to become a standard ISO method for the food detection by PCR of the five major STEC serogroups involved in severe human cases.

Commercialized DNA-Based Methods

Management of STEC-associated risks in foodstuffs is critical for the food industry since these emerging pathogens may have a significant public health and economic impacts. To guarantee the safety of their products, the food industry is demanding fast and accurate automated methods for STEC detection. Several DNA-based detection kits are available for STEC. Most of them are targeting *stx* genes and the *E. coli* O157:H7 serotype, as it is responsible for the major part of outbreaks and sporadic cases.

The Assurance GDS shiga-toxin and GDS O157:H7 methods developed by Biocontrol Systems use specific probes and primers to amplify a highly conserved sequence of the target genes with a centrifugal air-exchange thermal cycler. Both methods have been validated by AOAC-RI (AOAC Official Method 2005.05) after trials in orange juice, raw ground beef, and fresh lettuce (Feldsine et al. 2005). The BAX system with automated detection is a commercial PCR-based assay developed by Dupont Qualicon and validated by AFNOR. Tableted reagents comprising primers, DNA polymerase and deoxynucleotides for PCR, a positive control and an intercaling dye are rehydrated with the DNA solution extracted from food after an overnight enrichment (Johnson et al. 1998). Automatic detection is performed by analysis of the fluorescent signal emitted during a melting curve generated after the amplification step (Deisingh and Thompson 2004). Shearer et al. (2001) have evaluated the BAX system into 15 food samples including alfalfa sprouts, radish, leaf lettuce and apples, inoculated with *E. coli* O157:H7. They reported that the BAX was more sensitive than the culture-based method for some samples. However, both BAX and the culture method were unable to recover low numbers of *E. coli* O157:H7 from alfalfa sprouts. In a study performed by Johnson et al. (1998), the BAX showed a detection rate of 96.5% compared to 39% for the best cultural method and 71.5% for the immunodiffusion method. In fruit juices samples spiked with *E. coli* O157:7 and enriched in modified EC medium, the BAX system enabled the detection of less than 5 cfu/25 mL (Bouvet and Vernozy-Rozand 2000).

The Probelia PCR System *E. coli* O157:H7 (Biocontrol System) use primers and probes specific for *E. coli* O157:H7, a synthetic DNA as IAC and a revealing system on microtiter plates. After PCR amplification of the target, the detection is performed by sandwich hybridization. The specificity of this method was assessed on a collection of 98 STEC and 40 non-STEC bacterial strains and detection of 1–10 cfu/25 g

after enrichment was possible (Bouvet and Vernozy-Rozand 2000). The iQ-Check *E. coli* O157:H7 (Biorad) is a real-time PCR method validated by the AOAC and the French association for standardization (AFNOR). This method uses specific primers and probe for the detection of *E. coli* O157:H7 from food samples after an enrichment step. GeneSystems have developed an automated and miniaturized real-time PCR instrument which perform gene amplification in a disposable device, the GeneDisc. The rim of this device is engraved with 36 reaction microchambers preloaded with desiccated PCR amplification mixtures. Every reaction wells contain specific primers and fluorescent-labeled probes which enable the detection of two targets in duplex assay. A Genedisc for the detection of *E. coli* O157:H7 and *Salmonella spp.* (GeneDisc DUO *E. coli* O157 & *Salmonella*) have been validated by AFNOR and a second Genedisc for detection of genes encoding Shiga toxins (*stx*), intimins (*eae*), *E. coli* O157 (*rfbE*-O157) and H7 (*fliC*-H7) antigens as well as genes specific for EHEC O26 (*wzx*-O26), O103 (*wzx*-O103), O111 (*wbd1*-O111), O145 (*ihp1*-O145) and O157 (*ihp1*-O157) have been evaluated recently (Beutin et al. 2009). The authors were able to detect reliably 2–3 CFU of target bacteria in a background of approximately 50,000 *E. coli* K-12 from agar plates.

Other Techniques and Emerging Technologies

Biosensors

Current methods to detect and confirm the presence of STEC in a sample are time consuming, especially if they require enrichment protocols. They also might be too complex and costly for use in routine analysis. Rapid detection and confirmation PCR assays have recently been developed but inhibition of PCR by compounds present in matrices such as food or feces can lead to difficulties and false-negative results. New approaches based on the use of biosensors have been described. Biosensors combine a biological recognition element in contact with a physico-chemical detection component. They convert an optical, piezoelectric or electrochemical signal modified by a biological response into an electrical signal. They aim for a rapid and reliable detection of pathogens in complex matrix without the use of additional (eg: washing) steps.

Demarco and Lim (2002) have used an optic fiber-based biosensor for the detection of *E. coli* O157:H7 in 10 and 25 g of grounded meat. The detection system comprises a 635 nm laser diode that directs light onto optical fiber probes and generates an evanescent wave. Fluorescent molecules present in the evanescent field are excited and a part of the emission re-coupled into the fiber probe. The signal is then quantified by a photodiode. *E. coli* O157:H7 detection was performed with an immunological sandwich test using antibodies against O157:H7 labelled with cyanine 5 and attached to the optic fiber. This method allows the detection of 9×10^3 CFU g⁻¹ and 5.2×10^2 CFU g⁻¹ for 25 and 10 g of meat, respectively. Results are obtained in 25 min after sample processing.

An other biosensor using an optic fiber aimed at detecting *E. coli* species was described by Almadidy et al. (2002). This system used a *lacZ*-specific 25-mer DNA probe which is covalently linked to the optic fiber. Detection of probe hybridization with the target was performed with ethidium bromide (BET). Moreover, regeneration of this system can be performed with 90°C-heated water and formamid 90% in TE buffer. Detection tests were performed in the presence of *E. coli* genomic DNA fragments of 300 pb obtained by sonication, or 25-mer complementary *lacZ* sequence, or 100-mer PCR products containing the *lacZ* sequence at various locations, and finally non-complementary DNA including genomic samples from salmon sperm. Detection of genomic fragments containing the *lacZ* sequence was possible in as little as 20 s by observation of the steady-state fluorescence intensity. No inhibition of the hybridization between the probe and the target was observed in the presence of non specific DNA.

A rapid and specific assay for the detection of *E. coli* O157:H7 using Resonant Acoustic Profiling (RAP) has been tested by Huang and Cooper (2006). The RAP experiment applies the principle of quartz crystal microbalance, whereby high-frequency voltage applied to a piezoelectric crystal induced the latter to resonate, followed by resonance frequency monitoring in real-time. This frequency is modified when analytes (eg: biomolecules) bind to the sensor surface. Anti-O157 antibodies were covalently attached to activated sensors for the detection of *E. coli* O157:H7. Both direct binding (bacteria only) and sandwich assays (bacteria followed by antibody) were tested. This system was able to detect 6.10^3 bacteria mL^{-1} for direct O157 binding and 6.10^2 bacteria mL^{-1} for the sandwich assay. The same principle was used by Deisingh and Thompson (2001), who developed a PCR-acoustic wave sensor combined approach to detect a 509-bp O157:H7 specific sequence captured by a biotinylated DNA probe bound to a sensor by biotin-neutravidin interaction. This approach was promising but it has not yet been tested on clinical samples.

Surface Plasmon Resonance (SPR) has also been used as a platform for biosensor development. This method measures refractive index changes close to the interface of a sensor chip in real-time. Such changes occur when biomolecules interact with immobilized ligands, replacing water from binding sites on the sensor surface. The refractive index differences are detected via angle changes of a reflected laser beam and are directly related to the amount of sensor surface-bound biomolecules. The main application of SPR in the field of biomedical science is to analyze antibody-antigen interaction dynamics and to sense specific antigen through the chemical binding of the corresponding antibody (Chiang et al. 2009). A SPR biacore biosensor using antibodies against *E. coli* O157:H7 was found to have a detection limit of 5×10^7 CFU mL^{-1} (Deisingh and Thompson 2004) which is still far from the limit of detection of biosensors described above. Miyachi et al. (2000) have constructed a chimeric oligonucleotide primer comprising 21 RNA nucleotides with six DNA nucleotides at the 3' hydroxyl end. This primer was used to amplify a DNA fragment by PCR, prior to analysis by SPR. This technique allowed the detection of *E. coli* O157:H7 and its distinction from other bacteria. Another application of SPR biosensor was also described by Medina et al., 2001 for the analysis

of interactions between *E. coli* O157:H7 immobilized cells and extracellular molecules such as collagen I, laminin and fibronectin. Using this SPR biosensor model, the authors have evaluated the inhibition of the interactions between collagen/laminin and *E. coli* cell surface with the use of carrageenan, sodium alginate and pectin. The results indicated that the bindings was inhibited from 89% to 100% with carrageenan, 50% with sodium alginate and 10% with pectin. This study opened new perspectives to develop strategies in order to lower bacterial adhesion or to detach bacteria from meat.

Fluorescence and Microscopy

Detection of fluorescent *E. coli* O157:H7 by confocal scanning laser microscopy has been tested in a study carried out by Burnett and Beuchat (2002). *E. coli* O157:H7 cells labelled with an enhanced green fluorescent protein (EGFP) plasmid were submitted to treatments with chlorine, hydrogen peroxide and acetic acid. Changes in fluorescence intensity was measured with a spectrophotometer and individual cells were observed with a confocal scanning laser microscope. In order to differentiate viable and dead cells after surface treatment with sanitizers, three nucleic acid stains were compared. It was found that EGFP is not suitable for the differentiation of viable and dead cells following treatments with sanitizers. On the contrary, the use of SYTOX GREEN to stain dead O157:H7 cells in combination with Alexa Fluor 594 antibodies anti-O157:H7 to stain total cells was preferable.

Yamaguchi et al. (2003) have investigated a flow cytometric (FCM) approach to detect respiring *E. coli* O157:H7 in apple juice, milk and ground beef. This method used a double staining with fluorescein isothiocyanate (FITC)-labeled anti *E. coli* O157:H7 antibody and 5-cyano-2,3-ditoly tetrazolium chloride (CTC) used as an indicator of bacterial respiratory function. Respiring *E. coli* O157:H7 were clearly distinguished from respiring *E. coli* K12 or inactive cells. The detection limit of respiring *E. coli* O157:H7 with this flow cytometric method in apple juice and milk was 10^3 cells mL⁻¹. In ground beef, the detection limit was 10^3 cells g⁻¹ when the total bacterial number inoculated in the sample is more than 10^6 cells g⁻¹. Authors highlighted the fact that FCM allow the detection of 10^4 bacterial cells in a few minutes, which is faster than any other methods to detect pathogenic cells with viability in food samples. A laser induced fluorescence coupled with cytometry approach to detect *E. coli* O157:H7 in ground beef has been described by Johnson et al. (2001). Authors have demonstrated that this strategy was feasible and could bring some advantages such as examination of an important volume of food or water in real time, capability to detect a single organism, sensitivity to the selected bacteria and automatic detection.

Stender et al. (2001) have described a method based on fluorescence *in situ* hybridization assay (FISH) coupled with array scanner for the detection of *E. coli*. A Cy3-labeled peptide nucleic acid (PNA) probe complementary to a specific 16s rRNA sequence of *E. coli* was employed. PNA probes are DNA mimics with

improved hybridization characteristics. After filtration on a membrane, bacterial cells were incubated for 5 h at 35°C prior to the PNA FISH assays and transferred onto Petri dishes for standard colony count. Membranes were then read with an array scanner. Following a short incubation step, *E. coli* microcolonies were distinguished before they became confluent and provided a much broader dynamic range than standard plate counting. This method has combined the large volume analysis of membrane filtration, the specificity of the PNA probe and the speed and high resolution of the array scanner. However, it should be noted that two *Shigella* species were also detected, due to the very high level of similarity between rRNA of *E. coli* and *Shigella* species.

Microarrays

Microarrays allow thousands or tens of thousands of specific DNA or RNA sequences to be detected simultaneously on a small glass or silica slide only 1–2 cm² (Aitman 2001). This has allowed high-throughput screening applications and rapid analyses but this technology is not free from disadvantages. Microarrays instruments are expensive and of limited availability. The huge amounts of data generated by this technology requires specialist knowledge, training and a software support to extract useful informations (Deisingh and Thompson 2001). However, several studies using microarrays for the detection of *E. coli* have been conducted.

Call et al. (2001) have developed a multiplex PCR and microarray approach to detect and characterize *E. coli* O157:H7. Specific 25–30 mer probes complementary to four sequences of virulence genes were bound to microarrays. Target DNA was amplified by multiplex PCR and hybridized to the microarrays without further modification or purification. The authors have reported that this system was 32-fold more sensitive than gel electrophoresis and it was capable to detect 1 fg of genomic DNA. The combination of immunomagnetic capture, PCR and microarray detection make it possible to detect 55 CFU ml⁻¹ of O157:H7 from chicken rinse without enrichment. This microarray was also employed in an electromagnetic flow cell and fluidic system for automated immunomagnetic separation (IMS) of *E. coli* O157:H7 from poultry carcass rinse (Chandler et al. 2001). The flow cell incorporated highly porous nickel foam to immobilize the magnetic beads throughout the fluid thus enabling an efficient perfusion and target binding. O157:H7 cells were reproducibly isolated directly from poultry carcass rinse without enrichment, with 39% recovery efficiency at 10³ CFU ml⁻¹ inoculum. Recovered beads were then used for PCR amplification and microarrays detection.

For quality control of drinking water, Wolter et al. (2008) have developed the first flow-through chemiluminescence microarray for the rapid detection of *E. coli* O157:H7, *Salmonella typhimurium*, and *Legionella pneumophila* in water samples. Microarrays were coated with species-specific antibodies for the capture of target bacteria. Cell recognition and chemiluminescence detection were carried out by binding of species-specific biotinylated antibodies, and streptavidin-horseradish peroxi-

dase (HRP) catalyzed reaction of luminol and hydrogen peroxide. Chemiluminescence was measured by a sensitive charge-coupled device (CCD) camera. *E. coli* O157:H7 was detected with a sensitivity of 3.10^3 cells ml^{-1} . Authors presented this semi automated readout system as well adapted for quick and automatic analysis.

Viable but non culturable (VBNC) *E. coli* may also represent a threat, especially in drinking water and source water because of the potential of water distribution to transmit this pathogen. Liu et al. (2008) have developed a sensitive method to detect VBNC *E. coli* O157:H7 in water. This method involved capture of the bacterial cells by a low-protein-binding membrane filter, followed by RNA extraction and purification. RNA encoding the *rfbE* and *fliC*-H7 genes were chosen as viability markers and were detected with an electronic microarray. This type of array uses an electric field to deliver DNA probes or target to defined positions on the chip, which enables concentration of negatively charged DNA resulting in an improvement of detection sensitivity and specificity. Fifty VBNC *E. coli* O157:H7 were detected in 1 L of river water and as few as 1 CFU was detected in diluted culture.

Conclusion

Shiga-toxins producing *E. coli*, and especially *E. coli* O157:H7, are foodborne pathogens that can cause severe and life threatening diseases in humans. Moreover, the number of O157 and non-O157 STEC outbreaks and individual cases are increasing from years to years (EFSA 2007) so rapid and reliable detection methods are needed. The severity of the disease, the lack of effective treatments and the potential for large outbreaks from contaminated food have led to proceed to intensive research on pathogenesis and detection of *E. coli* O157:H7 (Perna et al. 2001). Furthermore, the low infectious dose needed, the low amounts of STEC within products and the wide range of serotypes implicated in human disease make the detection strategies even more difficult. Phenotypical and immunological methods do not allow STEC strains to be easily distinguished from competitor organisms which are often present at high level in the samples.

Standard or validated methods are available for the detection of *E. coli* O157:H7 and their use is recommended for the detection and isolation of pathogenic strains belonging to this serotype. However it is not the case yet for the other STEC serotypes. An outcome is now possible with the PCR-based sequential strategy proposed for the detection of the five major STEC serogroups which is currently under evaluation in order to become an ISO method. PCR-based methods and multiplex PCR in particular are valuable approaches as pathogenesis is mediated by a specific combination of virulence genes. As no single genetic marker is yet known whose detection would report for the presence of a pathogenic STEC strain, multiplex PCR enabling the rapid and simultaneous detection of several virulence-associated markers is well-suited to rapidly screen suspected samples. However, several genes that are known to be implicated in STEC pathogenesis still need further characterization. More studies on STEC pathogenesis are required in order to discover new virulence genetic

markers and make the detection methods more accurate. Optimization of an enrichment protocol adapted to all serotypes of STEC strains is also needed and similar optimizations are required regarding the isolation of STEC strains from suspected samples with PCR-positive results. Isolation remains a challenge as STEC strains do not share the same biochemical properties and, as mentioned above, may be present in low amount in foods. The promising DNA-based methods may facilitate STEC detection in foodstuffs and screening of virulence markers might allow pathogenic STEC strains to be detected without the need to isolate them. To date however, this isolation step still remains essential in order to assess that the virulence genes detected by PCR are present within the same strain.

References

- AFSSA (2003) Bilan des connaissances relatives aux *Escherichia coli* producteurs de shiga-toxines (pdf). <http://www.afssa.fr/Documents/MIC-Ra-STECC.pdf>
- Almadidy A, Watterson J, Piuino PAE, Raha S, Foulds IV, Horgen PA, Castle A, Krull U (2002) Direct selective detection of genomic DNA from coliform using a fibre optic biosensor. *Anal Chim Acta* 461:37–47
- Aitman TJ (2001) DNA microarrays in medical practice. *BMJ* 323:611–615
- Auvray F, Lecureuil C, Dilasser F, Tache J, Derzelle S (2009) Development of a real-time PCR assay with an internal amplification control for the screening of Shiga toxin-producing *Escherichia coli* in foods. *Lett Appl Microbiol* 48:554–559
- Auvray F, Lecureuil C, Tache J, Leclerc V, Deperrois V, Lombard B (2007) Detection, isolation and characterization of Shiga toxin-producing *Escherichia coli* in retail-minced beef using PCR-based techniques, immunoassays and colony hybridization. *Lett Appl Microbiol* 45:646–651
- Auvray F, Lecureuil C, Tache J, Perelle S, Fach P (2008) Development of a 5'-nuclease PCR assay for the identification of *Escherichia coli* strains expressing the flagellar antigen H21 and their detection in food after enrichment. *J Appl Microbiol* 104:899–905
- Begum D, Jackson MP (1995) Direct detection of Shiga-like toxin-producing *Escherichia coli* in ground beef using the polymerase chain reaction. *Mol Cell Probes* 9:259–264
- Belanger SD, Boissinot M, Menard C, Picard FJ, Bergeron MG (2002) Rapid detection of Shiga toxin-producing bacteria in feces by multiplex PCR with molecular beacons on the smart cycler. *J Clin Microbiol* 40:1436–1440
- Bellin T, Pulz M, Matussek A, Hempen HG, Gunzer F (2001) Rapid detection of enterohemorrhagic *Escherichia coli* by real-time PCR with fluorescent hybridization probes. *J Clin Microbiol* 39:370–374
- Besser RE, Griffin PM, Slutsker L (1999) *Escherichia coli* O157:H7 gastroenteritis and the hemolytic uremic syndrome: an emerging infectious disease. *Annu Rev Med* 50:355–367
- Bettelheim KA (1998a) Reliability of CHROMagar O157 for the detection of enterohaemorrhagic *Escherichia coli* (EHEC) O157 but not EHEC belonging to other serogroups. *J Appl Microbiol* 85:425–428
- Bettelheim KA (1998b) Studies of *Escherichia coli* cultured on Rainbow Agar O157 with particular reference to enterohaemorrhagic *Escherichia coli* (EHEC). *Microbiol Immunol* 42:265–269
- Bettelheim KA (2005) Reliability of O157:H7 ID agar (O157 H7 ID-F) for the detection and isolation of verocytotoxigenic strains of *Escherichia coli* belonging to serogroup O157. *J Appl Microbiol* 99:408–410
- Beutin L, Jahn S, Fach P (2009) Evaluation of the 'GeneDisc' real-time PCR system for detection of enterohaemorrhagic *Escherichia coli* (EHEC) O26, O103, O111, O145 and O157 strains

- according to their virulence markers and their O- and H-antigen-associated genes. J Appl Microbiol 106:1122–1132
- Beutin L, Kaulfuss S, Cheasty T, Brandenburg B, Zimmermann S, Gleier K, Willshaw GA, Smith HR (2002a) Characteristics and association with disease of two major subclones of Shiga toxin (Verocytotoxin)-producing strains of *Escherichia coli* (STEC) O157 that are present among isolates from patients in Germany. Diagn Microbiol Infect Dis 44:337–346
- Beutin L, Montenegro MA, Orskov I, Orskov F, Prada J, Zimmermann S, Stephan R (1989) Close association of verotoxin (Shiga-like toxin) production with enterohemolysin production in strains of *Escherichia coli*. J Clin Microbiol 27:2559–2564
- Beutin L, Zimmermann S, Gleier K (1996) Rapid detection and isolation of shiga-like toxin (verocytotoxin)-producing *Escherichia coli* by direct testing of individual enterohemolytic colonies from washed sheep blood agar plates in the VTEC-RPLA assay. J Clin Microbiol 34:2812–2814
- Beutin L, Zimmermann S, Gleier K (2002) Evaluation of the VTEC-Screen “Seiken” test for detection of different types of Shiga toxin (verotoxin)-producing *Escherichia coli* (STEC) in human stool samples. Diagn Microbiol Infect Dis 42:1–8
- Bielaszewska M, Schmidt H, Karmali MA, Khakhria R, Janda J, Blahova K, Karch H (1998) Isolation and characterization of sorbitol-fermenting Shiga toxin (Verocytotoxin)-producing *Escherichia coli* O157:H- strains in the Czech Republic. J Clin Microbiol 36:2135–2137
- Bouvet J, Vernozzy-Rozand C (2000) Méthodes génétiques de détection des *Escherichia coli* vérotoxiques (STEC) et de *E. coli* O157:H7 dans les aliments. Rev Med Vet 151:907–914
- Brian MJ, Frosolono M, Murray BE, Miranda A, Lopez EL, Gomez HF, Cleary TG (1992) Polymerase chain reaction for diagnosis of enterohemorrhagic *Escherichia coli* infection and hemolytic-uremic syndrome. J Clin Microbiol 30:1801–1806
- Brown JE, Sethabutr O, Jackson MP, Lolekha S, Echeverria P (1989) Hybridization of *Escherichia coli* producing Shiga-like toxin I, Shiga-like toxin II, and a variant of Shiga-like toxin II with synthetic oligonucleotide probes. Infect Immun 57:2811–2814
- Burnett SL, Beuchat LR (2002) Comparison of methods for fluorescent detection of viable, dead, and total *Escherichia coli* O157:H7 cells in suspensions and on apples using confocal scanning laser microscopy following treatment with sanitizers. Int J Food Microbiol 74:37–45
- Call DR, Brockman FJ, Chandler DP (2001) Detecting and genotyping *Escherichia coli* O157:H7 using multiplexed PCR and nucleic acid microarrays. Int J Food Microbiol 67:71–80
- Callaway TR, Carr MA, Edrington TS, Anderson RC, Nisbet DJ (2009) Diet, *Escherichia coli* O157:H7, and Cattle: A review after 10 years. Curr Issues Mol Biol 11:67–80
- Campbell GR, Prosser J, Glover A, Killham K (2001) Detection of *Escherichia coli* O157:H7 in soil and water using multiplex PCR. J Appl Microbiol 91:1004–1010
- Catarame TM, O’Hanlon KA, Duffy G, Sheridan JJ, Blair IS, McDowell DA (2003) Optimization of enrichment and plating procedures for the recovery of *Escherichia coli* O111 and O26 from minced beef. J Appl Microbiol 95:949–957
- Cebula TA, Payne WL, Feng P (1995) Simultaneous identification of strains of *Escherichia coli* serotype O157:H7 and their Shiga-like toxin type by mismatch amplification mutation assay-multiplex PCR. J Clin Microbiol 33:248–250
- Chandler DP, Brown J, Call DR, Wunschel S, Grate JW, Holman DA, Olson L, Stottlemire MS, Bruckner-Lea CJ (2001) Automated immunomagnetic separation and microarray detection of *E. coli* O157:H7 from poultry carcass rinse. Int J Food Microbiol 70:143–154
- Chapman PA, Siddons CA, Zadik PM, Jewes L (1991) An improved selective medium for the isolation of *Escherichia coli* O157. J Med Microbiol 35:107–110
- Chapman PA, Wright DJ, Siddons CA (1994) A comparison of immunomagnetic separation and direct culture for the isolation of verocytotoxin-producing *Escherichia coli* O157 from bovine faeces. J Med Microbiol 40:424–427
- Chassagne L, Pradel N, Robin F, Livrelli V, Bonnet R, Delmas J (2009) Detection of stx1, stx2, and eae genes of enterohemorrhagic *Escherichia coli* using SYBR Green in a real-time polymerase chain reaction. Diagn Microbiol Infect Dis 64:98–101

- Chen S, Xu R, Yee A, Wu KY, Wang CN, Read S, De Grandis SA (1998) An automated fluorescent PCR method for detection of shiga toxin-producing *Escherichia coli* in foods. *Appl Environ Microbiol* 64:4210–4216
- Chiang YL, Lin CH, Yen MY, Su YD, Chen SJ, Chen HF (2009) Innovative antimicrobial susceptibility testing method using surface plasmon resonance. *Biosens Bioelectron* 24:1905–1910
- China B, Pirson V, Mainil J (1996) Typing of bovine attaching and effacing *Escherichia coli* by multiplex in vitro amplification of virulence-associated genes. *Appl Environ Microbiol* 62:3462–3465
- DeGrandis S, Law H, Brunton J, Gyles C, Lingwood CA (1989) Globotetraosylceramide is recognized by the pig edema disease toxin. *J Biol Chem* 264:12520–12525
- Deisingh AK, Thompson M (2001) Sequences of *E. coli* O157:H7 detected by a PCR-acoustic wave sensor combination. *Analyst* 126:2153–2158
- Deisingh AK, Thompson M (2004) Strategies for the detection of *Escherichia coli* O157:H7 in foods. *J Appl Microbiol* 96:419–429
- Demarco DR, Lim DV (2002) Detection of *Escherichia coli* O157:H7 in 10- and 25-gram ground beef samples with an evanescent-wave biosensor with silica and polystyrene waveguides. *J Food Prot* 65:596–602
- Desmarchelier PM, Bilge SS, Fegan N, Mills L, Vary JC Jr, Tarr PI (1998) A PCR specific for *Escherichia coli* O157 based on the *rfb* locus encoding O157 lipopolysaccharide. *J Clin Microbiol* 36:1801–1804
- Dorak T (2006) Real-time PCR. (Advanced Methods Series). Oxford: Taylor & Francis
- Drysdale M, MacRae M, Strachan NJ, Reid TM, Ogden ID (2004) The detection of non-O157 *E. coli* in food by immunomagnetic separation. *J Appl Microbiol* 97:220–224
- EFSA (2007) Monitoring of verotoxigenic *Escherichia coli* (VTEC) and identification of human pathogenic VTEC types: Scientific opinion of the panel on biological hazards. *EFSA J* 579:1–61
- Fach P, Perelle S, Dilasser F, Grout J (2001) Comparison between a PCR-ELISA test and the vero cell assay for detecting Shiga toxin-producing *Escherichia coli* in dairy products and characterization of virulence traits of the isolated strains. *J Appl Microbiol* 90:809–818
- Farmer JJ 3rd, Davis BR (1985) H7 antiserum-sorbitol fermentation medium: a single tube screening medium for detecting *Escherichia coli* O157:H7 associated with hemorrhagic colitis. *J Clin Microbiol* 22:620–625
- Feldsine PT, Green ST, Lienau AH, Stephens J, Jucker MT, Kerr DE (2005) Evaluation of the assurance GDS for *E. coli* O157:H7 method and assurance GDS for shigatoxin genes method in selected foods: collaborative study. *J AOAC Int* 88:1334–1348
- Feng P (1993) Identification of *Escherichia coli* serotype O157:H7 by DNA probe specific for an allele of uid A gene. *Mol Cell Probes* 7:151–154
- Feng PC, Monday SR, Lacher DW, Allison L, Siitonen A, Keys C, Eklund M, Nagano H, Karch H, Keen J, Whittam TS (2007) Genetic diversity among clonal lineages within *Escherichia coli* O157:H7 stepwise evolutionary model. *Emerg Infect Dis* 13:1701–1706
- Foster G, Hopkins GF, Gunn GJ, Ternent HE, Thomson-Carter F, Knight HI, Graham DJ, Edge V, Syngé BA (2003) A comparison of two pre-enrichment media prior to immunomagnetic separation for the isolation of *E. coli* O157 from bovine faeces. *J Appl Microbiol* 95:155–159
- Fratamico PM, DebRoy C, Strobaugh TP Jr, Chen CY (2005) DNA sequence of the *Escherichia coli* O103 O antigen gene cluster and detection of enterohemorrhagic *E. coli* O103 by PCR amplification of the *wzx* and *wzy* genes. *Can J Microbiol* 51:515–522
- Gannon VP, D'Souza S, Graham T, King RK, Rahn K, Read S (1997) Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic *Escherichia coli* strains. *J Clin Microbiol* 35:656–662
- Hamabata T, Tanaka T, Ozawa A, Shima T, Sato T, Takeda Y (2002) Genetic variation in the flanking regions of Shiga toxin 2 gene in Shiga toxin-producing *Escherichia coli* O157:H7 isolated in Japan. *FEMS Microbiol Lett* 215:229–236
- Heijnen L, Medema G (2006) Quantitative detection of *E. coli*, *E. coli* O157 and other shiga toxin producing *E. coli* in water samples using a culture method combined with real-time PCR. *J Water Health* 4:487–498

- Hepburn NF, MacRae M, Johnston M, Mooney J, Ogden ID (2002) Optimizing enrichment conditions for the isolation of *Escherichia coli* O157 in soils by immunomagnetic separation. *Lett Appl Microbiol* 34:365–369
- Hiramatsu R, Matsumoto M, Miwa Y, Suzuki Y, Saito M, Miyazaki Y (2002) Characterization of Shiga toxin-producing *Escherichia coli* O26 strains and establishment of selective isolation media for these strains. *J Clin Microbiol* 40:922–925
- Hu Y, Zhang Q, Meitzler JC (1999) Rapid and sensitive detection of *Escherichia coli* O157:H7 in bovine faeces by a multiplex PCR. *J Appl Microbiol* 87:867–876
- Huang L, Cooper MA (2006) Real-time label-free acoustic technology for rapid detection of *Escherichia coli* O157:H7. *Clin Chem* 52:2148–2151
- Ibekwe AM, Watt PM, Grieve CM, Sharma VK, Lyons SR (2002) Multiplex fluorogenic real-time PCR for detection and quantification of *Escherichia coli* O157:H7 in dairy wastewater wetlands. *Appl Environ Microbiol* 68:4853–4862
- Ibekwe AM, Grieve CM (2003) Detection and quantification of *Escherichia coli* O157:H7 in environmental samples by real-time PCR. *J Appl Microbiol* 94:421–431
- Ito K, Iida M, Yamazaki M, Moriya K, Moroishi S, Yatsuyanagi J, Kurazono T, Hiruta N, Ratchranchai OA (2007) Intimin types determined by heteroduplex mobility assay of intimin gene (*eae*)-positive *Escherichia coli* strains. *J Clin Microbiol* 45:1038–1041
- Jenkins C, Pearce MC, Smith AW, Knight HI, Shaw DJ, Cheasty T, Foster G, Gunn GJ, Dougan G, Smith HR, Frankel G (2003) Detection of *Escherichia coli* serogroups O26, O103, O111 and O145 from bovine faeces using immunomagnetic separation and PCR/DNA probe techniques. *Lett Appl Microbiol* 37:207–212
- Jinneman KC, Yoshitomi KJ, Weagant SD (2003) Multiplex real-time PCR method to identify Shiga toxin genes *stx1* and *stx2* and *Escherichia coli* O157:H7/H-serotype. *Appl Environ Microbiol* 69:6327–6333
- Johnson JL, Brooke CL, Fritschel SJ (1998) Comparison of the BAX for screening *E. coli* O157:H7 method with conventional methods for detection of extremely low levels of *Escherichia coli* O157:H7 in ground beef. *Appl Environ Microbiol* 64:4390–4395
- Johnson PE, Lund ML, Shorthill RW, Swanson JE, Kellogg JL (2001) Real time biotransformation of individual pathogenic microorganisms in food and water. *Biomed Sci Instrum* 37:191–196
- Jothikumar N, Griffiths MW (2002) Rapid detection of *Escherichia coli* O157:H7 with multiplex real-time PCR assays. *Appl Environ Microbiol* 68:3169–3171
- Karch H, Bielaszewska M (2001) Sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157:H(-) strains: epidemiology, phenotypic and molecular characteristics, and microbiological diagnosis. *J Clin Microbiol* 39:2043–2049
- Karch H, Bohm H, Schmidt H, Gunzer F, Aleksic S, Heesemann J (1993) Clonal structure and pathogenicity of Shiga-like toxin-producing, sorbitol-fermenting *Escherichia coli* O157:H. *J Clin Microbiol* 31:1200–1205
- Karch H, Janetzki-Mittmann C, Aleksic S, Datz M (1996) Isolation of enterohemorrhagic *Escherichia coli* O157 strains from patients with hemolytic-uremic syndrome by using immunomagnetic separation, DNA-based methods, and direct culture. *J Clin Microbiol* 34:516–519
- Karch H, Meyer T (1989) Evaluation of oligonucleotide probes for identification of shiga-like-toxin-producing *Escherichia coli*. *J Clin Microbiol* 27:1180–1186
- Karmali MA, Mascarenhas M, Shen S, Ziebell K, Johnson S, Reid-Smith R, Isaac-Renton J, Clark C, Rahn K, Kaper JB (2003) Association of genomic O island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. *J Clin Microbiol* 41:4930–4940
- Karmali MA, Petric M, Bielaszewska M (1999) Evaluation of a microplate latex agglutination method (Verotox-F assay) for detecting and characterizing verotoxins (Shiga toxins) in *Escherichia coli*. *J Clin Microbiol* 37:396–399
- Karmali MA, Petric M, Lim C, Cheung R, Arbus GS (1985) Sensitive method for detecting low numbers of verotoxin-producing *Escherichia coli* in mixed cultures by use of colony sweeps and polymyxin extraction of verotoxin. *J Clin Microbiol* 22:614–619

- Karmali MA, Steele BT, Petric M, Lim C (1983) Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. *Lancet* 1:619–620
- Kehl KS, Havens P, Behnke CE, Acheson DW (1997) Evaluation of the premier EHEC assay for detection of Shiga toxin-producing *Escherichia coli*. *J Clin Microbiol* 35:2051–2054
- Konowalchuk J, Speirs JJ, Stavric S (1977) Vero response to a cytotoxin of *Escherichia coli*. *Infect Immun* 18:775–779
- Levine MM, Xu JG, Kaper JB, Lior H, Prado V, Tall B, Nataro J, Karch H, Wachsmuth K (1987) A DNA probe to identify enterohemorrhagic *Escherichia coli* of O157:H7 and other serotypes that cause hemorrhagic colitis and hemolytic uremic syndrome. *J Infect Dis* 156:175–182
- Lin Z, Kurazono H, Yamasaki S, Takeda Y (1993) Detection of various variant verotoxin genes in *Escherichia coli* by polymerase chain reaction. *Microbiol Immunol* 37:543–548
- Lindgren SW, Melton AR, O'Brien AD (1993) Virulence of enterohemorrhagic *Escherichia coli* O91:H21 clinical isolates in an orally infected mouse model. *Infect Immun* 61:3832–3842
- Lingwood CA, Law H, Richardson S, Petric M, Brunton JL, De Grandis S, Karmali M (1987) Glycolipid binding of purified and recombinant *Escherichia coli* produced verotoxin in vitro. *J Biol Chem* 262:8834–8839
- Liu Y, Gilchrist A, Zhang J, Li XF (2008) Detection of viable but nonculturable *Escherichia coli* O157:H7 bacteria in drinking water and river water. *Appl Environ Microbiol* 74:1502–1507
- Mackenzie A, Orrbine E, Hyde L, Benoit M, Chan F, Park C, Alverson J, Lembke A, Hoban D, Kennedy W (2000) Performance of the ImmunoCard STAT! *E. coli* O157:H7 test for detection of *Escherichia coli* O157:H7 in stools. *J Clin Microbiol* 38:1866–1868
- Mackenzie AM, Lebel P, Orrbine E, Rowe PC, Hyde L, Chan F, Johnson W, McLaine PN (1998) Sensitivities and specificities of premier *E. coli* O157 and premier EHEC enzyme immunoassays for diagnosis of infection with verotoxin (Shiga-like toxin)-producing *Escherichia coli*. The SYNORB Pk Study investigators. *J Clin Microbiol* 36:1608–1611
- March SB, Ratnam S (1986) Sorbitol-MacConkey medium for detection of *Escherichia coli* O157:H7 associated with hemorrhagic colitis. *J Clin Microbiol* 23:869–872
- Medina MB (2001) Binding of collagen I to *Escherichia coli* O157:H7 and inhibition by carrageenans. *Int J Food Microbiol* 69:199–208
- Monday SR, Beisaw A, Feng PC (2007) Identification of Shiga toxigenic *Escherichia coli* seropathotypes A and B by multiplex PCR. *Mol Cell Probes* 21:308–311
- Miyachi H, Yano K, Ikebukuro K, Kono M, Hoshina S, Karube I (2000) Application of chimeric RNA–DNA oligonucleotides to the detection of pathogenic microorganisms using surface plasmon resonance. *Analytica Chimica Acta* 407:1–10
- Nagano H, Hirochi T, Fujita K, Wakamori Y, Takeshi K, Yano S (2004) Phenotypic and genotypic characterization of beta-D-glucuronidase-positive Shiga toxin-producing *Escherichia coli* O157:H7 isolates from deer. *J Med Microbiol* 53:1037–1043
- Nataro JP, Kaper JB (1998) Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* 11:142–201
- Nielsen EM, Andersen MT (2003) Detection and characterization of verocytotoxin-producing *Escherichia coli* by automated 5' nuclease PCR assay. *J Clin Microbiol* 41:2884–2893
- O'Brien AD, LaVeck GD, Thompson MR, Formal SB (1982) Production of Shigella dysenteriae type 1-like cytotoxin by *Escherichia coli*. *J Infect Dis* 146:763–769
- O'Hanlon KA, Catarama TM, Duffy G, Blair IS, McDowell DA (2004) RAPID detection and quantification of *E. coli* O157/O26/O111 in minced beef by real-time PCR. *J Appl Microbiol* 96:1013–1023
- Okrend AJG, Rose BE, Lattuda (1990) C.P. 5-bromo-4-chloro-3-indoxyl- β -D-glucuronide in sorbitol MacConkey agar to aid in the isolation of *Escherichia coli* O157: H7 from ground beef. *J Food Protect* 53:941–943
- Park CH, Kim HJ, Hixon DL, Bubert A (2003) Evaluation of the duopath verotoxin test for detection of shiga toxins in cultures of human stools. *J Clin Microbiol* 41:2650–2653
- Paton AW, Paton JC (1998a) Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1*, *stx2*, *eaeA*, enterohemorrhagic *E. coli* *hlyA*, *rfbO111*, and *rfbO157*. *J Clin Microbiol* 36:598–602

- Paton JC, Paton AW (1998b) Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clin Microbiol Rev* 11:450–479
- Paton AW, Paton JC (2002) Direct detection and characterization of Shiga toxigenic *Escherichia coli* by multiplex PCR for *stx1*, *stx2*, *eae*, *ehxA*, and *saa*. *J Clin Microbiol* 40:271–274
- Paton AW, Paton JC (2005) Multiplex PCR for direct detection of Shiga toxigenic *Escherichia coli* strains producing the novel subtilase cytotoxin. *J Clin Microbiol* 43:2944–2947
- Paton AW, Paton JC, Goldwater PN, Manning PA (1993) Direct detection of *Escherichia coli* Shiga-like toxin genes in primary fecal cultures by polymerase chain reaction. *J Clin Microbiol* 31:3063–3067
- Perelle S, Dilasser F, Grout J, Fach P (2004) Detection by 5'-nuclease PCR of Shiga-toxin producing *Escherichia coli* O26, O55, O91, O103, O111, O113, O145 and O157:H7, associated with the world's most frequent clinical cases. *Mol Cell Probes* 18:185–192
- Perelle S, Dilasser F, Grout J, Fach P (2007) Screening food raw materials for the presence of the world's most frequent clinical cases of Shiga toxin-encoding *Escherichia coli* O26, O103, O111, O145 and O157. *Int J Food Microbiol* 113:284–288
- Perna NT, Plunkett G 3rd, Burland V, Man B, Glasner JD, Rose DJ, Mayhew GF, Evans PS, Gregor J, Kirkpatrick HA, Posfai G, Hackett J, Klink S, Boutin A, Shao Y, Miller L, Grotbeck EJ, Davis NW, Lim A, Dimalanta ET, Potamousis KD, Apodaca J, Anantharaman TS, Lin J, Yen G, Schwartz DC, Welch RA, Blattner FR (2001) Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7 *Nature* 409:529–533
- Pollard DR, Johnson WM, Lior H, Tyler SD, Rozee KR (1990) Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction. *J Clin Microbiol* 28:540–545
- Possé B, De Zutter L, Heyndrickx M, Herman L (2008a) Novel differential and confirmation plating media for Shiga toxin-producing *Escherichia coli* serotypes O26, O103, O111, O145 and sorbitol-positive and -negative O157. *FEMS Microbiol Lett* 282:124–131
- Possé B, De Zutter L, Heyndrickx M, Herman L (2008b) Quantitative isolation efficiency of O26, O103, O111, O145 and O157 STEC serotypes from artificially contaminated food and cattle faeces samples using a new isolation protocol. *J Appl Microbiol* 105:227–235
- Pulz M, Matussek A, Monazahian M, Tittel A, Nikolic E, Hartmann M, Bellin T, Buer J, Gunzer F (2003) Comparison of a shiga toxin enzyme-linked immunosorbent assay and two types of PCR for detection of shiga toxin-producing *Escherichia coli* in human stool specimens. *J Clin Microbiol* 41:4671–4675
- Rahn K, Wilson JB, McFadden KA, Read SC, Ellis AG, Renwick SA, Clarke RC, Johnson RP (1996) Comparison of Vero cell assay and PCR as indicators of the presence of verocytotoxinogenic *Escherichia coli* in bovine and human fecal samples. *Appl Environ Microbiol* 62:4314–4317
- Read SC, Clarke RC, Martin A, De Grandis SA, Hii J, McEwen S, Gyles CL (1992) Polymerase chain reaction for detection of verocytotoxinogenic *Escherichia coli* isolated from animal and food sources. *Mol Cell Probes* 6:153–161
- Reischl U, Youssef MT, Kilwinski J, Lehn N, Zhang WL, Karch H, Strockbine NA (2002) Real-time fluorescence PCR assays for detection and characterization of Shiga toxin, intimin, and enterohemolysin genes from Shiga toxin-producing *Escherichia coli*. *J Clin Microbiol* 40:2555–2565
- Riley LW, Remis RS, Helgeson SD, McGee HB, Wells JG, Davis BR, Hebert RJ, Olcott ES, Johnson LM, Hargrett NT, Blake PA, Cohen ML (1983) Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N Engl J Med* 308:681–685
- Riordan JT, Viswanath SB, Manning SD, Whittam TS (2008) Genetic differentiation of *Escherichia coli* O157:H7 clades associated with human disease by real-time PCR. *J Clin Microbiol* 46:2070–2073
- Rosser T, Dransfield T, Allison L, Hanson M, Holden N, Evans J, Naylor S, La Ragione R, Low JC, Gally DL (2008) Pathogenic potential of emergent sorbitol-fermenting *Escherichia coli* O157:NM. *Infect Immun* 76:5598–5607
- Russmann H, Schmidt H, Heesemann J, Caprioli A, Karch H (1994) Variants of Shiga-like toxin II constitute a major toxin component in *Escherichia coli* O157 strains from patients with haemolytic uraemic syndrome. *J Med Microbiol* 40:338–343

- Scotland SM, Smith HR, Rowe B (1985) Two distinct toxins active on Vero cells from *Escherichia coli* O157. *Lancet* 2:885–886
- Sekse C, Solberg A, Petersen A, Rudi K, Wasteson Y (2005) Detection and quantification of Shiga toxin-encoding genes in sheep faeces by real-time PCR. *Mol Cell Probes* 19:363–370
- Sharma VK, Dean-Nystrom EA, Casey TA (1999) Semi-automated fluorogenic PCR assays (TaqMan) for rapid detection of *Escherichia coli* O157:H7 and other shiga toxinogenic *E. coli*. *Mol Cell Probes* 13:291–302
- Shearer AE, Strapp CM, Joerger RD (2001) Evaluation of a polymerase chain reaction-based system for detection of *Salmonella enteritidis*, *Escherichia coli* O157:H7, *Listeria* spp., and *Listeria monocytogenes* on fresh fruits and vegetables. *J Food Prot* 64:788–795
- Smith HR, Scotland SM (1993) ACP Broadsheet 135: January 1993. Isolation and identification methods for *Escherichia coli* O157 and other Vero cytotoxin producing strains. *J Clin Pathol* 46:10–17
- Stefan A, Scaramagli S, Bergami R, Mazzini C, Barbanera M, Perelle S, Fach P (2007) Real-time PCR and enzyme-linked fluorescent assay methods for detecting Shiga-toxin-producing *Escherichia coli* in mincemeat samples. *Can J Microbiol* 53:337–342
- Stender H, Oliveira K, Rigby S, Bargoot F, Coull J (2001) Rapid detection, identification, and enumeration of *Escherichia coli* by fluorescence in situ hybridization using an array scanner. *J Microbiol Methods* 45:31–39
- Thomas A, Smith HR, Willshaw GA, Rowe B (1991) Non-radioactively labelled polynucleotide and oligonucleotide DNA probes, for selectively detecting *Escherichia coli* strains producing Vero cytotoxins VT1, VT2 and VT2 variant. *Mol Cell Probes* 5:129–135
- Thompson JS, Hodge DS, Borczyk AA (1990) Rapid biochemical test to identify verocytotoxin-positive strains of *Escherichia coli* serotype O157. *J Clin Microbiol* 28:2165–2168
- Tyagi S, Kramer FR (1996) Molecular beacons: Probes that fluoresce upon hybridization. *Nat Biotechnol* 14:303–308
- Vernozy-Rozand C, Mazuy C, Ray-Gueniot S, Boutrand-Loei S, Meyrand A, Richard Y (1998) Evaluation of the VIDAS methodology for detection of *Escherichia coli* O157 in food samples. *J Food Prot* 61:917–920
- Vernozy-Rozand C, Bouvet J, Montet MP, Bavai C, Ray-Gueniot S, Mazuy-Cruchaudet C, Richard Y (2002) Survey of retail raw milk cheeses for Verotoxin-producing *E. coli* (VTEC) and *E. coli* O157:H7 in France (Poster). In 102th General Meeting of American Society for Microbiology May, 19–20 Salt-Lake City, USA
- Vimont A, Vernozy-Rozand C, Montet MP, Lazizzera C, Bavai C, Delignette-Muller ML (2006) Modeling and predicting the simultaneous growth of *Escherichia coli* O157:H7 and ground beef background microflora for various enrichment protocols. *Appl Environ Microbiol* 72:261–268
- Willford J, Mills K, Goodridge LD (2009) Evaluation of three commercially available enzyme-linked immunosorbent assay kits for detection of Shiga toxin. *J Food Prot* 72:741–747
- Wolter A, Niessner R, Seidel M (2008) Detection of *Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Legionella pneumophila* in water using a flow-through chemiluminescence microarray readout system. *Anal Chem* 80:5854–5863
- Yamaguchi N, Sasada M, Yamanaka M, Nasu M (2003) Rapid detection of respiring *Escherichia coli* O157:H7 in apple juice, milk, and ground beef by flow cytometry. *Cytometry A* 54:27–35
- Yaron S, Matthews KR (2002) A reverse transcriptase-polymerase chain reaction assay for detection of viable *Escherichia coli* O157:H7: investigation of specific target genes. *J Appl Microbiol* 92:633–640
- Yoshitomi KJ, Jinneman KC, Weagant SD (2003) Optimization of a 3'-minor groove binder-DNA probe targeting the uidA gene for rapid identification of *Escherichia coli* O157:H7 using real-time PCR. *Mol Cell Probes* 17:275–280
- Yoshitomi KJ, Jinneman KC, Weagant SD (2006) Detection of Shiga toxin genes stx1, stx2, and the +93 uidA mutation of *E. coli* O157:H7/H-using SYBR Green I in a real-time multiplex PCR. *Mol Cell Probes* 20:31–41
- Yu J, Kaper JB (1992) Cloning and characterization of the eae gene of enterohaemorrhagic *Escherichia coli* O157:H7. *Mol Microbiol* 6:411–417
- Zadik PM, Chapman PA, Siddons CA (1993) Use of tellurite for the selection of verocytotoxinogenic *Escherichia coli* O157. *J Med Microbiol* 39:155–158

Genetic Diversity of Enterococci in Bryndza Cheese

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Abstract Enterococci are gram-positive bacteria occurring in a remarkable array of environments. They can be found in soil, food, and water, and make up a significant portion of the normal gut flora of humans and animals. As other bacteria of the gut flora, enterococci can also cause infectious diseases. On the other hand enterococci are used as probiotics to improve the microbial balance of the intestine, or as a treatment for gastroenteritis in humans and animals. So far, 35 species have been proposed for inclusion in the genus *Enterococcus* considered the most controversial group of lactic acid bacteria. Studies on the microbiota of many traditional cheeses, especially in the Mediterranean countries, have indicated that enterococci play an important role in the ripening of these cheeses, hence contributing to their typical taste and flavour. Adaptability of enterococci to different substrates and growth conditions (low and high temperature, extreme pH, and salinity), allows them to increase in number during milk refrigeration, survive pasteurisation and fermentation. The presence and growth of enterococci in cheeses results in organoleptically unique products, which contribute to the local cuisine and the region's heritage. Due to interlinked European and worldwide markets, these cheeses are widely distributed and are internationally considered as delicacies. In addition to these technological properties, numerous strains of enterococci associated with cheeses, mainly *E. faecium* and *E. faecalis*, produce one or more bacteriocins, and may be considered as protective towards spoilage and pathogenic bacteria. Slovak Bryndza is a natural, white, spreadable cheese manufactured according to the traditional method by milling a lump of matured sheep cheese. The cheese samples were obtained from five different commercial distributors in Slovakia and were taken at three different seasonal intervals. Enterococci are found in high levels; an average value among Bryndza cheese samples ranged between 10^7

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and 10^8 CFU g^{-1} . Three hundred and eight presumed enterococcal isolates were recovered from Bryndza cheese. All isolates were identified to the species level using phenotypical methods and commercial biochemical sets and by genotypic tools, i.e. polymerase chain reaction (PCR) using *ddl* genes and repetitive element sequence (GTG)₅ in combination with phenylalanyl-tRNA synthase gene (*pheS*) sequence analysis and by whole-cell protein analysis (SDS-PAGE). Enterococcal strains were identified as *E. faecium*, *E. durans*, *E. faecalis*, *E. italicus*, *E. casseliflavus*, *E. gallinarum*, *E. hirae*, and eight strains were members of the species *Lactococcus lactis*. Of the seven enterococcal species isolated, three of them, *E. faecium*, *E. faecalis* and *E. durans* were present in all samples studied, with *E. faecium* as the predominant one (50% or more in cheese samples from all producers and seasons as well). Results of biochemical and molecular identification of enterococcal species were in agreement in more than 90%. Since *E. faecium* was found to be a dominant species in all analyzed Bryndza cheese samples, this species was studied in more details. Pulsed-field gel electrophoresis of macrorestriction fragments (PFGE), (GTG)₅-PCR and ERIC-PCR were applied to evaluate genetic diversity within this species. Among 176 *E. faecium* isolates 82 were plasmid positive. Their plasmid DNA was isolated and digested by *EcoRI* and *HindIII* restriction endonucleases. The patterns obtained were compared with those obtained by PFGE, (GTG)₅-PCR and ERIC-PCR. Molecular approaches revealed that there is not only a considerable genetic variability among *E. faecium* isolates among various Bryndza distributors, but even at one distributor at different intervals during 1 year. Plasmid profiling and ERIC-PCR have offered a higher resolution than PFGE and (GTG)₅-PCR. PCR was also used for assessment of presence of *vanA* and *vanB* genes and virulence determinants *gelE*, *agg* and cytolysin genes, namely: *cylL_L*, *cylL_S*, *cylM*, *cylB* and *cylA*. Vancomycin resistance genes *vanA* and *vanB* were not detected. Agar plate testing confirmed the sensitivity to vancomycin. Gene *gelE*, was found in 20 *E. faecalis* isolates, but only 13 of them showed gelatinase-positive phenotype. Seven isolates had five cytolysin genes, but none of the isolates exhibited a positive haemolytic phenotype. Four isolates possessed the *agg* gene. All enterococcal isolates from Bryndza cheese were susceptible to ampicillin, streptomycin, gentamicin, vancomycin, and teicoplanin as determined by the disk diffusion method. Resistance rates of enterococcal isolates to rifampicin, erythromycin, ciprofloxacin, and nitrofurantoin were 24%, 26%, 2%, and 1%, respectively. Thirty percent of the *E. faecium* isolates, 3% of the *E. durans* isolates, and 12% of the *E. faecalis* isolates exhibited multidrug resistance. The highest frequency of resistant enterococci was observed in Bryndza produced in winter season. In addition to this, in a close collaboration with clinics, we have shown that application of non-pathogenic *E. faecium* have an important immunostimulatory and antimutagenic properties and can be a promising method for elimination of pathogenic bacteria in the case of some diseases. Assumption of health benefit effects of Bryndza cheese was confirmed by results of our historically first clinical tests based on a daily consumption of Bryndza cheese during 8 weeks. Statistically significant decrease of total cholesterol and LDL-cholesterol was observed. Since enterococci occur in a remarkable array of environments incl. food and water, are

the most abundant Gram-positive cocci in humans (considered at the same time as the most controversial group of lactic acid bacteria) any study of genetic diversity of enterococci could be useful to evaluate their potential risks or benefits.

Keywords Enterococci • cheese • PFGE • ERIC-PCR • (GTG)5-PCR • probiotics • Bryndza • intraspecies variability

Introduction

Enterococci are gram-positive bacteria and fit within the general definition of lactic acid bacteria. Enterococci occur in a remarkable array of environments. Their notable resistance to adverse environmental conditions explains their ability to colonise different ecological niches. They can be found in soil, food, and water, and make up a significant portion of the normal gut flora of humans and animals. As other bacteria of the gut flora, enterococci can also cause infectious diseases. On the other hand enterococci are used as probiotics to improve the microbial balance of the intestine, or as a treatment for gastroenteritis in humans and animals. So far, more than 30 species have been included in the genus *Enterococcus* considered the most controversial group of lactic acid bacteria. Studies on the microbiota of many traditional cheeses, especially in the Mediterranean countries, have indicated that enterococci play an important role in the ripening of these cheeses, hence contributing to their typical taste and flavour. Slovak Bryndza is a natural, white, spreadable cheese manufactured according to the traditional method by milling a lump of matured sheep cheese. Since enterococci are found in high levels also in the Bryndza cheese their precise identification in this traditional food is an essential step in the process of evaluation of their functional properties.

Enterococci – Occurrence and Properties

Enterococci live as commensals of the gastrointestinal tract of warm-blooded animals, and are the most abundant Gram-positive cocci in humans (Tannock and Cook 2002). In the human intestine *E. faecium* and *E. faecalis* are the most frequent species (Franz et al. 1999). They represent 10^5 – 10^7 CFU g⁻¹ of stool (Kayser 2003). In production animals like cattle, pigs and fowl (Table 1) beside of *E. faecalis* occurs *E. gallinarum* and *durans/hirae* (Klein 2003).

Once released in the environment by means of human faeces or animal defecation, enterococci are able to colonise different ecological niches because of their resistance to adverse environmental conditions. Enterococci are widely spread in nature, in soil, food, on plant and vegetables and surface water. They are used as indicators of faecal pollution in environmental waters (Godfree et al. 1997).

Table 1 Occurrence of enterococci in the gastro-intestinal tract

Species	Human	Cattle	Pig	Fowl
<i>E. faecalis</i>	++	(+)	+	++
<i>E. faecium</i>	++	++	+	++
<i>E. durans/hirae</i>	(+)	–	(+)	(+)
<i>E. gallinarum</i>	(+)	–	–	(+)
<i>E. casseliflavus</i>	(+)	–	–	–
<i>E. cecorum/columbae</i>	–	+	+	++

++: usual; + : frequent; (+): occasional; – : not mentioned (According to Klein (2003))

Table 2 Enterococci in food

Species	Cheese	Fish Crustacea	Meat	Pork carcasses	Sausage	Minced beef	Minced pork
<i>E. faecalis</i>	(+)	+	+	++	++	++	++
<i>E. faecium</i>	++	(+)	++	(+)	–	(+)	(+)
<i>E. durans/hirae</i>	(+)	–	(+)	(+)	(+)	(+)	(+)
<i>E. gallinarum</i>	–	(+)	(+)	ND	ND	(+)	(+)
<i>E. casseliflavus</i>	–	–	(+)	ND	ND	–	(+)
<i>E. mundtii</i>	–	(+)	(+)	ND	ND	ND	ND
<i>E. avium</i>	ND	ND	ND	ND	ND	–	(+)
<i>E. malodoratus</i>	ND	ND	ND	(+)	(+)	ND	ND
<i>E. pseudoavium</i>	ND	ND	ND	(+)	–	ND	ND
<i>E. raffinosus</i>	ND	ND	ND	(+)	–	ND	ND

++: usual; + : frequent; (+): occasional; – : not mentioned; ND: not investigated (Adapted from Klein 2003)

By intestinal or environmental contamination they can colonize raw foods like milk and meat and due to their ability to survive heat treatment, extreme pH and salinity they multiply in these materials during fermentation. Moreover they can contaminate finished products. Overview of enterococci in food is in Table 2 (adapted from Klein 2003).

The genus *Enterococcus* is the most controversial group of lactic acid bacteria. Unlike most of lactic acid bacteria, enterococci are not considered as GRAS (generally recognized as safe). They are potentially dangerous because can be involved in food deterioration (Franz et al. 1999), in food poisoning (Gardini et al. 2001), in nosocomial infections (Kayser 2003) and in the spreading of antibiotic resistance through the food chain (Giraffa 2002). On the other hand numerous strains of enterococci of food origin produce bacteriocins with activity against some pathogenic bacteria in food (Lauková and Czikková 2001). Some enterococcal strains are used as probiotics to improve the microbial balance of the intestine or as a treatment for gastroenteritis in humans and animals (Franz et al. 2003; Eaton and Gasson 2001). Well-known is the technological role of enterococci in the manufacture of

meat and dairy products where they play important role in the ripening of different cheeses and contribute to their typical taste and flavour (Giraffa 2003; Foulquie-Moreno et al. 2006).

Genus Enterococcus – Taxonomy and Identification

Enterococci were originally classified as group D streptococci according to the scheme established by Lancefield. In 1984 DNA-DNA hybridization and 16S rRNA sequencing studies indicated that the species *Streptococcus faecalis* and *S. faecium* were sufficiently distinct from other streptococci and were transferred to the genus *Enterococcus* (Schleifer and Kilpper-Balz 1984). Members of the genus *Streptococcus* were subdivided into three separate genera: *Streptococcus*, *Lactococcus* and *Enterococcus* (Schleifer and Kilpper-Bälz 1987). Enterococci are Gram-positive, non-spore forming, catalase-negative, facultatively anaerobic cocci that occur as a singlet, in pairs or in short chains. They are chemoorganotrophic in nature and produce L-lactic acid from hexoses by homofermentative lactic acid fermentation (Franz et al. 2003).

The typical enterococci can be easily distinguished from other G-positive, catalase-negative cocci by their ability to grow both at 10°C and 45°C, in 6.5% NaCl, in the presence of 40% bile and pH 9.6 and to survive heating at 60°C for 30 min (Foulquie-Moreno et al. 2006). However, it is difficult to unequivocally categorise clinical and environmental isolates into one of the *Enterococcus* species only by physiological tests because of the heterogeneity in phenotypic features. Phenotypic traits can differ, regardless the origin of the isolate (Devriese et al. 1993; Ulrich and Muller 1998). Moreover, some of the recently described enterococcal species exhibit deviations of phenotypical properties from classical enterococci (Domig et al. 2003). Numerous enterococcal isolates, especially from environmental source, often remain unidentified when their identification is based on phenotypic traits alone. Therefore for a reliable and fast identification, especially from sources with a heterogeneous microflora, molecular-based methods are essential.

Molecular Identification

A variety of conserved genes, including 16S rRNA genes, the rRNA intergenic spacer, the D-alanine:D-alanine ligase gene (*ddl* gene), the *sodA* gene encoding superoxide dismutase, and the *tuf* gene for the elongation factor EF-Tu have been used for identification of enterococci (Tyrrell et al. 1997; Monstein et al. 1998; Patel et al. 1998; Ke et al. 1999; Poyart et al. 2000). Teng et al. (2001) used PCR-Restriction fragment length polymorphism (PCR-RFLP) for the *groESL* genes to differentiate *Enterococcus* species. Amplification of the putative transcriptional regulator gene *Ej0027* (Liu et al. 2005), sequencing of the

ddl genes (Ozawa et al. 2000), *cpn60* gene (Goh et al. 2000) and *atpA* gene (Naser et al. 2005) were also used.

Ribotyping, restriction endonuclease analysis (REA), pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP) have been applied to the investigations of *Enterococcus* strains (Murray et al. 1990; Kuhn et al. 1995; Descheemaeker et al. 1997; Pryce et al. 1999; Andrighetto et al. 2001; Švec et al. 2001; Drahovská et al. 2002; Vancanneyt et al. 2002). Differentiation of strains within a single species is carried out using of sets of primers for repetitive DNA sequences, e.g.: BOX, ERIC, (GTG)_n, etc. (Gevers et al. 2001; Švec et al. 2005). The powerful technique for such studies is multilocus sequence typing (MLST) based on identifying alleles from DNA sequences of internal fragments of housekeeping genes (Homan et al. 2002) and multilocus variable analysis (MLVA), based on variable-number tandem repeats (Top et al. 2004; Titze-de-Almeida et al. 2004).

Overview of the division of enterococi into eight species groups is in Franz et al. (2003). So far 35 species (<http://www.bacterio.cict.fr/e/enterococcus.html>, 10 December 2008) have been proposed for inclusion in the genus *Enterococcus*: *Enterococcus aquimarinus*, *E. asini*, *E. avium*, *E. caccae*, *E. camelliae*, *E. canintestini*, *E. canis*, *E. casseliflavus*, *E. cecorum*, *E. columbae*, *E. devriesei*, *E. dispar*, *E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. gilvus*, *E. haemoperoxidus*, *E. hermanni*, *E. hirae*, *E. italicus*, *E. malodoratus*, *E. moraviensis*, *E. mundtii*, *E. pallens*, *E. phoeniculicola*, *E. pseudoavium*, *E. raffinosus*, *E. ratti*, *E. saccharolyticus*, *E. silesiacus*, *E. sulfureus*, *E. termitis*, *E. thailandicus*, *E. villorum*.

E. faecium and *E. faecalis* remain the two most prominent species found especially in food and related habitats (Franz et al. 1999).

Enterococci and Human Health

Over the last decades, enterococci formerly viewed as organisms of minimal clinical impact, have emerged as important hospital-acquired pathogens in immunosuppressed patients and intensive care units. Even though the enterococci are not regarded as highly pathogenic organisms, they are among the most prevalent organisms encountered in nosocomial infections. Enterococci are currently third most common bacterial pathogen associated with nosocomial infections in United States (Wisplinghoff et al. 2004) and the fourth in Europe (<http://www.earss.rivm.nl/>). They are cause of infections such as endocarditis, urinary tract, central nervous system, neonatal, intraabdominal and pelvic infections and hospital acquired bacteremia (Franz et al. 1999). Enterococcal infections may be due to at least 12 enterococcal species (Mundy et al. 2000). Most clinical infections are predominantly caused by *E. faecalis* which accounts for 80–90% of clinical isolates and *E. faecium* which accounts for 5–10% of such isolates (Kayser 2003). However, recent studies indicate (Coque et al. 2005; Treitman et al. 2005; Mundy et al. 2000) that the proportion of *E. faecium*, especially the

multiresistant strains has increased. An increasing incidence was also reported for infections with *E. durans* and *E. hirae* as causative agents (Franz et al. 1999; Perlada et al. 1997; Descheemaeker et al. 2000).

Enterococci as Probiotics

Enterococci have featured in dairy fermentations for decades and isolates with histories of safe usage are being promoted as probiotic cultures. A variety of probiotic supplements are available for human and veterinary use. These range from fermented milks to lyophilised forms, containing both single and multiple strains. Actually about 20% of probiotic preparations in the market contains strains of enterococci – which in 50% consist from *E. faecium* (Ewing and Haresign 1989; Franz et al. 1999; Hamilton-Miller et al. 1996).

Numerous studies have proved the efficacy of the probiotic *Enterococcus faecium* SF68, which is produced in Switzerland (Cylactin® or Bioflorin®). The efficacy of *E. faecium* SF68 in the treatment of antibiotic-associated as well as acute diarrhea in adults and in children was demonstrated (Bellomo et al. 1980; Wunderlich et al. 1989). The strain has been tested in adults with acute diarrhea in two hospitals (Buydens and Debeuckelaere 1996). Loguercio et al. (1995) determined the effect on chronic encephalopathy of long-term administration of *E. faecium* SF68 versus lactulose. The reduction in both blood ammonia concentrations and Reitan's test times was more enhanced in patients on *E. faecium* SF68 than in patients on lactulose.

E. faecium SF68 has also been studied as a feed probiotic. Benyacoub et al. (2005) showed that the probiotic *E. faecium* SF68 offered specific humoral and cellular responsis (increased CD₄⁺ in Peyer's patches and spleen) against *Giardia intestinalis* infection in mice. *E. faecium* SF68 enhance specific immune functions also in young dogs (Benyacoub et al. 2003). The efficacy of a probiotic strain *E. faecium* on the reduction of both the rate of natural infection and the shedding of chlamydiae in swine was demonstrated (Pollmann et al. 2005).

Numerous studies have proven the probiotic efficacy of the strain *E. faecium* M-74 in humans and livestock. The strain was isolated from the intestinal tract of a human infant. It is a proprietary strain of probiotic bacteria patented and marketed by Swedish companies.

It was found that *E. faecium* strain M-74 has an important immunostimulatory and antimutagenic properties presented both in vitro and also in vivo (Ebringer et al. 1995; Mikeš et al. 1995; Ferenčík et al. 1999; Belicová et al. 2004, 2007). The effects of the *E. faecium* enriched with organic selenium was studied in methotrexate treatment in rats with adjuvant arthritis. The results indicate that *E. faecium* may increase the preventive effects of methotrexate in rat adjuvant arthritis by improving its anti-inflammatory and antiarthritic effects (Rovenský et al. 2002).

Clinical evaluation of the activity of *E. faecium* enriched with organic selenium in oncologic patients showed that this probiotic enterococcal strain eliminated intraepithelial pathogenic bacteria in patients with colonic adenoma (Mego et al. 2005b).

Administration of the probiotic *E. faecium* strain probably leads to the competition with and elimination of pathogenic bacteria from the intraepithelial niche. Therefore it can be considered that application of non-pathogenic *E. faecium* can be a promising method for elimination of pathogenic bacteria in the case of inflammatory bowel disease and colon cancer. The same strain of *E. faecium* was used also for the prevention of febrile neutropenia in leukemic patients. Tolerance of therapy was excellent without significant adverse effects. However, the administration of the enterococcal strain was not effected in the prevention of febrile neutropenia but no febrile episode or infection provoked by the probiotic strain *E. faecium* was noticed (Mego et al. 2005a, 2006).

The effect of long-term application of diet containing *E. faecium* M-74 with organic selenium on tumor induction in transgenic mice carrying mutation in *Apc* gen was tested (Hlubinová et al. 2004). Feeding of transgenic mice with enriched diet with *E. faecium* M-74 during 8 months has shown a minor therapeutic effect on the clinical manifestation in small intestine in comparison with control group.

Favourable effect of *E. faecium* M-74 enriched with organic selenium on chronic hepatic encephalopathy was also demonstrated (Boča et al. 2004). The blood ammonia levels as well as the results from the number-connecting test after 8–9 weeks significantly approached the normal pattern. EEG results were improved and they were often normalised. In another clinical study, the administration of *E. faecium* M-74 probiotic strain was associated with reduction of serum cholesterol concentrations by 12% after 56 weeks of oral administration (Hlivak et al. 2005).

The probiotic strain *E. faecium* PR88 was studied in clinical trial (Allen et al. 1996). The consumption of this strain led to alleviation of the symptoms of irritable bowel syndrome in humans. The efficacy and safety of live combined *Bifidobacterium*, *Lactobacillus* and *Enterococcus* capsules in treatment of irritable bowel syndrome was also demonstrated (Fan et al. 2006). *E. faecium* functions as a probiotic strain inhibiting biofilm formation by *Streptococcus* mutants, which is an etiological agent for dental caries (Kumada et al. 2008).

Antibiotic Resistance

The role of enterococci in nosocomial infections is probably due to a variety of factors of which antimicrobial resistance appears to be a primary cause. Enterococci possess a broad spectrum of both natural (intrinsic) resistance and acquired (transferable) resistance (Franz et al. 2003). Examples of antibiotics to which the enterococci present an intrinsic resistance include the β -lactam antibiotics (third generation cephalosporins), sulphonamides and clindamycin and aminoglycosides in low levels (Franz et al. 2003). Acquired resistance based on plasmids or transposons acquisition has relevance for chloramphenicol, erythromycin, high levels of clindamycin, aminoglycosides, tetracycline, high levels of β -lactam antibiotics, fluoroquinolones and glycopeptides like vancomycin (Murray 1990; Leclercq 1997). In particular, vancomycin-resistant enterococci (VRE) pose a major problem

in treating human infections (Cetinkaya et al. 2000). Vancomycin resistance is of special concern because this antibiotic was considered a last resort for treatment of multiple resistant enterococcal infections. The emergence of VRE in hospitals has led to infections that cannot be treated with conventional antibiotic therapy. Of the six different vancomycin resistances known to date, VanC phenotype are type of intrinsic resistance while *vanA*, *vanB*, *vanD*, *vanE* and *vanG* genes encode transferable vancomycin resistant phenotypes (Perichon et al. 1997; Fines et al. 1999). VanA and VanB phenotypes are of the highest clinical importance as they are most frequently observed in two predominant species *E. faecalis* and *E. faecium* (Cetinkaya et al. 2000).

Virulence Factors

Antibiotic resistance alone cannot explain the virulence of enterococci. Enterococci may carry different genes directly or indirectly contributing to their virulence (Eaton and Gasson 2001; Kayser 2003). Some of virulence factors such as aggregation substance or cytolysin are encoded by conjugative plasmid genes, and genes for others factors are arranged in different chromosomal regions (Shankar et al. 2002; Hufnagel et al. 2004). Adherence factors allow attachment to host cells and to extracellular matrix protein. Aggregation substance (AS) is an adhesin that is encoded on pheromone-responsive plasmids. AS contributes to pathogenicity by enhancing adhesion and to internationalization by cultured human cells and facilitate the conjugative exchange of plasmids (carrying virulence and/or antibiotic resistance genes) (Clewell 1993). Enterococcal surface protein (Esp) is a surface protein involved in the ability to colonize and in the immune evasion (Shankar et al. 2001). The presence of Esp also increase cell hydrophobicity, adherence to abiotic surfaces and biofilm formation in vitro (Toledo-Arana et al. 2001). Esp exhibits characteristics of surface protein receptors designated “microbial surface components recognising adhesive matrix molecules” (MSCRAMMs) that mediate binding to extracellular matrix proteins (Toledo-Arana et al. 2001). Production of the adhesin-like endocarditis antigens EfaA is considered to be potential virulence determinant (Singh et al. 1998). Ace (adhesion of collagen from *E. faecalis*) is similar to MSCRAMMs of other Gram-positive bacteria, particularly to the collagen-binding protein Cna of *S. aureus* (Rich et al. 1999). Ace not only binds to collagen (types I and IV) but also to laminin (Nallapareddy et al. 2000). In addition to adhesion function, aggregation substance favors intracellular survival of enterococci in host immune cells such as macrophages and neutrophils (Süßmuth et al. 2002). The major determinants of virulence known so far to play some role in the pathogenesis are β -haemolysin or cytolysin and secreted proteases – gelatinase and serin protease. Cytolysin is a confirmed enterococcal virulence factor, which enhances virulence in animal models (Ike et al. 1984; Gilmore et al. 1994). Cytolysin genes are carried on a plasmid or are integrated into the bacterial chromosome (Jett et al. 1994). The cytolysin operon consists of five genes, of which

cylL1, *cylL2*, *cylM*, and *cylB* are relevant to the expression of component L (lysin), whereas *cylA* is necessary for the expression of component A (activator) (Gilmore et al. 1990; Ike et al. 1987). In Japan 60% of clinical strains had a haemolytic phenotype, compared with only 17% isolates from healthy individuals (Ike et al. 1987). Similar trends were observed in USA (Huycke et al. 1991), but in Germany only 16% *E. faecalis* strains isolated from blood had haemolytic activity (Elsner et al. 2000). Gelatinase is an extracellular metallo-endopeptidase involved in the hydrolysis of gelatin, collagen, haemoglobin and other bioactive peptides (Su et al. 1991). Hyaluronidase is linked to pathogenesis of other microorganisms, but there is no direct evidence for the role of hyaluronidase in disease caused by enterococci (Jett et al. 1994). Three genes *fsr* regulate the expression of *gelE* and *sprE*. Interestingly, there are no data on the presentation of the Fsr system in enterococci from foods or probiotic strains (Franz et al. 2003).

Different and distinct patterns of virulence incidence were found for the *E. faecalis* and *E. faecium* strains. *Enterococcus faecalis* which is known to be most responsible for enterococcal infections has been reported to generate a variety of factors important for virulence, including aggregation substance (Galli et al. 1989), sex pheromones (Sannomiya et al. 1990), MSCRAMM protein (Rich et al. 1999), cytolysin (Coburn and Gilmore 2003), extracellular proteases (gelatinase and serine protease) (Engelbert et al. 2004) and hyaluronidase (Kayaoglu and Orstavik 2004). *E. faecium* strains were generally free of virulence determinants, however *esp* and *gelE* determinants were identified in *E. faecium* medical strains (Eaton and Gasson 2001).

Medical *E. faecalis* strains had more virulence determinants than the food strains, which in turn, had more virulence determinants than the starter strains (Eaton and Gasson 2001).

Enterococci in Food

Enterococci may contaminate the milk either directly from contact with animal faeces or indirectly from a contaminated water sources, milking equipment or the bulk storage tank (Giraffa 2003). Adaptability of enterococci to different substrates and growth conditions i.e. (low and high temperature, extreme pH, and salinity), allows them to increase in number during milk refrigeration, survive pasteurisation and fermentation. Thus they can be found either in food products manufactured from raw materials (milk and meat) or in heat treated food products. Moreover during food processing they can cause contamination of finished products (Foulquie-Moreno et al. 2006).

The contribution of enterococci to the organoleptic properties of fermented food products and their ability to produce bacteriocins (enterocins) are important characteristic for their application in food technology. In recent years there have been considerably increased reporting about enterococci used as starter cultures or co-cultures (adjuncts) (Foulquie-Moreno et al. 2006). Therefore enterococci can be an important part of the fermented food products such as fermented cheeses and meats.

Enterococci in Cheese

Enterococci occur in a variety of cheeses, especially artisan cheeses produced in mostly Mediterranean countries from raw or pasteurized goat's, ewe's, water-buffalo's or bovine milk. Numbers of enterococci in different cheese curd range from 10^4 to 10^5 CFU g⁻¹, and in the fully ripened cheeses their range is from 10^5 to 10^7 CFU g⁻¹ (Franz et al. 2003). The most frequently isolated species are *E. faecalis* and *E. faecium* (Gelsomino et al. 2001), followed by *E. durans* (Cosentino et al. 2004; Suzzi et al. 2000). The European cheeses in which enterococci have been studied are in Table 3.

Table 3 European cheese products which involved enterococci

Origin	Product	Reference
France	Comté	Bouton et al. 1998
	Roquefort	Devoyod 1969
	Saint nectaire cheeses	Duthoit et al. 2003
	Venaco	Casalta and Zennaro 1997
Greece	Feta cheeses	Sarantinopoulos et al. 2002; Manolopoulou et al. 2003; Litopolou-Tzanetaki et al. 1993
	Feta and teleme	Tzanetakis and Litopolou-Tzanetaki 1992
	Kefalotyri	Litopolou-Tzanetaki 1990
	Orinotyri	Prodromou et al. 2001
	Pichtogalo chanion	Papageorgiou et al. 1998
Ireland	Cheddar cheeses	Gelsomino et al. 2001
Italy	Artisanal Fiore Sardo cheese	Cosentino et al. 2004
	Artisanal goat's cheese (semicotto caprino)	Suzzi et al. 2000
	Artisanal Montasio cheeses	Marino et al. 2003
	Buffalo Mozarella	Parente et al. 1989
	Caprino d' áspromonte	Caridi et al. 2003
	Fiore Sardo	Ledda et al. 1994
	Fontina	Battistotti et al. 1977
	Montasio	Basso et al. 1994
	Monte Veronese	Torriani et al. 1998
	Mozarella	Morea et al. 1999
	Pecorino Sardo	Mannu and Paba 2002
	Semicotto Caprino	Suzzi et al. 2000
	Traditional goat and buffalo cheeses	Andrighetto et al. 2001
	Traditional Alps (Vanoi and Rolle) cheeses	Poznanski et al. 2004
Portugal	Waterbuffalo mozzarella cheese	Villani and Coppola 1994
	Model curdled goat's milk	Pintado et al. 2001
	Serra da Estrela	Macedo et al. 1995, Tavaría and Malcata 1998
	Picante de beira baixa cheese	Freitas et al. 1996

(continued)

Table 3 (continued)

Origin	Product	Reference
Slovakia	Bryndza	Jurkovič et al. 2006a, b, 2007; Drahovská et al. 2004
Slovenia	Tolminc cheeses	Majhenič et al. 2005
Spain	Armada	Tornadijo et al. 1995
	Arzuar	Centeno et al. 1995
	Cebreiro	Centeno et al. 1996, 1999
	Five different artisanal cheeses	Delgado et al. 2002
	La Serena	Del Pozo et al. 1988
	Majorero	Fontecha et al. 1990
	Manchego	Ordoñez et al. 1978
	Roncal-Idiazabal	Arizcun et al. 1997
	San Simon	García et al. 2002
	Serra ewe's cheeses	Macedo et al. 1995
	Tenerife goat cheese	Zárate et al. 1997
	Tetila	Menéndez et al. 2001

The enterococci contribute to the ripening and aroma development of cheese due to their proteolytic and esterolytic activities, as well as their diacetyl production by citrate metabolism (Centeno et al. 1999). The presence and growth of enterococci in cheeses and sausages results in organoleptically unique products, which contribute to the local cuisine and the region's heritage. Due to interlinked European and worldwide markets, these cheeses are widely distributed and are internationally considered as delicacies (Franz et al. 1999).

In addition to these technological properties, numerous strains of enterococci associated with cheeses, mainly *E. faecalis* and *E. faecium*, produce one or more bacteriocins, which may be considered as protection against spoilage and pathogenic bacteria (De Vuyst et al. 2004; Giraffa et al. 1995). Different enterocins possess activity against *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium* spp., including *Clostridium botulinum* and *Clostridium perfringens*, and *Vibrio cholerae* (Ennahar et al. 1998; Foulquie-Moreno et al. 2003; Giraffa et al. 1995; Maisnier-Patin et al. 1996; Núñez et al. 1997; Lauková and Czikková 1999; Sarantinopoulos et al. 2002; Simonetta et al. 1997).

Bryndza Cheese – History and Production

Slovak Bryndza is a traditional Slovak product which is closely bound up with the history of Slovakia. Whilst it is hard to know who and when produced the first sheep cheese there exist several versions about the origin of Bryndza production. Some of them indicate the Vallachian or Rumanian origin. In Rumania the term Bryndza is used to identify virtually all kinds of cheese but none of those is identical with the Slovak Bryndza. In Russia Bryndza denotes certain salt cheese.

Originally, Bryndza was produced from unpasteurized sheep cheese. In the sixteenth and seventeenth centuries sheep cheese was stored for winter consumption in sheep's or goat's skin and food stability was improved by salting and smoking. Later the sheep cheese was crushed, salted and pressed into clay vessels or wooden barrels which called it barrel cheese "sudovka". The industrial production of Bryndza began in the second half of the eighteenth century when Slovakia was a part of Austro-Hungarian empire. Ján Vagač, a merchant and butcher from Stará Turá, started to produce the local variety of Bryndza and in 1787 he founded the first Bryndza factory in Detva. The second Bryndza factory was founded in Zvolenská Slatina by Peter Molec in 1797. In the nineteenth century there was a proliferation of other Bryndza factories. For example, Peter Makovický founded one in Ružomberok (1884) and another one in Tisovec (1897). Since 1921 the Tisovec production has been in hands of Manica family and produces Bryndza even at present. Several small factories started in Liptov, Horehronie (valley along upper part of the river Hron), Považie (valley along the river Váh). The Bryndza processing was improved by Teodor Vallo who introduced the soft and spreadable Bryndza cheese in the kind known today. He modified the production by using a cylinder mill with the different speeds of the cylinders and addition of the salt as a salt solution.

Specific Attributes of the Slovak Bryndza Cheese

Today the production of Bryndza is carried out in Bryndza factories, which buy the cheese. The farmstead artisan sheep cheese made from raw milk is placed on a wooden or stainless-steel shelf to ferment. During fermentation for 2–3 days, the lump of cheese is turned over regularly and temperature may not be lower than 18–20°C. The cheese reaches maturity in Bryndza factory at a temperature around 15°C, over a period of 3–6 days. The matured lump cheese is carefully cleaned by scraping away the dried layer and placed in a pressing vat for 2 days. Lump sheep cheese alone or mixed with cow's cheese is crushed and ground in predetermined and the required proportions which contain minimum 50% lump sheep cheese. The ground cheese is homogenized by careful stirring in the mixing device. During the stirring, salt or a saturated salt solution is added in order to reach the required salt and dry matter contents.

In July 2008 "Slovenská bryndza" (Slovak Bryndza) was granted the Protected Geographical Indications – PGI by EU and was registered in the Register of Protected Designations of Origin and Protected Geographical Indications. 'Slovenská bryndza' is characterized as a natural, white, spreadable cheese manufactured according to the traditional method, by milling a lump of matured sheep cheese or by milling a mixture of lump sheep cheese and lump cow cheese. The percentage of lump sheep cheese is greater than 50%. It has a delicate odour and taste and has a pleasantly sour sheep cheese taste that is slightly spicy and salty. In winter, the only way of producing Bryndza was using surplus summer reserves of sheep cheese. A special preservation method was

introduced where sheep cheese was thoroughly salted (5–6% of salt) and stored in wooden barrels in cellars for several months. Sheep cheese preserved this way (barrel cheese) is ground and mixed with cow cheese and processed to produce so called “winter” Bryndza.

The specific attribute of the production of Slovenská bryndza is the crushing and milling of matured lump cheese in a cutting machine and then in a cylinder mill with the different speeds of the cylinders, then cheese is mixed with salt or a saturated salt solution to process the ingredients into a delicate, spreadable cheese. This specific procedure diversifies Slovenská bryndza from other sheep cheese products made in other countries. Characteristic sensory properties originate from the natural microflora present in the lump sheep cheese, which is produced from raw sheep milk using a specific production technique.

During the first stage of cheese production – the fermentation, microorganisms ferment mainly milk sugar whereby lactic acid as well as other organic acids in small amount are formed. These acids and inhibition compounds (bacteriocins, H_2O_2) destroy many of potential food pathogens mainly *Salmonella*, *Shigella*, *Listeria*, and *Escherichia*. In the second stage of cheese production – the maturation, further microbiological and biochemical processes, especially proteolytic and lipolytic, take place. All these microbial metabolites generate and ensure specific organoleptic properties of Bryndza.

The specific microflora of Bryndza and its connection to the quality of the product were already noted in 1917 in the Codex Alimentarius Austriacus. The old name ‘Karpthenkokkus’ was used by Professor Otakar Laxa in 1924 for the natural mixture of lactic acid bacteria. He also used the name ‘Oidium Lactis’ for a noble mould known as *Galactomyces geotrichum* in the current classification (EU Official Journal C 232, 04/10/2007). Slovenská bryndza contains a broad range of naturally occurring microorganisms, predominantly: lactic acid bacteria of the genera *Lactobacillus*, *Enterococcus*, *Lactococcus* (Drahovská et al. 2004; Jurkovič et al. 2006a, b; Jurkovič et al. 2007) and microscopic fungi *Geotrichum* sp., *Kluyveromyces* (*K. lactis*/*K. marxianus*) as the most abundant yeasts and *Mucor circinelloides* as the predominant filamentous fungus. Occasionally other yeasts, such as *Candida inconspicua*, *Candida silvae*, *Pichia fermentans* and *Trichosporon domesticum* were found (Laurenčík et al. 2008).

Enterocci in Bryndza

A high number of enterococci were found in Slovak Bryndza cheese. An average value of enterococci in Bryndza cheese samples ranged between 10^7 and 10^8 CFU g^{-1} . Three hundred and eight strains were isolated during microbial analysis of Bryndza cheese from five different commercial distributors from middle and south-west Slovakia, namely Liptovský Mikuláš (LM), Ružomberok (R), Červený Kameň (CK), Tisovec (T), and Zvolenská Slatina (ZS), taken at three different seasons – June, October and January. June Bryndza was produced from fresh fermented sheep

cheese from raw sheep milk, October Bryndza was made from a mixture (1:1) of fresh fermented sheep cheese prepared from raw sheep milk and fermented cow cheese made from pasteurized cow milk, and January Bryndza was made from a mixture (1:1) of barrel salted sheep cheese and fermented cow cheese (Jurkovič et al. 2006a).

Species Composition – Phenotypic Versus Genotypic Identification

Using phenotypical methods and commercial biochemical sets five enterococcal species, namely *E. faecium*, *E. durans*, *E. faecalis*, *E. mundtii* and *E. casseliflavus* were identified (Jurkovič et al. 2006a). Using PCR with species specific primers for *ddl* gene (Dutka-Malen et al. 1995), 175 isolates were identified as *E. faecium* and 41 isolates as *E. faecalis*. The identity of other 92 isolates, non *E. faecium/faecalis* strains, was genotypically investigated using (GTG)₅-PCR and cluster analysis with reference strains according to a method of Versalovic et al. (1994). Each cluster comprised reference strain(s) belonging to one enterococcal species. The main cluster (No. 4, see Fig. 1) was assigned to the species *E. durans* (59 isolates), in which several subclusters were delineated according to their cheese origin. The next cluster (No. 2) was assigned to the species *E. faecalis* (nine isolates), followed by *E. italicus* (eight isolates, cluster No. 6), *E. casseliflavus* (three isolates, cluster No. 7), *E. gallinarum* (three isolates, cluster No. 8), *E. faecium* (1 isolate, cluster No. 3) and *E. hirae* (1 isolate, cluster No. 1). By means of (GTG)₅-PCR eight strains were identified as *L. lactis*, which were not distinguishable from *Enterococcus* sp. by conventional phenotypic methods. Isolates assigned to the species *Lactococcus lactis* were grouped in two clusters – *L. lactis* subsp. *lactis* (seven isolates, cluster No. 5) and *L. lactis* subsp. *hordinae* (1 isolate, cluster No. 9). Identification of enterococci on the species level based on (GTG)₅-PCR clusters was confirmed by *pheS* gene sequence analysis and by whole-cell protein profiles in SDS-PAGE (Jurkovič et al. 2006b).

Results of biochemical and molecular identification of enterococcal species were in agreement in more than 90%. Eleven isolates identified as *E. mundtii* using commercial biochemical tests (EN-COCCUStest, STREPTOtest 16 (Pliva-Lachema, Czech Republic), and BBL Crystal Gram-Positive ID kit (Becton Dickinson, MD, USA)) were shown to be *E. faecalis* (9 strains), *E. faecium* (one strain) and *E. hirae* (one strain) when using molecular approaches. Similarly, three isolates identified biochemically as *E. casseliflavus* were genotypically determined as *E. gallinarum*. Using molecular approaches resting 16 *Enterococcus* sp. isolates were proved to be *E. italicus* (8 strains) and *L. lactis* (8 strains).

The predominant enterococcal species was *E. faecium* (176 isolates), followed by *E. durans* (59), *E. faecalis* (50), *E. italicus* (8), *E. casseliflavus* (3), *E. gallinarum* (3) and *E. hirae* (1). *E. faecium* predominated in Bryndza samples obtained from all five distributors. *E. durans* was the second most frequently determined species,

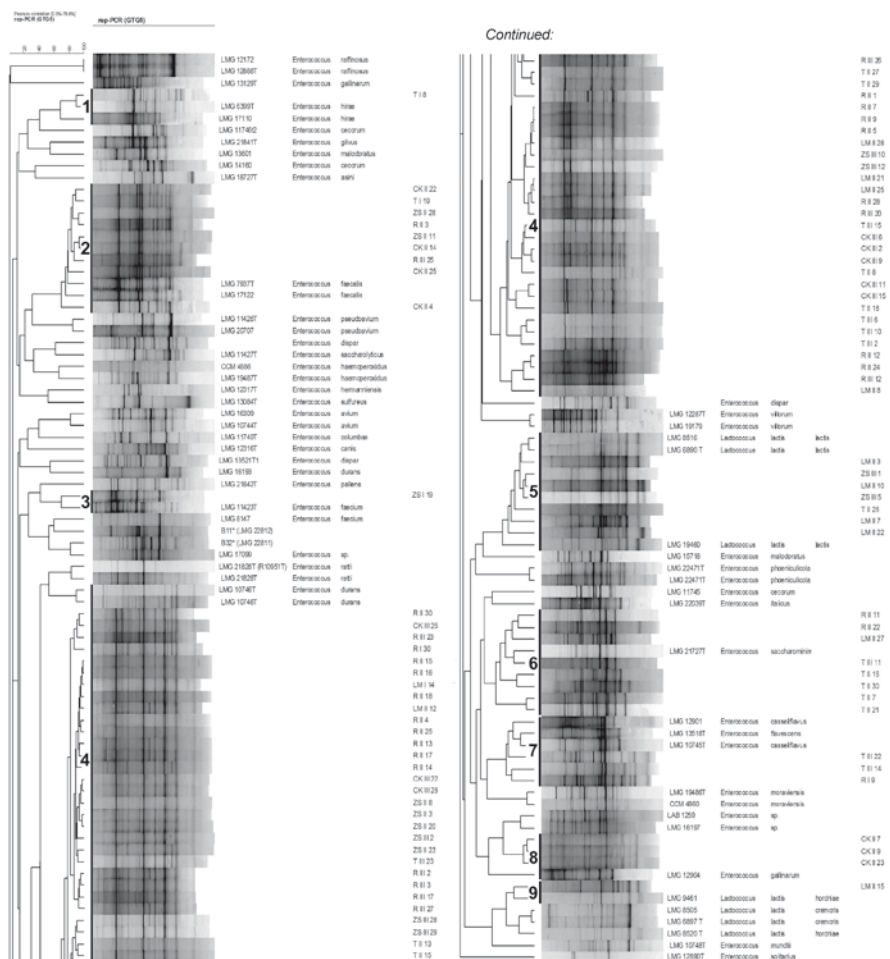


Fig. 1 Dendrogram based on cluster analysis of digitized (GTG)₅-PCR fingerprints of type, reference and Bryndza cheese strains. The dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using correlation levels expressed as percentage values of the Pearson correlation coefficient. Numbered clusters (1–9) correspond to identified species of *Enterococcus* sp. and *Lactococcus* sp.

in all except in Zvolenská Slatina samples. Other enterococcal species were found with lower frequencies (Fig. 2). The highest species diversity was detected in Bryndza cheese from Tisovec (six species), while in Bryndza cheese from Zvolenská Slatina only three enterococcal species were found. *E. faecium*, *E. durans* and *E. faecalis* were found in all three seasons (January, June and October), and *E. italicus* and *L. lactis* in two seasons (June and October). The distribution of other Bryndza species (*E. casseliflavus*, *E. gallinarum* and *E. hirae*) was not equal

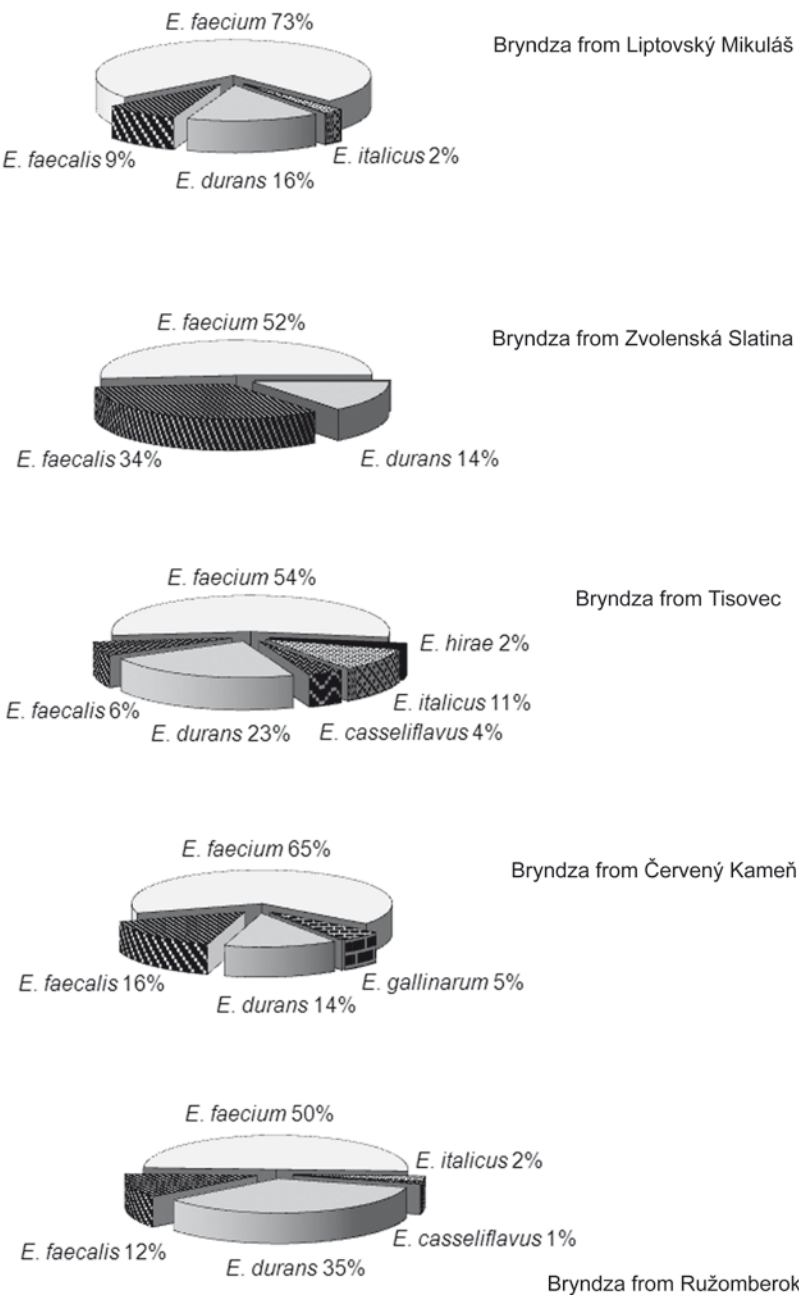


Fig. 2 Species composition of enterococci in Bryndza from different producers

through the seasons and regions. *E. faecium* was the dominant enterococcal species in Bryndza cheese and the most prevalent in the winter season product (Fig. 3).

Enterococci are a heterogeneous group of Gram-positive cocci sharing many characteristics with other lactic acid bacteria, e.g. genus *Lactococcus* (Gram staining, aesculin hydrolysis, growth at 10°C, 45°C, and in 6.5% NaCl, and in presence of 40% of bile salts) (Devriese et al. 1993; Facklam and Elliot 1995). As a result their identification is challenging, therefore *Lactococcus* sp. was not identified using conventional phenotypic tests. It is difficult to unequivocally categorize isolates into one of the *Enterococcus* species by only physiological tests because heterogeneity in phenotypic features is very high, regardless of the origin of the isolate (Giraffa 2002). Identification of enterococci using traditional phenotypic differentiation can be a tedious process requiring numerous tests. Although more than 20 species can be identified using these methods, tests often require long incubation

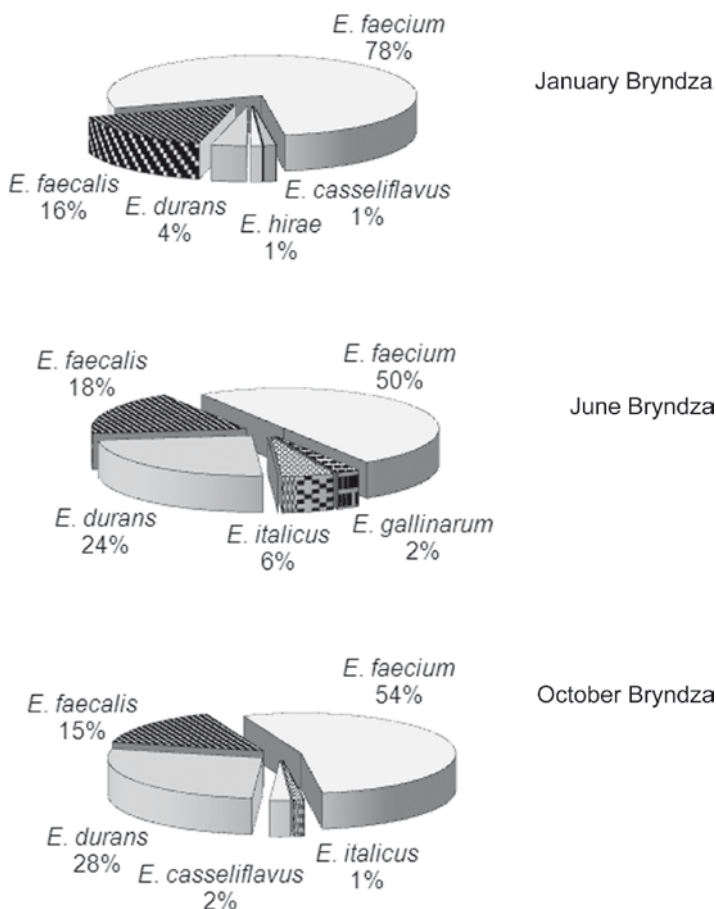


Fig. 3 Species composition of enterococci in Bryndza from different seasons

periods before results can be interpreted. Time constraints and number of samples to be processed can be overcome by using commercial biochemical kits. The accuracy of these, however, has been assessed using only type strains and strains from clinical sources, and they usually identify a maximum of ten enterococcal species (Jackson et al. 2004). Similarly, atypical biochemical profiles of Bryndza isolates may be due to different origin of strains (clinical versus environmental). Our results derived from biochemical tests did not reveal the accurate or comprehensive enterococcal composition of Bryndza cheese, namely 4.1% (12 of 292) of enterococci were not identified to species level and 7.2% (21 of 292) did not agree with genotypic findings. Andrighetto et al. (2001) isolated 124 enterococcal strains from traditional Italian cheeses. Identification using the rapid ID 32 Strep galleries gave different results from SDS-PAGE in 12.1% of the cases. Franzetti et al. (2004) showed that among 64 *Enterococcus* sp. from different origins, biochemical identification (API 20 Strep) correlated with PCR using species-specific primer only in 57.8%. Harwood et al. (2004) reported that among 139 *Enterococcus* sp. isolated from various sources, 58 (42.2%) *E. faecalis* determined biochemically were also confirmed by PCR. However, of 27 (19.5%) biochemically classified *E. faecium*, only seven (5.0%) showed PCR-positive result.

The enterococcal composition of Bryndza cheese corresponds with those of other European artisanal cheeses, where *E. faecium* and *E. faecalis* represent the dominant enterococcal microflora (Franz et al. 1999; Giraffa et al. 1997), although there are exceptions. Cosentino et al. (2004) showed that in artisanal Fiore Sardo cheese *E. durans* is more frequently detected (20.3%) than *E. faecalis* (8.5%). Arizcun et al. (1997) identified *E. faecalis* as the most frequent (85%) enterococcal species in Roncal and Idiazábal cheese in which *E. faecium*, *E. durans* and *E. avium* were present in lower counts, while in traditional Italian cheeses, *E. faecalis* was found to be predominant enterococcal species (Andrighetto et al. 2001) and *E. casseliflavus* predominates in Irish farmhouse cheese Baylough (Gelsomino et al. 2002).

Intraspecies Diversity – Molecular Approaches

Pulsed-Field Gel Electrophoresis (PFGE) and Rep-PCR

Because *E. faecium* was found to be a dominant species in Bryndza cheese in all analyzed samples, this species was studied in more details. The genetic variability of *E. faecium*, intraspecies strain relationships, and possible clonal dissemination of strains were analyzed in order to study the potential correlation of the occurrence of organisms with the geographic origin, the respective producers and the time of sampling. The method PFGE of genomic macrorestriction fragments, (GTG)₅-PCR and ERIC-PCR were applied for evaluation of genetic relatedness using total genomic DNA. PFGE was performed in accordance with method described by Gelsomino et al. (2002) (GTG)₅-PCR was performed according to a method of

Versalovic et al. (1994), ERIC-PCR was a modification of the method of Barbier et al. (1996), only ERIC1 primer was used.

Since PFGE of genomic macrorestriction fragments has gained wide acceptance for establishing of clonal relatedness within many bacterial species including *E. faecium* and *E. faecalis* (Murray et al. 1990; Gordillo et al. 1993; Chiew and Hall 1998; Dicuonzo et al. 2001), the dendrogram in Fig. 4 was constructed

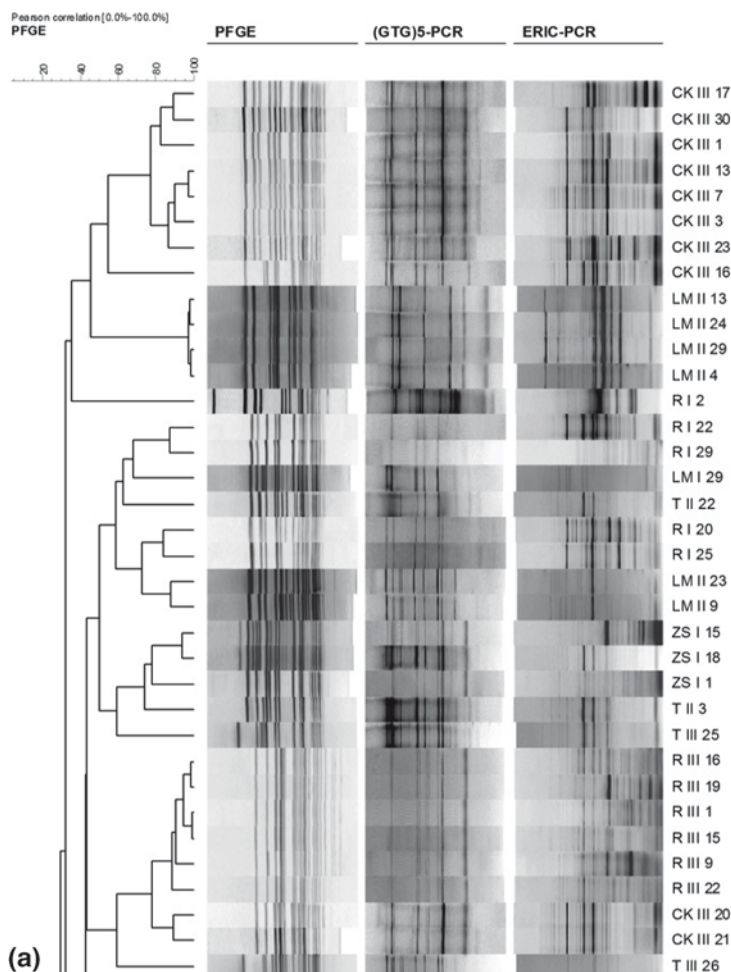


Fig. 4 (a) PFGE, (GTG)₅-PCR, ERIC-PCR fingerprints of 82 *E. faecium* isolates. The dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using correlation levels expressed as percentage values of the Pearson correlation coefficient. Bryndza producers: CK – Červený Kameň, LM – Liptovský Mikuláš, R – Ružomberok, T – Tisovec, ZS – Zvolenská Slatina. Seasons: I – January, II – June, III – October (continued on b).

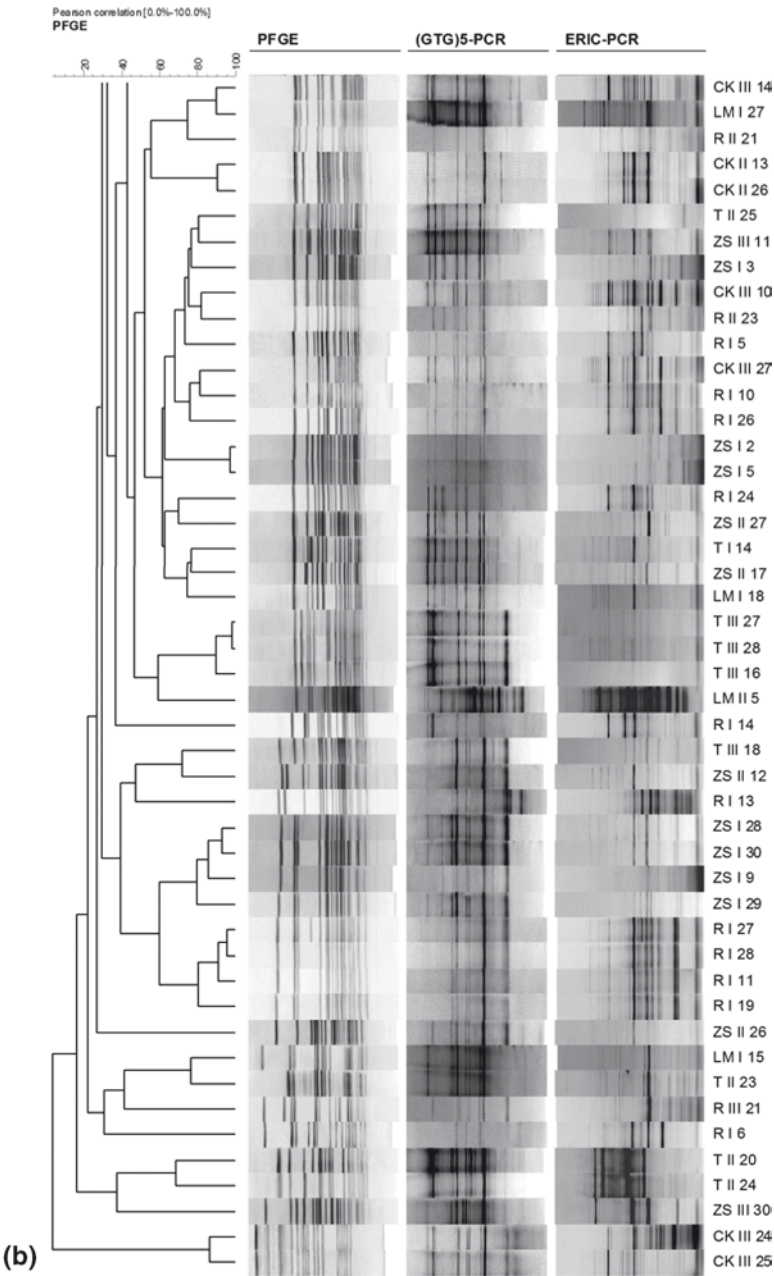


Fig. 4 (continued) **(b)** PFGE, (GTG)5-PCR, ERIC-PCR fingerprints of 82 *E. faecium* isolates. The dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using correlation levels expressed as percentage values of the Pearson correlation coefficient (For further legends see Fig. 4a)

using PFGE profiles. We next evaluated the use of (GTG)₅-PCR for intraspecies differentiation of *E. faecium*. As illustrated in Fig. 4 the grouping of (GTG)₅-PCR fingerprints was highly similar with those from PFGE and shows that both analytical approaches yield an analogous taxonomic resolution. Higher discriminatory power was revealed by using ERIC-PCR which even allowed the differentiation of the isolates within clusters generated using PFGE, e.g. of isolates from Červený Kameň sampled in September (CK III) or Ružomberok sampled in January (R I).

When studying intraspecies diversity of *E. faecium* isolates it was shown that most PFGE clusters contain isolates obtained from the same producer. This supports our hypothesis about the unique geographical effect on samples origin. When monitoring the strain dissemination of *E. faecium* during all three seasons in one type of Bryndza distributor, it seems that there was probably no strain circulation of *E. faecium* within 1 year in Bryndza cheese. Most types of these isolates originated from the same producer. However, the results are ambiguous as some clusters and subclusters comprise of the fingerprints originated from various producers and different time periods of sampling. It most probably means there was no strict connection between one type of producer and one particular season of sampling. Moreover, ERIC-PCR and (GTG)₅-PCR, showed that strain diversity within one cluster is even higher than that evaluated by PFGE.

Based on these findings, we can note that there is not only a considerable genetic variability among *E. faecium* isolates among various Bryndza distributors, but also at one distributor at different intervals during 1 year. When analyzing the intraspecies genotypic variability in *E. durans*, we discovered that few subclusters contain isolates originating from the same producer which supports our hypothesis about the uniqueness of samples of origin. Moreover, each cheese brand was dominated by strains from different subclusters confirming the high genetic diversity of enterococcal strains. This observation is interesting when monitoring the strain dissemination of *E. durans* during all three seasons. The evaluation of strain diversity of remaining enterococcal species using dendrogram based on (GTG)₅-PCR gave ambiguous results due to the low number of identified isolates.

Plasmid Profile Analysis

Eighty-two *E. faecium* isolates recovered from Bryndza were proven to possess the plasmid DNA (Jurkovič et al. 2007). pDNA was digested using *EcoRI* and *HindIII* restriction endonucleases and used for gel electrophoresis. Plasmid profile analysis carried out with 82 isolates confirmed the taxonomic value of this method as a supplementary one for typing of enterococci as described by several authors (Donabedian et al. 1992, Mannu et al. 1999, Son et al. 1999). The *HindIII* restriction endonuclease-digestion appears to have higher discriminatory power than that of *EcoRI* as more DNA fragments were obtained. The plasmid profile analysis should be only considered as an additional approach to diversity study because of possible loss of bacterial plasmids.

Antimicrobial Susceptibility and Virulence Determinants

Enterococcal strains isolated from Bryndza cheese were tested for susceptibility to nine antimicrobial agents by disk diffusion assay (Belicová et al. 2007) according to the methods recommended by the National Committee for Clinical Laboratory Standards (NCCLS 2003). Eighty-two isolates of *E. faecium* harboring plasmid for the presence of selected genes encoding resistance to vancomycin *vanA* (Dutka-Malen et al. 1995), streptomycin *aph(3')-IIIa*, gentamicin *aac(6')-Ie-aph(2'')-Ia* (Vakulenko et al. 2003), tetracycline *tet(L)*, *tet(M)*, chloramfenicol *cat* (Aarestrup et al. 2000) and erythromycin *erm(B)* (Volokhov et al. 2003) using PCR were screened. Gelatinase and haemolytic activity of enterococcal strains was evaluated by standard agar plate assays. PCR was also used for assessment of presence of virulence determinants *gelE*, *agg* and cytolysin genes namely: *cylL_L*, *cylL_S*, *cylM*, *cylB* and *cylA* (Jurkovič et al. 2006a).

The Antibiotic Resistance Patterns

Antibiotic resistance behaviour of 308 enterococci isolated from Bryndza cheese is shown in Fig. 5. All enterococcal isolates from Bryndza cheese were susceptible to ampicillin, streptomycin, gentamicin, vancomycin, and teicoplanin. For these same isolates, resistance rates to rifampicin, erythromycin, ciprofloxacin, and

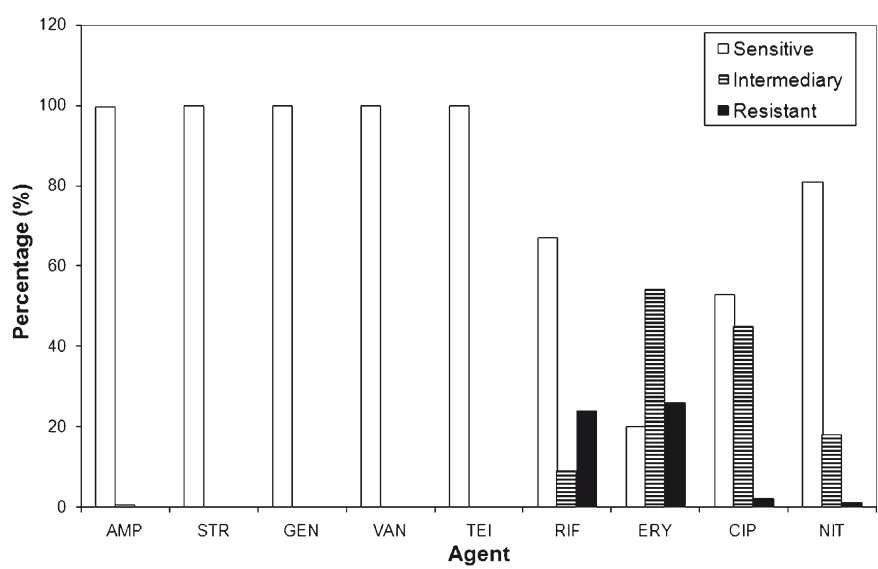


Fig. 5 Antibiotic resistance behaviour of 308 enterococci isolated from Bryndza. Ampicillin (AMP), streptomycin (STR), gentamicin (GEN), vancomycin (VAN), teicoplanin (TEI), rifampicin (RIF), erythromycin (ERY), ciprofloxacin (CIP), and nitrofurantoin (NIT)

nitrofurantoin were 24%, 26%, 2%, and 1%, respectively. Hundred percent susceptibility of enterococcal isolates from Bryndza to both β -lactam antibiotics and aminoglycosides have a great importance from medical point of view since in combination they are valuable therapeutics in enterococcal infections such as endocarditis and meningitis. Similarly the susceptibility of enterococcal isolates from Bryndza to vancomycin and teicoplanin (glycopeptide antibiotics) is very important as they are used as therapeutic alternatives against multiple-resistant enterococci and other Gram-positive bacteria (e.g. enterococci possessing ampicillin resistance, highly resistant enterococci to aminoglycosides or in patients allergic to penicillins). In enterococcus food isolates a moderate resistance to macrolides (erythromycin) and rifampicin is relatively common. The enterococcal isolates from Bryndza were also observed to be resistant to these antibiotics and this may be of some concern. On the other hand, only very low resistance to ciprofloxacin and nitrofurantoin was detected.

Figures 6–8 illustrate antibiotic resistance patterns of *E. faecium*, *E. faecalis*, and *E. durans* isolates from Bryndza cheese. No one of the *E. faecium*, *E. durans*, and *E. faecalis* isolates were resistant to ampicillin, streptomycin, gentamicin, vancomycin, and teicoplanin. Thirty six percent of the *E. faecium* isolates and 22% of the *E. faecalis* isolates were resistant to erythromycin. *E. faecium* showed similar resistance to rifampicin (31%) as *E. faecalis* (29%). Both *E. faecium* and *E. faecalis* strains exhibited the same resistance to ciprofloxacin (2%). *E. durans* isolates showed very low level of resistance to rifampicin, erythromycin, ciprofloxacin, and nitrofurantoin (1–4%). *E. faecium*, *E. faecalis* and *E. durans* were also found to be the predominant species recovered from naturally ripened European cheeses and

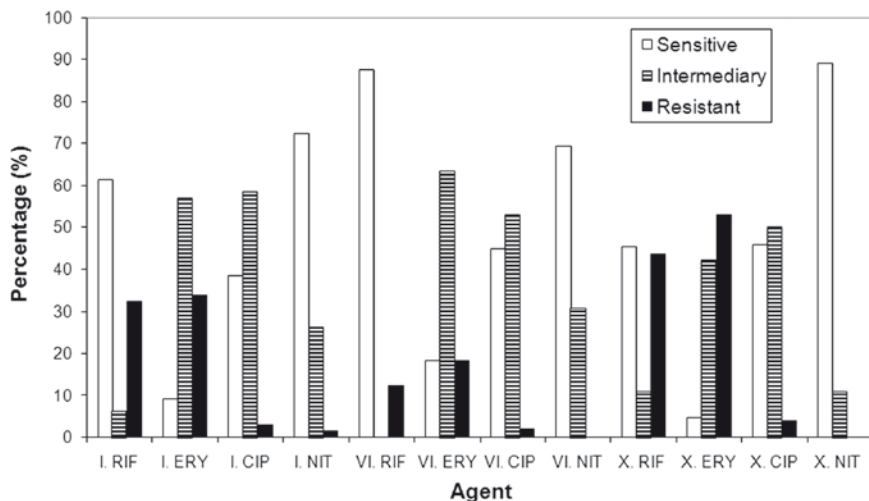


Fig. 6 Incidence of sensitive, intermediary and resistant *E. faecium* strains (%) isolated from Bryndza (I – January, VI – June and X – October) to rifampicin (RIF), erythromycin (ERY), ciprofloxacin (CIP), and nitrofurantoin (NIT)

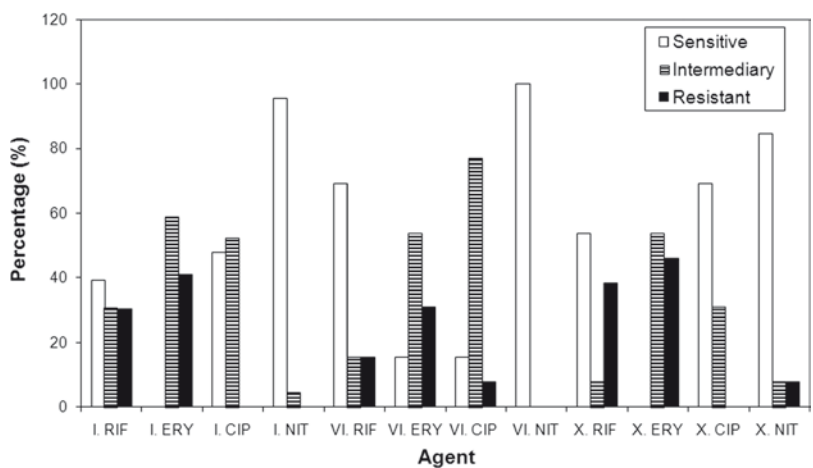


Fig. 7 Incidence of sensitive, intermediary and resistant *E. faecalis* strains (%) isolated from Bryndza (I – January, VI – June and X – October) to rifampicin (RIF), erythromycin (ERY), ciprofloxacin (CIP), and nitrofurantoin (NIT)

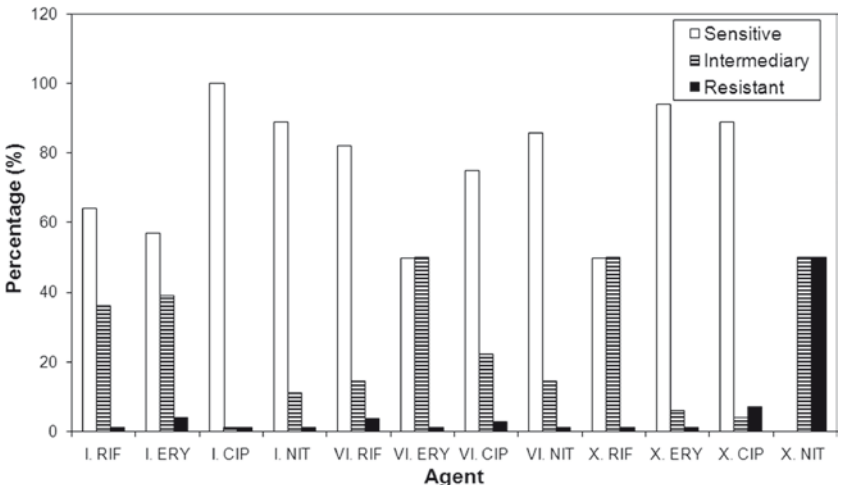


Fig. 8 Incidence of sensitive, intermediary and resistant *E. durans* strains (%) isolated from Bryndza (I – January, VI – June and X – October) to rifampicin (RIF), erythromycin (ERY), ciprofloxacin (CIP), and nitrofurantoin (NIT)

dairy products, with different sensitivity to antimicrobials (Teuber et al. 1999; Franz et al. 2001; Peters et al. 2003; Drahovská et al. 2004; Lopes et al. 2005). These results are in concordance with ours. Susceptibility of enterococcal species to antimicrobials decreased in the order *E. durans*, *E. faecalis* and *E. faecium*. In contrast, Teuber et al. (1999) reported that *E. faecium* cheese isolates were generally

more susceptible than *E. faecalis* cheese isolates and the rate of enterococcal resistance to vancomycin and other antimicrobials was <4%, and 7–80%, respectively. In a study undertaken by Franz et al. (2001) similar results were observed. The resistance of 95 cheese enterococcal isolates to vancomycin was <2.1% and the resistance to penicillin, ampicillin, erythromycin, chloramphenicol, tetracycline, streptomycin, and gentamicin increased from 2.1% to 63.8% of the strains. Results showed that the isolates from Bryndza are generally more susceptible to antimicrobials than above mentioned enterococci. These isolates from Bryndza displayed the same susceptibility to clinically important antibiotics such as ampicillin, vancomycin, and teicoplanin as was reported for 118 strains of *E. faecalis* and *E. faecium* isolated from food of animal origin in Germany (Peters et al. 2003). In contrast Majhenič et al. (2005) published that *E. faecalis* isolates from tolmic cheese possess very low antibiotic resistance to nine antibiotics, because they were resistant only to clindamycin.

The study of Belicová et al. (2007) proved a relatively low level of enterococcal resistance against antimicrobial in comparison to data concerning of most European cheeses. To our knowledge, the present results represent the first attempt to analyse the differences in the antibiotic resistance frequency of enterococcal microflora that have been affected by different composition of the Bryndza cheese. The results provide evidence about the present level of resistance to selected antibacterials in enterococci from Bryndza cheese in Slovakia.

None of the *Enterococcus* sp. strains had *vanA* or *vanB* genes (Jurkovič et al. 2006a). The *aph(3')* gene was found in 10% of streptomycin-resistant isolates. Six percent of gentamicin-resistant isolates harbored the *aac(6')-aph(2'')* gene. Resistance to tetracycline was mediated by *tet(L)* gene in 4% of enterococci, whereas *tet(M)* gene only in 1%. Gene *erm(B)* was determined in 11% of *E. faecium*. Frequency of chloramphenicol-resistant isolates with *cat* gene was 6% (not published data).

Multiple-Drug Resistance

Forty eight (30%) of the *E. faecium* isolates, two (3%) of the *E. durans* isolates, and six (12%) of the *E. faecalis* isolates exhibited multidrug resistance. Ten percent of *E. faecium*, 0% of *E. durans*, and 17% of *E. faecalis* strains showed simultaneous resistance to three drugs (rifampicin, erythromycin, and ciprofloxacin or nitrofurantoin). For all *E. faecium*, *E. durans*, and *E. faecalis* isolates, 55 (19%) multidrug resistant strains were resistant to rifampicin, 55 (19%) strains were resistant to erythromycin, nine (2%) strains were resistant to ciprofloxacin, and one (0.3%) strain was resistant to nitrofurantoin. The incidence of multiple resistance to five or more antimicrobials of enterococcal cheese isolates was confirmed by Teuber et al. (1999) and Franz et al. (2001). In this respect it may be stated that the enterococcal isolates from Bryndza cheese have simultaneous resistance to fewer antimicrobials compared to the above-mentioned cheese isolates.

Seasonal Differences in the Antibiotic Resistance Frequency

Differences in the antibiotic resistance frequency of enterococcal microflora affected by the changes in the Bryndza cheese composition are documented in Fig. 9. In June Bryndza cheese the resistance frequency of enterococci to rifampicin, erythromycin and nitrofurantoin was 22%, 19%, and 3%, respectively. In October Bryndza cheese, incidence for above-mentioned resistance of enterococcal isolates decreased, and in January Bryndza cheese the same resistance increased approximately two-fold. Also noticed was the fact that the different composition of the Bryndza cheese had a significant effect on antibiotic resistance to rifampicin, erythromycin, and nitrofurantoin of *E. faecium*, and *E. faecalis* but not *E. durans* isolates (Figs. 6–8). Frequency of rifampicin-, erythromycin-, and nitrofurantoin-resistant *E. faecium* and *E. faecalis* isolates was lower in October Bryndza than in June Bryndza. The highest frequency was in January Bryndza. The same course of changes in the rate of ciprofloxacin-resistant *E. faecium* isolates were documented. On the other hand, ciprofloxacin-resistant *E. faecalis* strains were detected only in October Bryndza (8%). From these results it can be concluded that the changes in the rates of antibiotic resistance of isolates depend on the number and distribution of enterococcal species in Bryndza samples. The *E. faecium*, *E. durans*, and *E. faecalis* species represented 49%, 29% and 17% of the isolates in June Bryndza, 53%, 30%, and 14% in October Bryndza, and 77%, 5%, and 16% of the isolates in January Bryndza. The changes in frequency of *E. faecium* species isolates had an overall effect on susceptibility data. The highest levels of resistance were found among enterococci harvested from January Bryndza, followed by enterococci from June Bryndza. The lowest level of resistance was in enterococci from October Bryndza.

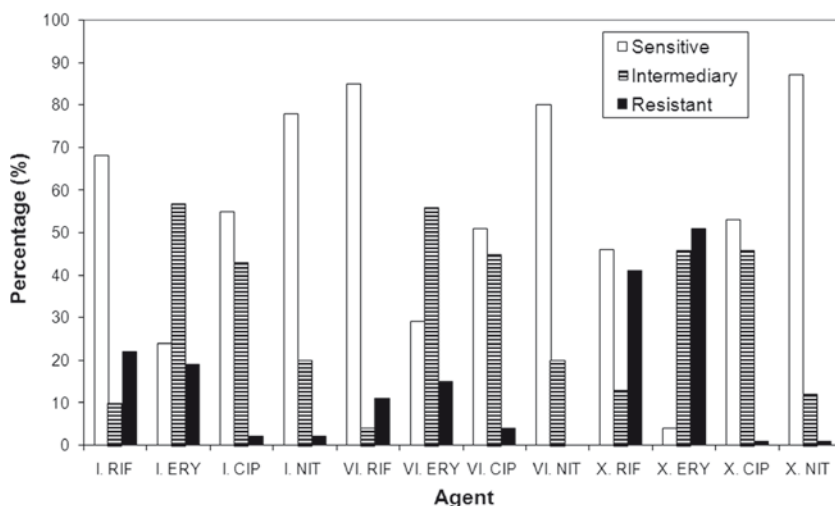


Fig. 9 Susceptibility (%) of enterococci isolated from Bryndza (I – January, VI – June and X – October) to rifampicin (RIF), erythromycin (ERY), ciprofloxacin (CIP) and nitrofurantoin (NIT)

Virulence Determinants

Virulence genes were found in several strains. Of the 308 *Enterococcus* sp. isolates, 20 (6.5%) contained *gelE* gene, but only 13 (4.2%) of them showed gelatinase-positive phenotype by agar plate assay. Seven isolates (2.3%) harboured all five cytolysin genes (*cylLL*, *cylLS*, *cylM*, *cylB* and *cylA*), but none showed a positive haemolytic phenotype. Four isolates (1.3%) contained the *agg* gene. Among the 308 *Enterococcus* sp. isolates, 20 (6.5%) possessed at least one virulence determinant and from them two isolates harboured three and five isolates harboured two virulence determinants. All these isolates were proved to be *E. faecalis*.

However, we detected virulence determinants in low number of Bryndza cheese isolates identified as *E. faecalis*. The frequency of *E. faecalis* in all Bryndza samples was relatively low and the presence of virulence genes was detected in 20 enterococcal isolates (6.5%). In addition, the presence of *gelE* and *cyl* genes did not correlate with its phenotypic expression. Comparison of β -haemolysis and detection of *cyl* genes revealed that cytolysin determinants behave as silent genes in most nonhaemolytic isolates, a finding already reported for some *E. faecalis* strains (Eaton and Gasson 2001). This discrepancy in both genes was also found in enterococci from different sources (Creti et al. 2004). Semedo et al. (2003) showed that virulence traits occur in enterococci irrespective of their origin (human and animal hosts, food and environment). Our results have shown that virulence factors, such as haemolysin, AS and Gel occur among enterococci in Bryndza cheese.

Since food enterococci do not seem to be associated with the potentially high virulence profiles, when compared with clinical strains, it seems that their presence in traditional cheeses results in a low health risk (Eaton and Gasson 2001; Semedo et al. 2003). Nevertheless, the interaction of such strains with susceptible hosts seems to be a concern mainly in high-risk population groups, i.e. immunocompromised patients. These findings, associated with the natural ability of enterococci to acquire, accumulate and share extrachromosomal elements within their genus and with other bacteria, require further studies to evaluate the consequent risk for human health. The question of whether enterococci possessing virulence determinants present in cheese are safe remains a question. However, Bryndza cheese has been consumed safely for more than 200 years and was never associated with foodborne diseases (Keresteš and Selecký 2003).

Health Effect of Bryndza

Long term tradition, as well as scientific knowledge, shows that consumption of fermented milk products is beneficial for human health. For instance, longevity in some Mediterranean areas is attributed to an increased consumption of sheep cheese and fermented milk. More than 100 years ago Russian biologist Ilya Mechnikov already mentioned health effects of fermented milk.

Assumption of health benefit effects of Bryndza is confirmed by results of historically first clinical tests performed at Comenius University in Bratislava. A group of 30 volunteers consumed daily 100 g of Bryndza made by traditional procedure during 8 weeks. Statistically significant decrease of total cholesterol and LDL-cholesterol was observed. In probands with low level of serum HDL-cholesterol there were documented its significant increase. As well, values of glucose, serum creatinine, C-reactive protein and blood pressure were decreased. Decrease of the C-reactive protein was interesting because this protein is an inflammation marker considered as the risk factor for cardiovascular diseases and colorectal carcinoma (Mikeš et al. 2005).

In Denmark, a fermented milk product GAIO® (MD Foods, Aarhus, Denmark) that contains *E. faecium* and two strains of *Streptococcus thermophilus* was investigated on risk factors for cardiovascular disease in overweight and obese subjects. GAIO® yogurt reduced LDL-cholesterol by 8.4% and increased fibrinogen in the overweight subjects at a 450 ml consumption daily for 8 weeks (Agerholm-Larsen et al. 2000). Authors suggest that *E. faecium* is the bacterial strain with the cholesterol-lowering effect. In another study, consumption of 200 ml GAIO® daily for 6 weeks reduced LDL-cholesterol in normal-weight men by 10% (Agerbaek et al. 1995). These findings are also supported by Bertolami et al. (1999) These authors tested non-obese moderately hypercholesterolaemic subjects who had not earlier shown an improvement in LDL-cholesterol level by dietetic modifications alone. Their results confirmed that GAIO® was able to reduce LDL-cholesterol by about 6% compared with placebo group.

Rossi et al. (1999) used the strain *E. faecium* CRL 183 for the development of a novel fermented soymilk product with potential probiotic properties. This strain in combination with *Lactobacillus jugurti* resulted in a 43% decrease in cholesterol in vitro.

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References

- Aarestrup FM, Agerso Y, Gerner-Smidt P, Madsen M, Jensen LB (2000) Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. *Diagn Microbiol Infect Dis* 37:127–137
- Agerbaek M, Gerdes LU, Richelsen B (1995) Hypocholesterolaemic effect of a new fermented milk product in healthy middle-aged men. *Eur J Clin Nutr* 49:346–352
- Agerholm-Larsen L, Raben A, Haulrik N, Haulrik N, Hansen AS, Manders M, Astrup A (2000) Effect of 8 week intake of probiotic milk products on risk factors for cardiovascular diseases. *Eur J Clin Nutr* 54:288–297
- Allen WD, Lingood MA, Porter P. (1996) *Enterococcus* organisms and their use as probiotics in alleviating irritable bowel syndrome symptoms. European Patent 0508701 (B1)
- Andrighetto C, Knijff E, Lombardi A, Torriani S, Vancanneyt M, Kersters K, Swings J, Dellaglio F (2001) Phenotypic and genetic diversity of enterococci isolated from Italian cheeses. *J Dairy Res* 68:303–316

- Arizcun C, Barcina Y, Torre P (1997) Identification and characterization of proteolytic activity of *Enterococcus* spp. isolated from milk and Roncal and Idiazabal cheese. *Int J Food Microbiol* 38:17–24
- Barbier N, Saulnier P, Chachaty E, Dumontier S, Andreumont A (1996) Random amplified polymorphic DNA typing versus pulsed-field gel electrophoresis for epidemiological typing of vancomycin-resistant enterococci. *J Clin Microbiol* 34:1096–1099
- Basso A, Goffo A, Rossi G, Conterno N (1994) A preliminary characterization of the microflora of Montasio cheese – Occurrence of galactose fermenting strains in cheese and in natural starter cultures. *Mic Aliments Nutri* 12:139–144
- Battistotti B, Bottazzi V, Vola G (1977) Impiego di *Str. faecium*, *Str. thermophilus* e bacilli lattici nella caseificazione del formaggio Fontina. *Scienza e Tecnica Lattiero-Casearia* 28:331
- Belicová A, Križková L, Dobias J, Krajčovič J, Ebringer L (2004) Synergic activity of selenium and probiotic bacterium *Enterococcus faecium* M-74 against selected mutagens in *Salmonella* assay. *Folia Microbiol* 49:301–305
- Belicová A, Križková L, Krajčovič J, Jurkovič D, Sojka M, Dušinský R (2007) Antimicrobial susceptibility of *Enterococcus* species isolated from Slovak Bryndza Cheese. *Folia Microbiol* 52:115–119
- Bellomo G, Mangiagli A, Nicastro L, Frigerio G (1980) A controlled doubleblind study of SF68 strain as a new biological preparation for the treatment of diarrhoea in pediatrics. *Curr Ther Res* 28:927–936
- Benyacoub J, Czarniecki-Maulden GL, Cavadini C, Sauthier T, Anderson RE, Schifffrin EJ, von der Weid T (2003) Supplementation of food with *Enterococcus faecium* (SF68) stimulates immune functions in young dogs. *J Nutr* 133:1158–1162
- Benyacoub J, Perez PF, Rochat F, Saudan KY, Reuteler G, Antille N, Humen M, De Antoni GL, Cavadini C, Blum S, Schifffrin EJ (2005) *Enterococcus faecium* SF68 enhances the immune response to *Giardia intestinalis* in mice. *J Nutr* 135:1171–1176
- Bertolami BC, Faludi AA, Batloumi M (1999) Evaluation of the effects of a new fermented milk product (Gaio) on primary hypercholesterolemia. *Eur J Clin Nutr* 53:97–101
- Boča M, Vyskočil M, Mikulecký M, Ebringer L, Kolibáš E, Kratochvířová H, Buzgová D (2004) Complex therapy of chronic hepatic encephalopathy with probiotic: Comparison of two studies (in Slovak). *Čas Lék Čes* 143:324–328
- Bouton Y, Guyot P, Grappin R (1998) Preliminary characterization of microflora of Comte cheese. *J Appl Microbiol* 85:123–131
- Buydens P, Debeuckelaere S (1996) Efficacy of SF68 in the treatment of acute diarrhea. A placebo-controlled trial. *Scand J Gastroenterol* 31:887–891
- Caridi A, Micari P, Foti F, Ramondino D, Sarullo V (2003) Ripening and seasonal changes in microbiological and chemical parameters of the artisanal cheese Caprino d'Aspromonte produced from raw or thermized goat's milk. *Food Microbiol* 20:201–209
- Casalta E, Zennaro R (1997) Effect of specific starters on microbiological, biochemical and sensory characteristics of Venaco, a Corsican soft cheese. *Sci Aliment* 17:79–94
- Centeno JA, Cepeda A, Rodríguez-Otero JL, Docampo F (1995) Estudio higienico-sanitario del queso de Arzúa. *Alimentaria* 33:91–96
- Centeno JA, Menendez S, Rodríguez-Otero JL (1996) Main microbial flora present as natural starters in Cebreiro raw cow's-milk cheese (northwest Spain). *Int J Food Microbiol* 33:307–313
- Centeno JA, Menendez S, Hermida M, Rodríguez-Otero JL (1999) Effects of the addition of *Enterococcus faecalis* in Cebreiro cheese manufacture. *Int J Food Microbiol* 48:97–111
- Cetinkaya Y, Falk P, Mayhall CG (2000) Vancomycin-resistant enterococci. *Clin Microbiol Rev* 13:686–707
- Chiew YF, Hall LM (1998) Comparison of three methods for the molecular typing of Singapore isolates of enterococci with high-level aminoglycoside resistances. *J Hosp Infect* 38:223–230
- Clewell DB (1993) Bacterial sex pheromone-induced plasmid transfer. *Cell* 73:9–12
- Coburn PS, Gilmore MS (2003) The *Enterococcus faecalis* cytolysin: a novel toxin active against eukaryotic and prokaryotic cells. *Cell Microbiol* 5:661–669

- Coque TM, Willems RJL, Fortun J, Top J, Diz S, Loza E, Canton R, Baquero F (2005) Population structure of *Enterococcus faecium* causing bacteremia in a Spanish University Hospital: setting the scene for a future increase in vancomycin resistance? *Antimicrob Agents Chemother* 49:2693–2700
- Cosentino S, Pisano MB, Corda A, Fadda ME, Piras C (2004) Genotypic and technological characterization of enterococci isolated from artisanal Fiore Sardo cheese. *J Dairy Res* 71:444–450
- Creti R, Imperi M, Bertuccini L, Fabretti F, Orefici G, Di Rosa R, Baldassarri L (2004) Survey for virulence determinants among *Ent. faecalis* isolated from different sources. *J Med Microbiol* 53:13–20
- De Vuyst L, Avonts L, Makras E (2004) Probiotics, prebiotics and gut health (Chap. 17). In: Remacle C, Reusens B (eds) *Functional foods, ageing and degenerative disease*. Woodhead Publishing, Cambridge, UK
- Del Pozo BF, Gaya P, Medina M, Rodriguez-Marin MA, Nuñez M (1988) Changes of microflora of La Serena ewe's milk cheese during ripening. *J Dairy Res* 55:449–455
- Delgado S, Delgado T, Mayo B (2002) Technological performance of several *Lactococcus* and *Enterococcus* strains of dairy origin in milk. *J Food Prot* 65:1590–1596
- Descheemaeker P, Lammens C, Pot B, Vandamme P, Goossens H (1997) Evaluation of arbitrarily primed PCR analysis and pulsed-field gel electrophoresis of large genomic DNA fragments for identification of enterococci important in human medicine. *Int J Syst Bacteriol* 47:555–561
- Descheemaeker P, Leven M, Chapelle S, Lammens C, Hauchecorne M, Wijdooghe M, Vandamme P, Goossens H (2000) Prevalence and molecular epidemiology of glycopeptide-resistant enterococci in Belgian renal dialysis units. *J Infect Dis* 181:235–241
- Devoyod JJ (1969) La flore microbienne du fromage de Roquefort. IV Les entérocoques. *Lait* 49:637–650
- Devriese LA, Pot B, Collins MD (1993) Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. *J Appl Bacteriol* 75:399–408
- Dicuonzo G, Gherardi G, Lorino G, Angeletti S, Battistoni F, Bertuccini L, Creti R, Di Rosa R, Venditti M, Baldassarri L (2001) Antibiotic resistance and genotypic characterization by PFGE of clinical and environmental isolates of enterococci. *FEMS Microbiol Lett* 201:205–211
- Domig KJ, Mayer HK, Kneifel W (2003) Methods used for the isolation, enumeration, characterization and identification of *Enterococcus* spp. 2. Pheno- and genotypic criteria. *Int J Food Microbiol* 88:165–188
- Donabedian SM, Chow JW, Boyce JM, McCabe RE, Markowitz SM, Coudron PE, Kuritza A, Pierson CL, Zervos MJ (1992) Molecular typing of ampicillin-resistant, non- β -lactamase-producing *Enterococcus faecium* isolates from diverse geographic areas. *J Clin Microbiol* 30:2757–2761
- Drahovská H, Kocíncová D, Seman M, Turna J (2002) PCR-based methods for identification of *Enterococcus* species. *Folia Microbiol* 47:649–653
- Drahovská H, Slobodníková L, Kocíncová D, Seman M, Končáková R, Trupl J, Turňa J (2004) Antibiotic resistance and virulence factors among clinical and food enterococci isolated in Slovakia. *Folia Microbiol* 49:763–768
- Duthoit F, Godon JJ, Montel MC (2003) Bacterial community dynamics during production of registered designation of origin Salers cheese as evaluated by 16S rRNA gene single-strand conformation polymorphism analysis. *Appl Environ Microbiol* 69:3840–3848
- Dutka-Malen S, Evers S, Courvalin P (1995) Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J Clin Microbiol* 33:24–27
- Eaton TJ, Gasson MJ (2001) Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl Environ Microbiol* 67:1628–1635

- Ebringer L, Ferencík M, Lahitová N, Kačani L, Michalková D (1995) Antimutagenic and immunostimulatory properties of lactic acid bacteria. *World J Microbiol Biotechnol* 11:294–298
- Elsner HA, Sobottka I, Mack D, Claussen M, Laufs R, Wirth R (2000) Virulence factors of *Enterococcus faecalis* and *Enterococcus faecium* blood culture isolates. *Eur J Clin Microbiol Infect Dis* 19:39–42
- Engelbert M, Mylonakis E, Ausubel FM, Calderwood SB, Gilmore MS (2004) Contribution of gelatinase, serine protease, and *fsr* to the pathogenesis of *Enterococcus faecalis* endophthalmitis. *Infect Immun* 72:3628–3633
- Ennahar S, Aoude-Werner D, Assobhei O, Hasselmann C (1998) Antilisterial activity of enterocin 81, a bacteriocin produced by *Enterococcus faecium* WHE 81 isolated from cheese. *J Appl Microbiol* 85:521–526
- Ewing W, Haresign W (1989) Probiotics UK. Chalcombe Publications, Bucks, UK
- Facklam R, Elliot JA (1995) Identification, classification, and clinical relevance of catalase-negative, Gram-positive cocci, excluding the streptococci and enterococci. *Clin Microbiol Rev* 8:479–495
- Fan Y, Chen S, Yu Y (2006) A probiotic treatment containing *Lactobacillus*, *Bifidobacterium* and *Enterococcus* improves IBS symptoms in an open label trial. *Zhejiang Univ Sci B* 7:987–991
- Ferencík M, Ebringer L, Mikeš Z, Jahnová E, Čižnár I (1999) Beneficial modification of the human intestinal microflora using orally administered lactic acid bacteria (in Slovak). *Bratisl Lek Listy* 100:238–245
- Fines M, Perichon B, Reynolds P, Sahm DF, Courvalin P (1999) Courvalin, VanE, a new type of acquired glycopeptide resistance in *Enterococcus faecalis* BM4405. *Antimicrob Agents Chemother* 43:2161–2174
- Fontecha J, Pelaez C, Juarez M, Requena T, Gomez C (1990) Biochemical and microbiological characteristics of artisanal hard goat's cheese. *J Dairy Res* 73:1150–1157
- Foulquie-Moreno MR, Callewaert R, Devreese B, Van Beeumen J, De Vuyst L (2003) Isolation and biochemical characterisation of enterocins produced by enterococci from different sources. *J Appl Microbiol* 94:214–229
- Foulquie-Moreno MR, Sarantinopoulos P, Tsakalidou E, De Vuyst L (2006) The role and application of enterococci in food and health. *Int J Food Microbiol* 106:1–24
- Franz CMAP, Holzapfel WH, Stiles ME (1999) Enterococci at the crossroads of food safety? *Int J Food Microbiol* 47:1–24
- Franz CMAP, Muscholl-Silberhorn AB, Yousif NMK, Vancanneyt M, Swings J, Holzapfel WH (2001) Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. *Appl Environ Microbiol* 67:4385–4389
- Franz CMAP, Stiles ME, Schleifer KH, Holzapfel WH (2003) Enterococci in foods — a conundrum for food safety. *Int J Food Microbiol* 88:105–122
- Franzetti L, Pompei M, Scarpellini M, Galli A (2004) Phenotypic and genotypic characterization of *Enterococcus* spp. of different origins. *Curr Microbiol* 49:225–260
- Freitas AC, Pais C, Malcata FX, Hogg TA (1996) Microbiological characterization of Picante da Beira Baixa cheese. *J Food Prot* 59:155–160
- Galli D, Wirth R, Wanner G (1989) Identification of aggregation substances of *Enterococcus faecalis* cells after induction by sex pheromones. An immunological and ultrastructural investigation. *Arch Microbiol* 151:486–490
- García MC, Rodríguez MJ, Bernardo A, Tornadijo ME, Carballo J (2002) Study of enterococci and micrococci isolated throughout manufacture and ripening of San Simón cheese. *Food Microbiol* 19:23–33
- Gardini F, Martuscelli M, Caruso MC, Galgano F, Crudele MA, Favati F, Guerzoni ME, Suzzi G (2001) Effects of pH, temperature and NaCl concentration on growth kinetics, proteolytic activity and biogenic amine production of *Enterococcus faecalis*. *Int J Food Microbiol* 64:105–117
- Gelsomino R, Vancanneyt M, Cogan TM, Condon S, Swings J (2002) Source of enterococci in a farmhouse raw-milk cheese. *Appl Environ Microbiol* 68:3560–3565

- Gelsomino R, Vancanneyt M, Condon S, Swings J, Cogan TM (2001) Enterococcal diversity in the environment of an Irish Cheddar-type cheesemaking factory. *Int J Food Microbiol* 71:177–188
- Gevers D, Huys G, Swings J (2001) Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol Lett* 205:31–36
- Gilmore MS, Segarra RA, Booth MC (1990) An HlyB-type function is required for expression of the *Enterococcus faecalis* hemolysin/bacteriocin. *Infect Immun* 58:3914–3923
- Gilmore MS, Segarra RA, Booth MC, Bogie CP, Hall LR, Clewell DB (1994) Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. *J Bacteriol* 176:7335–7344
- Giraffa G (2002) Enterococci from food. *FEMS Microbiol Lett* 26:163–171
- Giraffa G (2003) Functionality of enterococci in dairy products. *Int J Food Microbiol* 88:215–222
- Giraffa G, Carminati D, Neviani E (1997) Enterococci isolated from dairy products: a review of risks and potential technological use. *J Food Prot* 60:732–738
- Giraffa G, Picchioni N, Neviani E, Carminati D (1995) Production and stability of an *Enterococcus faecium* bacteriocin during Taleggio cheesemaking and ripening. *Food Microbiol* 12:301–307
- Godfree AF, Kay D, Wyer D (1997) Faecal streptococci as indicators of faecal contamination in water. *Soc Appl Bacteriol Symp Ser* 26:110S–119S
- Goh SH, Facklam RR, Chang M, Hill JE, Tyrrell GJ, Burns EC, Chan D, He C, Rahim T, Shaw C, Hemmingsen SM (2000) Identification of *Enterococcus* species and phenotypically similar *Lactococcus* and *Vagococcus* species by reverse checkerboard hybridization to chaperonin 60 gene sequences. *J Clin Microbiol* 38:3953–3959
- Gordillo ME, Sigh KV, Murray BE (1993) Comparison of ribotyping and pulsed-field gel electrophoresis for subspecies differentiation of strains of *Enterococcus faecalis*. *J Clin Microbiol* 31:1570–1574
- Hamilton-Miller J, Shah S, Smith CT (1996) Probiotic remedies are not what they seem. *BMJ* 312:55–56
- Harwood VJ, Delahoya NC, Ulrich RM, Kramer MF, Whitlock JE, Garey JR, Lim DV (2004) Molecular confirmation of *Enterococcus faecalis* and *E. faecium* from clinical, faecal and environmental sources. *Lett Appl Microbiol* 38:476–482
- Hlivak P, Odraška J, Ferenčík M, Ebringer L, Jáhnová E, Mikeš Z (2005) One year application of probiotic strain *Enterococcus faecium* M74 decreases serum cholesterol levels. *Bratisl Lek Listy* 106:67–72
- Hlubinová K, Rychlý B, Altanerová V, Ebringer L, Altaner Č (2004) Influence of diet containing lyophilized *Enterococcus faecium* M-74 with organic selenium on tumor incidence in *Apc*^{1638N} mice. *Neoplasma* 51:341–344
- Homan WL, Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, Van Embden JD, Willems RJ (2002) Multilocus sequence typing scheme for *Enterococcus faecium*. *J Clin Microbiol* 40:1963–1971
- Hufnagel M, Hancock LE, Koch S, Theilacker C, Gilmore MS, Huebner J (2004) Serological and genetic diversity of capsular polysaccharides in *Enterococcus faecalis*. *J Clin Microbiol* 42:2548–2557
- Huycke MM, Spiegel CA, Gilmore MS (1991) Bacteremia caused by hemolytic, high-level gentamicin-resistant *Enterococcus faecalis*. *Antimicrob Agents Chemother* 35:1626–1634
- Ike Y, Hashimoto H, Clewell DB (1984) Hemolysin of *Streptococcus faecalis* subspecies zymogenes contributes to virulence in mice. *Infect Immun* 45:528–530
- Ike Y, Hashimoto H, Clewell DB (1987) High incidence of hemolysin production by *Enterococcus* (*Streptococcus*) *faecalis* strains associated with human parenteral infections. *J Clin Microbiol* 25:1524–1528
- Jackson CR, Fedorka-Cray PJ, Barrett JB (2004) Use of a genus- and species-specific multiplex PCR for identification of enterococci. *J Clin Microbiol* 42:3558–3565
- Jett BD, Huycke MM, Gilmore MS (1994) Virulence of enterococci. *Clin Microbiol Rev* 7:462–478

- Jurkovič D, Križková L, Dušínský R, Belicová A, Sojka M, Krajčovič J, Ebringer L (2006a) Identification and characterization of enterococci from bryndza cheese. *Lett Appl Microbiol* 42:553–559
- Jurkovič D, Križková L, Sojka M, Belicová A, Dušínský R, Krajčovič J, Snauwaert C, Naser S, Vandamme P, Vancanneyt M (2006b) Molecular identification and diversity of enterococci isolated from slovak bryndza cheese. *J Gen Appl Microbiol* 52:329–337
- Jurkovič D, Križková L, Sojka M, Takáčová M, Dušínský R, Krajčovič J, Vandamme P, Vancanneyt M (2007) Genetic diversity of *Enterococcus faecium* isolated from bryndza cheese. *Int J Food Microbiol* 116:82–87
- Kayaoglu G, Orstavik D (2004) Virulence factors of *Enterococcus faecalis*: relationship to endodontic disease. *Crit Rev Oral Biol Med* 15:308–320
- Kayser FH (2003) Safety aspects of enterococci from the medical point of view. *Int J Food Microbiol* 88:255–262
- Ke D, Picard FJ, Martineau F, Ménard Ch, Roy PH, Ouellette M, Bergeron MG (1999) Development of a PCR assay for rapid detection of enterococci. *J Clin Microbiol* 37:3497–3503
- Keresteš J, Selecký J (2003) Slovak bryndza cheese. In: Keresteš, J (ed) *Dairy industry in Middle Slovakia*, pp 109–122. Eminent, Považská Bystrica, Slovak
- Klein G (2003) Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *Int J Food Microbiol* 88:123–131
- Kuhn I, Burman LG, Haeggman S, Tullus K, Murray BE (1995) Biochemical fingerprinting compared with ribotyping and pulsed-field gel electrophoresis of DNA for epidemiological typing of enterococci. *J Clin Microbiol* 33:2812–2817
- Kumada M, Senpuku H, Motegi M, Nkao R, Yonezawa H, Yamamura H, Watanabe H, Tagami J (2008) Effects of *Enterococcus faecium* on *Streptococcus mutans* biofilm formation using flow cell system. *J Oral Biosci* 50:68–76
- Lauková A, Czikková S (1999) The use of enterocin CCM 4231 in soy milk to control the growth of *Listeria monocytogenes* and *Staphylococcus aureus*. *J Appl Microbiol* 87:182–186
- Lauková A, Czikková S (2001) Antagonistic effect of enterocin CCM 4231 from *Enterococcus faecium* on “bryndza”, a traditional Slovak dairy product from sheep milk. *Microbiol Res* 156:31–34
- Laurenčík M, Sulo P, Slavikova E, Pieckova E, Seman M, Ebringer L (2008) The diversity of eukaryotic microbiota in the traditional Slovak sheep cheese – Bryndza. *Int J Food Microbiol* 127:176–179
- Leclercq R (1997) Enterococci acquire new kinds of resistance. *Clin Infect Dis* 24:S80–S84
- Ledda A, Scintu MF, Pirisi A, Sanna S, Mannu L (1994) Technological characterization of lactococci and enterococci for the manufacture of Fiore Sardo sheep cheese. *Scienza e Tecnica Lattiero-Casearia* 45:443–456
- Litopolou-Tzanetaki E (1990) Changes in numbers and kinds of lactic acid bacteria during ripening of Kefalotyri cheese. *J Food Sci* 55:111–113
- Litopolou-Tzanetaki E, Tzanetakis N, Vafopoulou-Mastrojiannaki A (1993) Effect of type of lactic starter on microbiological chemical and sensory characteristics of feta cheese. *Food Microbiol* 10:31–41
- Liu D, Wang C, Swiatlo EJ, Lawrence ML (2005) PCR amplification of a species-specific putative transcriptional regulator gene reveals the identity of *Enterococcus faecalis*. *Microbiol Res* 156:944–948
- Loguerio C, Abbiati R, Rinaldi M, Romano A, Vecchio D, Blanco C, Coltorti M (1995) Long-term effects of *Enterococcus faecium* SF68 versus lactulose in the treatment of patients with cirrhosis and grade 1-2 hepatic encephalopathy. *J Hepatol* 23:39–46
- Lopes MFS, Ribeiro T, Abrantes M, Marques JJF, Tenreiro R, Crespo MTB (2005) Antimicrobial resistance profiles of dairy and clinical isolates and type strains of enterococci. *Int J Food Microbiol* 103:191–198
- Macedo AC, Malcata FX, Hogg TA (1995) Microbiological profile in Serra ewe’s cheese during ripening. *J Appl Microbiol* 79:1–11

- Maisnier-Patin S, Forni E, Richard J (1996) Purification, partial characterisation and mode of action of enterococcin EFS2, an antilisterial bacteriocin produced by a strain of *Enterococcus faecalis* isolated from a cheese. *Int J Food Microbiol* 30:255–270
- Majhenič AČ, Rogelj I, Perko B (2005) Enterococci from Tolmic cheese: Population structure, antibiotic susceptibility and incidence of virulence determinants. *Int J Food Microbiol* 102:239–244
- Mannu L, Paba A (2002) Genetic diversity of lactococci and enterococci isolated from home-made Pecorino Sardo ewes' milk cheese. *J Appl Microbiol* 92:55–62
- Mannu L, Paba A, Pes M, Floris R, Scintu MF, Morelli L (1999) Strain typing among enterococci isolated from home-made Pecorino Sardo cheese. *FEMS Microbiol Lett* 170:25–30
- Manolopoulou E, Sarantinopoulos P, Zoidou E, Aktypis A, Moschopoulou E, Kandarakis IG, Anifantakis EM (2003) Evolution of microbial populations during traditional Feta cheese manufacture and ripening. *Int J Food Microbiol* 82:153–161
- Marino M, Maifreni M, Rondinini G (2003) Microbiological characterization of artisanal Montasio cheese: analysis of its indigenous lactic acid bacteria. *FEMS Microbiol Lett* 229:133–140
- Mego M, Ebringer L, Drgoňa L, Mardiak J, Trupl J, Greksak R, Nemová I, Oravcová E, Zajac V, Koza I (2005a) Prevention of febrile neutropenia in cancer patients by probiotic strain *Enterococcus faecium* M-74. Pilot study phase I. *Neoplasma* 52:76–80
- Mego M, Končerková R, Mikušková E, Drgoňa L, Ebringer L, Demitrovičová L, Nemová I, Trupl J, Mardiak J, Koza I, Zajac V (2006) Prevention of febrile neutropenia in cancer patients by probiotic strain of *Enterococcus faecium* M-74. Phase II study. *Supp Care Cancer* 14:285–290
- Mego M, Májek J, Končerková R, Ebringer L, Čierniková S, Rauko P, Kováč M, Trupl J, Slezák P, Zajac V (2005b) Intramucosal bacteria in colon cancer and their elimination by probiotic strain *Enterococcus faecium* M-74 with organic selenium. *Folia Microbiol* 50:443–447
- Menéndez S, Godínez R, Centeno JA, Rodríguez-Otero JL (2001) Microbiological, chemical and biochemical characteristics of “Tetilla” raw cows-milk cheese. *Food Microbiol* 18:151–158
- Mikeš Z, Ebringer L, Boča M, Dušínský R, Jahnová E (2005) Some cardiovascular factors after consumption of the traditional Slovak bryndza cheese – pilot study (in Slovak). *Geriatrics* 1:29–36
- Mikeš Z, Ferencík M, Jahnová E, Ebringer L, Čižnár I (1995) Hypcholesterolemic and immunostimulatory effects of orally applied *Enterococcus faecium* M-74 in man. *Folia Microbiol* 40:639–646
- Monstein HJ, Quednau M, Samuelsson A, Ahrné S, Isaksson B, Jonasson J (1998) Division of the genus *Enterococcus* into species groups using PCR-based molecular typing methods. *Microbiology* 144:1171–1179
- Morea M, Baruzzi F, Cocconcelli PS (1999) Molecular and physiological characterization of dominant bacterial populations in traditional Mozzarella cheese processing. *J Appl Microbiol* 87:574–582
- Mundy LM, Sahm DF, Gilmore MS (2000) Relationships between enterococcal virulence and antimicrobial resistance. *Clin Microbiol Rev* 13:513–522
- Murray BE (1990) The life and times of the *Enterococcus*. *Clin Microbiol Rev* 3:46–65
- Murray BE, Singh KV, Heath JD, Sharma BR, Weinstock GM (1990) Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. *J Clin Microbiol* 28:2059–2063
- Nallapareddy SR, Qin X, Weinstock GM, Höök M, Murray BE (2000) *Enterococcus faecalis* adhesin, ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. *Infect Immun* 68:5218–5224
- Naser S, Thompson FL, Hoste B, Gevers D, Vandemeulebroecke K, Cleenwerck I, Thompson CC, Vancanneyt M, Swings J (2005) Phylogeny and identification of enterococci by *atpA* gene sequence analysis. *J Clin Microbiol* 43:2224–2230

- NCCLS (2003) National Committee for Clinical Laboratory Standards Document M100-S13: Performance standards for antimicrobial susceptibility testing. 13th Informational Supplement NCCLS, Villanova, PA
- Núñez M, Rodríguez JL, García E, Gaya P, Medina M (1997) Inhibition of *Listeria monocytogenes* by enterocin 4 during the manufacture and ripening of Manchego cheese. *J Appl Microbiol* 83:671–677
- Ordoñez JA, Barneto R, Ramos M (1978) Studies on Manchego cheese ripened in olive oil. *Milchwissenschaft* 33:609–612
- Ozawa Y, Courvalin P, Gaiimand M (2000) Identification of enterococci at the species level by sequencing of the genes for D-alanine: D-alanine ligases. *Sys Appl Microbiol* 23: 230–237
- Papageorgiou DK, Abraham A, Bori M, Doundounakis S (1998) Chemical and bacteriological characteristics of Pichtogalo Chanion cheese and mesophilic starter cultures for its production. *J Food Prot* 61:688–692
- Parente E, Villani F, Coppola R, Coppola S (1989) A multiple strain starter for water-buffalo Mozzarella cheese manufacture. *Lait* 69:271–279
- Patel R, Piper KE, Rouse MS, Steckelberg JM, Uhl JR, Kohner P, Hopkins MK, Cockerill FR, Kline BC (1998) Determination of 16S rRNA sequences of enterococci and application to species identification of nonmotile *Enterococcus gallinarum* isolates. *J Clin Microbiol* 36:3399–3407
- Perichon B, Reynolds P, Courvalin P (1997) VanD-type glycopeptide-resistant *Enterococcus faecium* BM4339. *Antimicrob Agents Chemother* 41:2016–2018
- Perlada DE, Smulian AG, Cushion MT (1997) Molecular epidemiology and antibiotic susceptibility of enterococci in Cincinnati, Ohio: a prospective citywide survey. *J Clin Microbiol* 35:2342–2347
- Peters J, Mac K, Wichmann-Schauer H, Klein G, Ellerbroek L (2003) Species distribution and antibiotic resistance patterns of enterococci isolated from food of animal origin in Germany. *Int J Food Microbiol* 88:311–314
- Pintado ME, Santos CC, Malcata FX (2001) Activity of adventitious *Enterococcus* strains on model curdled caprine milk: microbial growth and evolution of concentration of organic acids and lactose throughout time. *Meded Rijksuniv Gent Fak Landbouwk Toegep Biol Wet* 66:613–616
- Pollmann M, Nordhoff M, Pospischil A, Tedin K, Wieler LH (2005) Effect of a probiotic strain of *Enterococcus faecium* on the rate of natural chlamydia infection in swine. *Infect Immun* 73:4346–4353
- Poyart C, Quesnes G, Trieu-Cuot P (2000) Sequencing the gene encoding Manganese Dependant superoxyde dismutase for rapid species identification of enterococci. *J Clin Microbiol* 38:415–418
- Poznanski E, Cavazza A, Cappa F, Cocconcelli PS (2004) Indigenous raw milk microbiota influences the bacterial development in traditional cheese from an alpine natural park. *Int J Food Microbiol* 92:141–151
- Prodromou K, Thasitou P, Haritonidou E, Tzanetakis N, Litopoulou-Tzanetaki E (2001) Microbiology of “Orinotryi” a ewe’s milk cheese from the Greek mountains. *Food Microbiol* 18:319–328
- Pryce TM, Wilson RD, Kulski JK (1999) Identification of enterococci by ribotyping with horse-radish-peroxidase-labelled 16S rDNA probes. *J Microbiol Methods* 36:147–155
- Rich RL, Kreikemeyer B, Owens RT, LaBrenz S, Narayana SV, Weinstock GM, Murray BE, Hook M (1999) Ace is a collagen-binding MSCRAMM from *Enterococcus faecalis*. *J Biol Chem* 274:26939–26945
- Rossi EA, Vendramini RC, Carlos IZ, Pei YC, de Valdez GF (1999) Development of a novel fermented soymilk product with potential probiotic properties. *Eur Food Res Technol* 209:305–307
- Rovenský J, Švik K, Stančíková M, Ebringer L, Ferenčík M (2002) Treatment of experimental adjuvant arthritis with the combination of methotrexate and lyophilized *Enterococcus faecium* enriched with organic selenium. *Folia Microbiol* 47:573–578

- Sannomiya PRA, Craig DB, Clewell A, Suzuki M, Fujino G, Till O, Marasco WA (1990) Characterization of a class of nonformylated *Enterococcus faecalis*-derived neutrophil chemotactic peptides: the sex pheromones. *Proc Natl Acad Sci USA* 87:66–70
- Sarantinopoulos P, Leroy F, Leontopoulou E, Georgalaki MD, Kalantzopoulos G, Tsakalidou E, De VL (2002) Bacteriocin production by *Enterococcus faecium* FAIR-E 198 in view of its application as adjunct starter in Greek Feta cheese making. *Int J Food Microbiol* 72: 125–136
- Schleifer KH, Kilpper-Bälz R (1984) Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. *Int J Syst Bacteriol* 34:31–34
- Schleifer KH, Kilpper-Bälz R (1987) Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci and lactococci: a review. *Syst Appl Microbiol* 6:1–19
- Semedo T, Santos MA, Lopes MFS, Marques JJF, Crespo MTB, Tenreiro R (2003) Virulence factors in food, clinical and reference enterococci: a common trait in the genus? *Syst Appl Microbiol* 26:13–22
- Shankar N, Baghdayan AS, Gilmore MS (2002) Modulation of virulence within a pathogenicity island in vancomycin-resistant *Enterococcus faecalis*. *Nature* 417:746–750
- Shankar N, Lockatell CV, Baghdayan AS, Drachenberg C, Gilmore MS, Johnson DE (2001) Role of *Enterococcus faecalis* surface protein Esp in the pathogenesis of ascending urinary tract infection. *Infect Immun* 69:4366–4372
- Simonetta AC, Moragues de Velasco LG, Frisón LN (1997) Antibacterial activity of enterococci strains against *Vibrio cholerae*. *Lett Appl Microbiol* 24:139–143
- Singh KV, Coque TM, Weinstock GM, Murray BE (1998) In vivo testing of an *Enterococcus faecalis* efaA mutant and use of efaA homologs for species identification. *FEMS Immunol Med Microbiol* 21:323–331
- Son R, Nimita F, Rusul G, Nasreldin E, Samuel L, Nishibuchi M (1999) Isolation and molecular characterization of vancomycin-resistant *Enterococcus faecium* in Malaysia. *Lett Appl Microbiol* 29:118–122
- Su A, Sulavik MC, He P, Mäkinen KK, Mäkinen P, Fiedler S, Wirth R, Clewell DB (1991) Nucleotide sequence of the gelatinase gene (*gelE*) from *Enterococcus faecalis* subsp. *Liquefaciens* *Infect Immun* 59:415–420
- Süßmuth SD, Muscholl-Silberhorn A, Wirth R, Susa M, Marre R, Rozdzinski E (2002) Aggregation substance promotes adherence, phagocytosis, and intracellular survival of *Enterococcus faecalis* within human macrophages and suppresses respiratory burst. *Infect Immun* 68:4900–4906
- Suzzi G, Caruso M, Gardini F, Lombardi A, Vannini L, Guerzoni ME, Andrighetto C, Lanorte MT (2000) A survey of the enterococci isolated from an artisanal Italian goat's cheese (semicotto caprino). *J Appl Microbiol* 89:267–274
- Švec P, Devriese LA, Sedláček I, Baele M, Vancanneyt M, Haesebrouck F, Swings J, Dožkař J (2001) *Enterococcus haemoperoxidus* sp. nov. and *Enterococcus moraviensis* sp. nov., isolated from water. *Int J Syst Evol Microbiol* 51:1567–1574
- Švec P, Vancanneyt M, Seman M, Snauwaert C, Lefebvre K, Sedlacek I, Swings J (2005) Evaluation of (GTG)₅-PCR for identification of *Enterococcus* spp. *FEMS Microbiol Lett* 247:59–63
- Tannock GW, Cook G (2002) Enterococci as members of the intestinal microflora of humans. In: Gilmore MS, Clewell DB, Courvalin P, Dunne GM, Murray BE, Rice LB (eds) *The enterococci: pathogenesis, molecular biology, and antibiotic resistance*. ASM, Washington, DC
- Tavaria FK, Malcata FX (1998) Microbiological characterization of Serra da Estrela cheese throughout its Appellation d'Origine Protégée region. *J Food Prot* 61:601–607
- Teng LJ, Hsueh PR, Wang YH, Lin HM, Luh KT, Ho SW (2001) Determination of *Enterococcus faecalis* groESL Full-Length sequence and application for species identification. *J Clin Microbiol* 39:3326–3331
- Teuber M, Meile L, Schwarz F (1999) Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie van Leeuwenhoek* 76:115–137

- Titze-de-Almeida R, Willems RJL, Top J, Pereira Rodrigues I, Fonseca Ferreira R, Boelens H, Brandileone MCC, Zanella RC, Soares Felipe MS, van Belkum A (2004) Multilocus variable-number tandemrepeat polymorphism among Brazilian *Enterococcus faecalis* strains. *J Clin Microbiol* 42:4879–4881
- Toledo-Arana A, Valle J, Solano C, Arrizubieta MJ, Cucarella C, Lamata M, Amorena B, Leiva L, Penadés JR, Lasa I (2001) The enterococcal surface protein, esp, is involved in *Enterococcus faecalis* biofilm formation. *Appl Environ Microbiol* 67:4538–4545
- Top J, Schouls LM, Bonten MJ, Willems RJ (2004) Multiple-locus variable-number tandem repeat analysis, a novel typing scheme to study the genetic relatedness and epidemiology of *Enterococcus faecium* isolates. *J Clin Microbiol* 42:4503–4511
- Tornadizo ME, Fresno JM, Bernardo A, Marin Sarmiento R, Carballo J (1995) Microbiological changes throughout the manufacturing and ripening of Spanish goat's raw milk cheese (Armada variety). *Lait* 75:551–570
- Torriani S, Dellaglio F, Lombardi A (1998) Microbiological contribution to the study of Monte Veronese cheese. Proceedings of the Symposium "Quality and Microbiology of Traditional and Raw Milk Cheeses", Dijon, France, pp 239–250
- Treitman AN, Yarnold PR, Warren J, Noskin GA (2005) Emerging incidence of *Enterococcus faecium* among hospital isolates (1993 to 2002). *J Clin Microbiol* 43:462–463
- Tyrrell GJ, Bethune RN, Willey B, Low DE (1997) Species identification of enterococci via intergenic ribosomal PCR. *J Clin Microbiol* 35:1054–1060
- Tzanetakis N, Litopoulou-Tzanetaki E (1992) Changes in numbers and kinds of lactic acid bacteria in Feta and Teleme, two Greek cheeses from ewe's milk. *J Dairy Sci* 75:1389–1393
- Ulrich A, Muller T (1998) Heterogeneity of plant-associated streptococci as characterized by phenotypic features and restriction analysis of PCR-amplified 16S rDNA. *J Appl Microbiol* 84:293–303
- Vakulenko SB, Donabedian SM, Voskresenskiy AM, Zervos MJ, Lerner SA, Chow JW (2003) Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. *Antimicrob Agents Chemother* 47:1423–1426
- Vancanney M, Lombardi A, Andrighetto C, Knijff E, Torriani S, Bjorkroth KJ, Franz CM, Foulquie Moreno MR, Revets H, De Vuyst L, Swings J, Kersters K, Dellaglio F, Holzapfel WH (2002) Intraspecies genomic groups in *Enterococcus faecium* and their correlation with origin and pathogenicity. *Appl Environ Microbiol* 68:1381–1390
- Versalovic J, Schneider M, De Bruijn FJ, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* 5:25–40
- Villani F, Coppola S (1994) Selection of enterococcal strains for water-buffalo Mozzarella cheese manufacture. *Annali di Microbiologia ed Enzimologia* 44:97–105
- Volokhov VD, Chizhikov V, Chumakov K, Rasooly A (2003) Microarray analysis of erythromycin resistance determinants. *J Appl Microbiol* 95:787–798
- Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB (2004) Nosocomial bloodstream infections in US hospitals: analysis of 24, 179 cases from a prospective nationwide surveillance study. *Clin Infect Dis* 39:309–317
- Wunderlich PF, Braun L, Fumagalli I, D'Apuzzo V, Heim F, Karli M, Lodi R, Politta G, Vonbank F, Zeltner L (1989) Double-blind report on the efficacy of lactic acid producing *Enterococcus* SF68 in the prevention of antibiotic-associated diarrhoea and in the treatment of acute diarrhoea. *J Int Med Res* 17:333–338
- Zárate V, Belda F, Pérez C, Cardell E (1997) Changes in the microbial flora of Tenerife goat's milk cheese during ripening. *Int Dairy J* 7:635–641

Salmonella Infections

Constantine Arvanitakis

Abstract The incidence of salmonella infections have increased dramatically in recent years, affecting all regions in developing, as well as developed countries.

This is particularly the case with the non-typhoid, non-paratyphoid enteric salmonella infections. Morbidity and mortality are high in developing countries, particularly in children. Salmonella infection is the commonest food borne disease causing gastroenteritis in developed countries affecting all ages and particularly patients with immunodeficiency who are at higher risk.

There are currently over 2,200 serotypes of salmonella identified. The commonest serotype in Europe is phagotype 4 (PT4) and in the USA phagotypes 8 and 13. Salmonella strains affect animal husbandry, especially poultry and their products, such as shell eggs, the main source of infection.

Pathogenesis of salmonellosis involves the following stages. After the entry of pathogen in the gastrointestinal tract, it adheres to epithelial cells of intestinal mucosa and generates endotoxin by activation of cyclic adenosinomonophosphate (cAMP), which causes electrolyte and water secretion from plasma to lumen. Bacterial infiltration of intestinal mucosa and inflammation contributes to diarrhea, the main clinical manifestation.

Other clinical manifestations are fever, abdominal cramps, tenesmus, headache, myalgia and occasionally nausea and vomiting.

Laboratory diagnosis is based on stool culture and isolation of salmonella serotype. Blood culture may be also positive in bacteremic phase. For typhoid fever, the Widal test is used to measure antibodies against O and H antigens *S. Typhi*.

Most cases of non-typhoid, salmonella infections are self-limited and do not require antimicrobial therapy. Oral electrolyte and glucose solution per os is the mainstay of management for dehydration. Oral antibiotics are indicated in protracted

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clinical course and high risk groups, children, elderly and immunocompromised patients. Trimethoprim – sulfamethoxazole and quinolones are the antibiotics administered.

Intravenous administration of fluids is indicated in severe cases with marked dehydration, electrolyte disturbances and metabolic acidosis. Antidiarrheal agents are used with caution, because they prolong bacterial contamination and induce carrier state. Antimicrobial agents are used selectively, only in severe cases with intractable diarrhea, bacteremia, systemic manifestations, immunodeficiency and valvular heart disease with valve replacement.

In the event of antibiotic administration, quinolones, macrolides and third generation cephalosporins are preferred.

Prevention of salmonella infection, as a bacterial food borne disease, is an issue of public health measures for effective control of food safety, including food production, processing, distribution, storage and food preparation. Primary prevention is the key strategy to control the incidence of salmonella infection and reduce morbidity and mortality.

Keywords Salmonellosis • Salmonella infection • typhoid fever • enterobacteriaceae • Salmonella serotypes • Salmonella serovars • Salmonella enterica • Salmonella enteritidis • Salmonella typhimurium • Salmonella heidelberg • quinolones

Introduction

Salmonellosis and Salmonella infection are due to genus *Salmonella*, family Enterobacteriaceae. *Salmonella* is a gram-negative bacterium, which is named after the veterinarian Daniel Salmon, who first isolated in the USA the species *S. Choleraesuis* in 1884, in an outbreak of cholera in pigs. Salmonellosis is the leading cause worldwide of food borne disease. *Salmonella* species are the etiological bacterial agents of enteric *Salmonella* infection, typhoid fever (*Salmonella typhi* and *Salmonella paratyphi*) and enterocolitis (*Salmonella typhimurium*, *Salmonella enteritidis*, *Salmonella Heidelberg*). The classification of *Salmonella* species has been modified several times.

The current nomenclature for *Salmonella typhi* is *Salmonella enterica*, serovar typhi. At the present over 2,500 serotypes or serovars of *Salmonella enterica* have been identified. Some serovars are host restricted.

Common clinical manifestations of salmonella infection are diarrhea, fever, abdominal cramps. Most cases resolve within 4–7 days and the majority of patients recover without treatment. In certain cases, however, *Salmonella* infection may run a severe course, particularly in infants, elderly and patients with immunodeficiency. Bacteremia may develop and infection may affect multiple organs and sites with increased morbidity and mortality.

Epidemiology

The incidence of non-typhoid, non-paratyphoid *Salmonella* infection has increased dramatically in the past 20 years and it is still increasing, affecting all geographic regions, developed, as well as, developing countries. It is currently the commonest cause of gastroenteritis in developed countries. The problem is particularly important in developing countries, with high morbidity and mortality, particularly in infants and children. Poor sanitation and ineffective infrastructure to control food processing from contamination are key factors for the spread of infection. Salmonellosis is a food borne infection and the fecal-oral route is the main mode of transmission. Food is the most common vehicle of salmonella enteric infection. Accordingly, preventive measures should be directed to effective sanitation works with sewage systems and tight control of the nutrition chain in all phases, since food is the most common vehicle.

Food borne diseases may effect a large number of persons in a short time from a common source. Previous outbreaks of Salmonellosis counted thousands of affected persons. Two representative examples of these outbreaks are the following. In the USA in 1985 contamination of pasteurized milk with *Salmonella typhimurium* caused an outbreak of salmonella infection which affected 170,000 people. Another outbreak in China in 1991, contamination of liquid ice-cream with *Salmonella enteritidis* affected 224,000 people.

Salmonella bacteria have been studied more extensively than the other enteropathogenic bacteria. The incidence of *Salmonella* isolation has increased over 10-fold in recent years, an observation which underscores the spread of salmonellosis, attaining epidemic proportions.

Salmonellae are isolated in the laboratory using low, medium and high selective media bacteria. Salmonellae are oxidase-negative, and predominantly lactose-negative. Isolates are characterized with serogrouping, pulse-field electrophoresis and bacteriophage serotyping methods.

The commonest serotype worldwide is *Salmonella enteritidis*. In Europe the prevalent phagotype is PT4 (Rampling 1993). In the USA the prevalent phagotypes are PT8 and PT13 (Mishu et al. 1991), but PT4 is also increasing in recent years. In UK there is a steady increase of the incidence of *Salmonella* infection, mainly due to *Salmonella enteritidis*, constituting over 50% of all *Salmonella* strains (Baird-Parker 1990). In Greece, studies have shown that there is a 10-fold increase of salmonella species isolates during the period 1984–1994, 85% of which were due to *Salmonella enteritidis* (Kansouzidou-Kanakoudi 1995).

This marked increase of *Salmonella enteritidis* in many countries, is attributed to wide spread contamination of food, mainly poultry, and grade A shell eggs, which constitute a common source of infection.

In the early 1990s due to intense publicity *Salmonella* infections received considerable attention by epidemiologists, state agencies and scientific societies. State agencies, responsible for food safety, instituted a tighter control on poultry units operating at a suboptimal level, imposing measures such as chicken extermination and destruction of large quantities of infected shell eggs.

These measures had some impact on the *Salmonella* infection rate, but the problem remained, since the causes are multiple and not confined only to contamination of poultry, but also to other foodstuffs, such as clams, vegetables and animal products.

The extent of the problem is greater than it is recorded in statistics. Not all cases of salmonellosis are reported and registered, even in countries with a developed network infrastructure of epidemiological surveillance. It is estimated that only 10–20% of Salmonellosis cases are reported.

From the epidemiological standpoint, several etiological factors have been analyzed to account for the massive increase of the incidence of *Salmonella* infection at a global scale.

1. Widespread dissemination of salmonella species in the environment.
2. Widespread contamination of animal food products and vegetables.
3. Infection of wide range of animal species and products, particularly poultry and eggs.
4. Mass production of canned foods without adequate safety controls.
5. Changes in social behavior regarding fast food consumption. The traditional way of preparing meals from fresh produce for daily consumption is a thing of the past for many people, with a hectic style of living.
6. Massive international travel, particularly visiting countries with high incidence of *Salmonella* infection has contributed to the increase of traveller's diarrhea with *Salmonella* and other enteric infections.

Transmission of Salmonellosis occurs in the majority of cases by contaminated food. The most common sources are poultry, eggs, fruits, vegetables, dairy products and shellfish. Peanut butter has been also implicated in an outbreak of salmonellosis in the USA and powdered infant formula in two outbreaks with *Salmonella* enteric in infants in France.

Transmission of *Salmonella* infection may also occur from man to man and animal to man. Salmonellosis outbreaks have been reported in person handling chicken, ducks, kittens and hedgehogs.

Pathophysiology

Transmission of infection usually requires a high bacterial load of the inoculum to survive in the gastric lumen with acidity and overcome the defensive mechanisms of the intestinal mucosa. Bacterial load of 10^6 bacterial cells are required to cause infection. High bacteriae load is usually associated with high rates of infection and short incubation period. Subjects with gastric atrophy and low acidity, as the elderly, may develop infection with a lower bacterial load of 10^3 cells.

Following the entry of *Salmonella* bacteria in the gastrointestinal tract, they adhere by fimbriae to the epithelial cells of intestinal mucosa. They are transported to the lamina propria by endocytosis where they are released inducing an influx of

macrophages. Epidemiological studies have shown that the commonest strain of Salmonellosis is *Salmonella enteritidis*, characterized by high infectivity and penetrance. These characteristics, particularly of the phagotype 4 are attributed to the presence of plasmid. Plasmid contributes to high infectivity by polysaccharide production, inhibition of antimicrobial function of host monocytes and release of toxins. Key factors of the pathogenesis of *Salmonella* infection are the production of endotoxins which are lipopolysaccharides and the ability of *Salmonella* bacteria to infiltrate macrophages. *Salmonella*, once inside the macrophage, inhibits the fusion of lysosomes and phagocytes and escapes the defensive mobilization of the host.

It is considered that the high incidence of *Salmonellae enteritidis* infection in poultry is due to plasmid and adherence to tissues (Gruner et al. 1994). Another factor regarding infectivity and transmission of infection is the resistance of *S. enteritidis* to high temperature compared with other serotypes of *Salmonellae*. It has been estimated that the temperature of boiling or frying eggs should exceed 80°C for the *Salmonellae* strains to be destroyed (Duguid and North 1991). Similarly shell fish containing *Salmonellae* is rendered safer by frying at high temperatures than by warming in soup preparation.

Salmonella typhi organisms affect the reticuloendothelial system through cytokine release and spread to the liver, spleen and bone marrow. The entrance into the bloodstream in the bacteremic phase following the intestinal phase, results in focal disease in spleen, endocardium, liver and gallbladder. This leads to a carrier state since *Salmonella typhi* or *Salmonella paratyphi* are secreted in the bile and through the enterohepatic circulation enter the intestine propagating infection through the carrier state.

In general, infection with *Salmonella paratyphi* elicits a localized immune response, whereas *Salmonella typhi* and other virulent strains elicit a major immune response with tissue invasion via lymphatics and capillaries.

The severity of *Salmonella* infection and the clinical course is influenced by host factors, as well as by virulent properties of *Salmonella* strains. Common risk factor for severe clinical course and virulent infection include immunodeficiency syndrome, HIV infection, immunosuppressive therapy, prior antimicrobial therapy which affect intestinal flora, malignant disease and diabetes mellitus (Hohmann 2001).

Other associated comorbidities, which predispose to Salmonellosis are sickle cell anemia, pernicious anemia due to achlorhydria, postoperative stomach due to hypochlorhydria, reduced motility of gastrointestinal tract due to a variety of conditions, promoting reduced stasis of intestinal contents and bacterial overgrowth.

The pathophysiology of *Salmonella* infection includes the following phases. After the entry of pathogen via the oral route into the gastrointestinal tract, it adheres to the epithelial cells of the gastrointestinal mucosa releasing endotoxins which activate intracellular cyclic adenosinomonophosphate (cAMP). Cyclic AMP causes water and electrolyte secretion from plasma to intestinal lumen. This is the type of secretory diarrhea due to release of endotoxin. It is characterized by a loss of large volume of fluids in a short period due to immediate action of toxins.

The other component of salmonella enteric infection, presenting with the main symptom of diarrhea, is bacterial infiltration of intestinal mucosa and acute inflammation, which contributes to the clinical manifestations of fever and diarrhea.

Mode of Transmission

As already indicated, the main source of Salmonella infection and transmission is by food, the main reservoir being poultry, eggs and egg products (Maki 2009).

In countries with increased incidence and frequency of Salmonella infection, poultry is infected by Salmonella enteritidis in 50–90% of all cases. The external and internal parts of egg shell are also infected by Salmonella enteritidis. Although the Salmonella cell count infecting the eggs is relatively small, taking into consideration the massive amount of eggs consumed, the cumulative bacterial count is substantial. Furthermore, during food processing or preparation the number of bacteria is increased by multiplication. Eggs stored in room temperature and infected by Salmonella carry greater infectivity than eggs stored in the refrigerator. Consumption of raw eggs should be avoided because of high risk of transmission. Similarly, foods which contain egg products, such as ice-cream and mayonnaise are frequent sources of Salmonella infection. Pasteurized milk has been also reported a source of Salmonella infection in children. Transmission of Salmonella has been also reported by consumption of vegetables, peanut butter and other foodstuffs. Transmission by water is rare. Transmission from person to person may occur, but it is not common. Particular attention should be given to food handlers who should wash their hands regularly and to nursing personnel caring for patients with enteric infections.

Types of Salmonella Infections

Salmonella infections cause two distinct clinical types, the non-typhoid and typhoid type. Non-typhoid is the commonest type and presents as gastroenteritis, bacteremia or focal disease. Typhoid fever is a systemic infectious disease and follows a more severe and protracted course.

Non-Typhoid Salmonella Infection

Clinical Manifestations

The most common manifestation of non-typhoid Salmonella infection is gastroenteritis. Less common manifestations are bacteremia and focal disease, following a

bacteremic phase. Most of the cases of non-typhoid Salmonellosis are due to *Salmonella enteritidis*. Other common serotypes of *Salmonella* causing non-typhoid infection are *S. typhimurium*, *S. Heidelberg*, *S. Newport*, *S. choleraesuis* and *S. Saint-Paul*.

The incubation period is usually 6–48 h after the entry of the organism. The symptoms are nausea, diarrhea, abdominal cramps, fever, myalgia and occasionally headache and vomiting. Stool is loose or watery and does not contain blood. If bloody diarrhea occurs the possibility of Shigellosis or enteric infection with *E. coli* enterohaemorrhagica should be considered. The majority of cases run a mild or medium severe course which lasts 3–4 days and symptoms subside completely not requiring hospitalization, unless there is more severe protracted course with fever and bacteremia. Bacteremia is uncommon in non-typhoid Salmonellosis. It has been reported, however, with infection with the serotypes *S. choleraesuis*, *S. typhimurium* and *S. Heidelberg*, which cause recurrent episodes of bacteremia.

Focal disease follows a bacteremic phase and involves a wide spectrum of organs. These include liver, gallbladder, aorta (aortic aneurysms), heart valves with stenosis or insufficiency, joints (septic arthritis), bones, urinary tract and soft tissue. The most common serotypes of *Salmonella* causing focal infection are *S. choleraesuis* and *S. typhimurium*.

Non-typhoid *Salmonella* infections resolve completely and do not pose a public health problem in outbreaks of non-typhoid gastroenteritis, as the carrier state occurs in a negligible incidence, less than 1%. Non-typhoid *Salmonella* infections affect all ages, but particularly vulnerable are children, elderly, patients with immunodeficiency syndrome and HIV infection. The clinical course in these groups is usually severe and characterized by higher morbidity and mortality.

Diagnosis of non-typhoid *Salmonella* infections is confirmed by isolation of the organism in stool culture and by blood culture in case of bacteremia.

Management

The majority of cases of non-typhoid *Salmonella* infections run a mild course. Replacement of fluid and electrolytes is required to prevent dehydration and electrolyte disturbances. In general there is no indication for administration of antibiotics. In fact, antibiotics may be deleterious, because they prolong the carrier state and contribute to the emergence of *Salmonella* strains resistant to antibiotics.

Antibiotics are indicated selectively in severe cases with protracted fever and in groups with increased risk, as children, elderly and patients with HIV infection. Trimethoprin-sulfamethoxazole or ciprofloxacin are given for a period of 5 days in non-immunocompromised patients.

In systemic or focal disease antibiotics should be administered for a longer period of 3–4 weeks. In the event of abscess formation, it should be drained surgically. Infected aneurysms, heart valves and joints usually require prolonged courses of antibiotics and surgical intervention.

Typhoid Fever

Typhoid fever is a systemic infection caused by *Salmonella enteric*, serotype typhi, member of Enterobacteriaceae family. It involves the reticuloendothelial system of multiple organs (Parry et al. 2002).

Salmonella typhi is a human pathogen. The mode of transmission is by contamination of water by fecal excretion, but it is also transmitted from person to person by hands and contact with infected patients. Food handlers who carry *Salmonella typhi* pose an increased risk of transmission of infection. *Salmonella typhi* are excreted in the stool of chronic carriers who may be asymptomatic as well as in the stool and urine in patients with active disease. Outbreaks of typhoid fever are associated with inadequate sanitary infrastructures in developing countries due to inadequate measures of hygiene. Contamination of water in the community is the main source of infection in endemic areas. In developed countries the main source of contamination is by food supplies infected by carriers. Flies and lack of adequate standards of proper disposal of waste may also account for the spread of infection.

Transmission of *Salmonella typhi* may also occur in day care centers and schools by direct contact with the fecal–oral route. Particularly attention should be taken by nursing and medical personnel in infectious units who care for patients with Salmonellosis and typhoid fever.

It is estimated that 3–5% of untreated patients become carriers of *S. typhi*. Chronic carriers pose a significant pool of infected individuals with *Salmonella typhi*, because they shed bacilli for a long period. The risk is enhanced since these carriers are difficult to identify because the majority are asymptomatic. Furthermore, *Salmonella typhi* colonize the gallbladder and biliary tree and they are excreted in the stool by the enterohepatic circulation. Even the removal of the gallbladder is not always an effective measure to abolish the carrier state, since *Salmonella typhi* spread to the intrahepatic bile ducts. Identification of carriers is important in order to contain and limit the transmission to healthy individuals. Carrier's state is confirmed by isolation of *Salmonella typhi* in stool culture. Treatment of carriers with amoxicillin, trimethoprim-sulfamethoxazole or ciprofloxacin is indicated.

Carriers of *Salmonella typhi* should be also evaluated for development of cholangiocarcinoma, because they carry an increased risk.

Predisposing Factors

The incidence and severity of Salmonellosis is increased in the following conditions or comorbidities. Achlorhydria, pernicious anemia, impaired immune response, HIV infection, malnutrition, chronic steroid administration, frequent administration of antibiotics which affects intestinal flora and lowers resistance of colonization, lymphoproliferative diseases, malignant tumors, schistosomiasis, chronic hemolytic syndromes (sickle cell disease), malaria and inflammatory bowel disease, mainly ulcerative colitis.

Clinical Manifestations

The organism enters the gastrointestinal tract by infected water or food and via the lymphatic vessels spread to blood. In the early stages of infection the small intestine and mainly the ileum is edematous and hyperemic due to inflammation. In severe cases hemorrhage and ulceration may develop. Incubation period ranges from 10 to 14 days from the entry of organism in the gastrointestinal tract. The onset of symptoms is gradual with fever, prostration, anorexia.

Pathogenesis

The entry of *Salmonella typhi* in the gastrointestinal tract passes through the stomach and colonize the intestine. Gastric acidity is the first line of defense. If conditions which cause hypo- or achlorhydria are present, *Salmonella typhi* with low bacterial load may be infective. Accordingly, *Salmonella typhi* entering the intestinal lumen, adheres to the mucosal epithelial cells and invades the mucosa. The M epithelial cells overlying Peyer's patches are the port of entry to lymphoid tissues and mesenteric lymph nodes spreading to reticuloendothelial system of the liver and spleen.

Salmonella microorganisms enter the mononuclear phagocyte and spleen and they multiply. At some point they are released in the bloodstream. In the bacteremic phase they cause focal disease in the liver, spleen, gallbladder, bone marrow and Peyer's patches of ileum. Increased levels of proinflammatory cytokines are observed in patients with systemic manifestations of typhoid fever.

Systemic and local immune responses, humoral and cellular, are mounted by the host but these are not associated with host protection. Necrosis of Peyer's patches, which may lead to intestinal perforation, is attributed to interaction of bacterial factors and host immune response. It is also interest that in typhoid fever there is no strong association with HIV infection, whereas in HIV infection there is an increased incidence of non-typhi *Salmonella* infection. Headache, pharyngitis and arthralgia may be present. Gastrointestinal symptoms are usually constipation, abdominal pain and tenderness. Other symptoms which may occur are diarrhea, the "pea soup" type containing inflammatory mononuclear cells. The biliary tree and liver is involved presenting cholecystitis and hepatitis. Splenomegaly may develop.

A characteristic rash (rose sports) may appear during the second week. Fever persists with high temperature, which fluctuates but does not return to normal level. Paradoxically, despite fever, there is bradycardia, which is characteristic of the clinical course. Temperature is usually high 39–40°C for the first 2 weeks. It begins to fall after the third week and gradually returns to normal during the fourth week. Laboratory tests show leucopenia, anemia, thrombocytopenia, elevation transaminases and bilirubin, proteinuria and abnormalities of coagulation.

Complications (Table 1)

Abdominal complications usually occur during the third week when intestinal lesions, namely infiltration of intestinal wall with mononuclear cells and macrophages and enlargement of lymphoid Peyer’s patches result in intestinal hemorrhage and occasionally perforation.

At this stage diarrhea may occur mixed with blood in 10% of cases. Severe bleeding occurs in a low percentage (2–3%), but the mortality is high (20%). Intestinal perforation presenting with signs of acute abdomen, leucocytosis and systemic manifestations of sepsis (SIRS) occurs in 2% of cases which follow a severe course.

Respiratory symptoms, cough and hemoptysis may occur due to pulmonary infiltrates of *Salmonella typhi* and secondary pneumococcal infection. Bacteremia leads to focal disease, which presents with osteomyelitis, endocarditis, particularly in valvular disease, meningitis, soft-tissue abscesses, glomerulonephritis and skin lesions. CNS complications, such as meningitis, mental disturbances, delirium or depressed level of consciousness may occur in severe cases.

Differential diagnosis of typhoid fever includes other *Salmonella* infections, leptospirosis, schistosomiasis, disseminated tuberculosis, malaria, brucellosis, viral hepatitis, *Yersinia enterocolitis*, influenza, lymphoma, toxoplasmosis, infectious mononucleosis, taphus, encephalopathy and connective-tissue disorders.

Table 1 Complications of typhoid fever

• Abdominal
Intestinal hemorrhage
Intestinal perforation
• Cholecystitis
• Hepatitis
• Bronchitis
• Pneumonia
• Hematologic
Anemia
Leucopenia
Thrombocytopenia
Coagulation disorder
• Neurological
Encephalopathy
Delirium
Meningitis
Impairment of coordination
• Miscellaneous
Focal abscesses
Relapse
Carrier state

Diagnosis

Diagnosis of typhoid fever is documented by the isolation of *Salmonella typhi* in blood, stool or urine cultures. Blood cultures should be obtained during the first 2 weeks of the disease when fever is high, indicating bacteremia. Typhoid fever is a consideration when patients present with prolonged period of high fever and it should be ruled out. Blood cultures are usually positive during the first 2 weeks, whereas stool cultures are positive at a later stage during the third and fourth week. If stool and blood cultures are negative but there is a strong clinical suspicion culture of bone marrow aspirate may yield a positive result to confirm the diagnosis.

Salmonella typhi contain antigen O (somatic) and antigen H (flagellar) which give rise to host antibodies. The antigens are lipopolysaccharides which are located in the outer membrane of *Salmonella* bacilli. *Salmonella typhi* is further identified by the additional presence of antigen Vi (capsular). A fourfold or over rise of antibody titers to O and H antigens. A 2-weeks apart in paired samples is indicative of typhoid fever.

However, serological diagnosis by Widal test has certain limitations. Sensitivity is modest (60–70%) and specificity is low, since several non-typhoid *Salmonella* strains cross-react and give false positive results.

Treatment

Antibiotics indicated in the treatment of typhoid fever are ceftriaxone 1 g/kg M or IV b.i.d. and fluoroquinolones such as ciprofloxacin 500 mg PO b.i.d. or levofloxacin 500 mg or IV for 10–14 days. Fluroquinolones may be also administered in children. Chloramphenicol is still used but aplastic anemia is a consideration. Other antibiotics which are used depending on sensitivity are trimethoprin-sulfamethoxazole, amoxicillin, ampicillin, azithromycin and aztreonam.

In severe cases with delirium and shock corticosteroids may be given combined with antibiotics to treat toxic effects. Fluid and electrolyte replacement and correction of metabolic disturbances are important part of the management.

Nutrition with adequate caloric intake for increased metabolic and protein catabolism should be maintained throughout the illness. Following the resolution of active infection, patients with typhoid fever should be under surveillance with successive stool cultures weekly, to exclude a carrier state.

Salmonella typhi may be excreted in the stool for a period of 3–6 months after the acute illness subsides. Those patients who become carriers should be treated with amoxicillin or trimethoprin-sulfamethoxazole and rifampin for a period of 4 weeks.

One of the worrisome issues in the treatment of typhoid fever is the emergence of *Salmonella typhi* strain resistant to ampicillin, amoxicillin and chloramphenicol. There are also reports of resistance to fluoroquinolones and multidrug resistance. This threat places a high priority for prevention and control of typhoid fever by sanitary measures, especially safe water and sewage disposal, as well as the development of new drugs.

Prevention of Salmonella Infections

The prevention of salmonellosis is closely associated with food safety and tight regulation of food industry by state agencies of public health. Legislation alone is not sufficient, although it is a legal prerequisite for food safety. The matter is complicated since it involves several phases in food production, processing, storage and transport in order to maintain hygienic standards, quality of animal feed and standards of personnel involved in food processing. As poultry and eggshells are the main source of infection particular attention should be directed to safety control and hygienic conditions in related industries and units.

The public should be well informed of the risks involved in certain foods and should also take measures, avoiding consumption of suspicious meat, chicken and seafood. Eggs should be fresh and stored in the refrigerator. Raw eggs should be avoided for consumption. Preparation of these foods requires high temperatures for cooking and sufficient time to avoid half-baking in order to eliminate potential bacterial contamination.

Food borne enteric infections constitute a significant problem in public health affecting millions of people, even in developed nations. In the USA in the 90s there have been several *E. coli* endemic outbreaks by leafy vegetables and an outbreak due to Shiga-toxin producing *E. coli*, related to spinach consumption. These outbreaks occurred despite the measures taken by state agencies of food safety, such as the US Department of Agriculture, the Food and Drug Administration and the Center for Disease Control (Maki 2006).

More recently, two major outbreaks of Salmonellosis occurred in the US related to eaten raw peppers and peanut butter (Maki 2009). These outbreaks underscore the fact that even in advanced countries with strict regulations of food processing and distribution, food borne outbreaks of enteric infections continue to occur. Safety controls should be mainly directed to milk and dairy products, eggs, egg products, poultry, fresh fruits and vegetables. These measures should extend to bacterial contamination of the products during storage and distribution of food to avoid contamination.

Another important aspect of prevention of food borne enteric infections is related to measures taken to ensure that restaurants comply with safety regulation in the proper food storage and preparation process. Regular control of personnel regarding harboring enteric infection or not following proper procedures of hygiene should be mandatory. Hygienic procedures of food preparation are not only limited to restaurants, but they should extend to hospitals, day care centers, nursing houses and private houses. Food safety refers to the condition of food at the time of consumption. This means that the food may be safe when it is selected, but may become unsafe by the time it is consumed.

It is estimated that one third of the total cases of food borne infections are due to suboptimal practices of storing, conservation and cooking. Food safety encompasses a broader concept which means that food consumed does not cause untoward effects at a short- or long-term.

There are four basic rules for the proper management and handling of food which apply to commercial establishments, private homes and institutions.

1. **Categorization** Avoid any mixing or direct contact of different types of food, that is mixing of cooked food or ready for consumption with raw food.
2. **Freezing** Foods should be stored and conserved in the proper temperature, usually below 5°C. Bacteriae, which are the main etiology of food borne infection, grow in temperatures ranging between +10 and +65°C.
3. **Cooking** Should be adequate regarding time and temperature for the reason stated above. It has been proven that sea food properly cooked reduces significantly the risk of food borne infection, compared with sea food cooked at lower temperature.
4. **Hygiene** Measures to maintain clean freezers and properly stored food reduce contamination. Avoid overloading of freezers.

Personal hygiene with frequent hand washing before food preparation is also important. These simple measures have proven effective to reduce the risk of infection in maintaining proper storage and handling.

One of the proposals put forward, in order to improve safety procedure of the food chain involving production, processing and cooking is irradiation (Osterholm and Norgan 2004). Irradiation of foodstuffs which carry a high risk of infection, such as poultry, egg and milk products, leafy vegetables, reduce the incidence of food borne infection. Food irradiation is safe and does not alter the nutritional value of food.

A major effort should be launched by agencies with the cooperation of consumers in order to reduce the risk of food borne infection with preventive measures, to reduce mortality, morbidity and burden of disease.

For the prevention and control of typhoid fever immunization is indicated in the endemic areas for residents and travelers (Engels et al. 1998). Regular hand washing, proper care in food handling and sound sanitary practices, that is safe drinking water and effective sewage disposal are important measures to limit the incidence and outbreaks of typhoid fever. Travelers should take precautions when visiting endemic areas, particularly with food and water. Drinking water should be boiled, food should be cooked thoroughly in high temperatures and ice-cream, fruits and vegetables should be avoided (Ivanoff 1995).

In developed countries the main source of typhoid fever is from travelers returning home having visited endemic areas and being exposed to infection. For travelers to endemic areas vaccines type Ty2Ia and Vi are recommended. The same applies to laboratory workers handling *Salmonella enteric* serotype and household members of typhoid carriers.

Mass immunization is also a consideration at times of natural disasters (floods, earthquakes) or during mass movement of refugees at times of war, combined with public health measure of safe water and food (Tarr et al. 1999).

References

- Baird-Parker AC (1990) Food borne salmonellosis. *Lancet* 336:1231–1235
- Duguid JP, North RAE (1991) Eggs and salmonella food poisoning: an evaluation. *J Med Microbiol* 34:65–72
- Engels EA, Falagas ME, Lau J, Bennish ML (1998) Typhoid fever vaccines – a metaanalysis of studies on efficacy and toxicity. *Br Med J* 316:110–116
- Gruner E, Martinetti Lucchini L, Hoops RK, Altwegg M (1994) Molecular epidemiology of salmonella enteritidis. *Eur J Epidemiol* 10:85–89
- Hohmann EL (2001) Non typhoid salmonellosis. *Clin Infect Dis* 32:263–269
- Ivanoff B (1995) Typhoid fever: global situation and WHO recommendations. *Southeast Asian J Trop Med Public Health* 26(2):1–6
- Kansouzidou-Kanakoudi A (1995) Salmonellosis – a current evaluation. *Helliniki Iatriki* 61:426–431
- Maki DG (2006) Don't eat the spinach – controlling food borne infectious disease. *N Engl J Med* 355:1952–55
- Maki DG (2009) Coming to grips with food borne infection – peanut butter, peppers and nation-wide salmonella outbreaks. *N Engl J Med* 360:949–953
- Mishu B, Kochler J, Lee LA, Rodrigue D, Hickman-Brenner F, Blake P, Tauxe RV (1991) Outbreaks of *Salmonella enteritidis* infections in the United States 1985–1991. *J Infect Dis* 169:547–552
- Osterholm MT, Norgan AP (2004) The role of irradiation in food safety. *N Engl J Med* 350:1898–1901
- Parry CM, Hien TT, Dongan G, White NJ, Farrar JJ (2002) Typhoid fever. *New Engl J Med* 347:1770–1782
- Rampling A (1993) *Salmonella enteritidis*, five years on. *Lancet* 342:317–318
- Tarr PE, Kuppens L, Jones TC, Ivanoff B, Aparin PG, Heymann DL (1999) Considerations regarding mass vaccination against typhoid fever as an adjunct to sanitation and public health measures: potential use in an epidemic in Tajikistan. *Am J Trop Med Hyg* 61:163–170

Molecular Diagnostics of *Staphylococcus aureus*

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Abstract The bacterial species of the genus *Staphylococcus* are important human and animal pathogens which cause severe infectious diseases. The most pathogenic species *Staphylococcus aureus* is an extraordinary versatile pathogen and the major causative agent of numerous hospital- and community acquired infections. The disease spectrum includes abscesses, bacteremia, central nervous system infections, endocarditis, osteomyelitis, pneumonia, urinary tract infections, and syndromes caused by exotoxins, including staphylococcal scaled skin syndrome, toxic shock syndrome and food poisoning. The spread, survival and prevalence of antibiotic resistant clones of *S. aureus* are immensely important problems for human health. Apart from the major pathogen *S. aureus*, other species of the genus *Staphylococcus* may also be involved in serious infections. The rapid and accurate identification of the disease causing agent is therefore a prerequisite for disease control as well as for epidemiological surveillance. Modern molecular methods for the identification and typing of bacterial species are based on their genomic and proteomic analysis, which is very advantageous compared to standard biotyping methods. For species identification of staphylococcal isolates, the most frequently used molecular method is amplification of conserved gene sequences by PCR. Genotypic methods focus on the characterization of chromosomal, plasmid, or total genomic DNA. The objectives of these methods are to assess relevant parameters of the staphylococcal genome and to detect polymorphism of DNA sequences with either

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direct or indirect methods. All the genotypic methods are aimed in generation of DNA fingerprints specific for individual species and/or isolates of distinct clonal lineages. *S. aureus* genomes comprise a core component consisting of genes present in all of the strains, and approximately 22% of the genome comprises an “accessory genome”, containing genes that encode a diverse array of non-essential functions, ranging from virulence, drug and metal resistance to substrate utilization and miscellaneous metabolism. The accessory genome is made up of a mobile genetic element (MGE), e.g. bacteriophages, pathogenicity islands, staphylococcal cassette chromosomes, plasmids and transposons. Given the steadily varying content of MGE and of their individual types in the *S. aureus* strains, these elements are the sources of genetic polymorphism and are one of the most important targets of molecular typing methods for *S. aureus*. Some *S. aureus* strains produce one or more exoproteins, which include toxic shock syndrome toxin-1, the staphylococcal enterotoxins, the exfoliative toxins and Panton-Valentine leukocidin. PCR for detection of relevant genes in *S. aureus* strains is the most commonly used method for the diagnostics of toxigenic staphylococci.

Keywords *Staphylococcus aureus* • molecular diagnostics • species identification • mobile genetics elements • bacteriophages • typing methods • toxigenic strains

Introduction

Staphylococci are among the most widespread pathogenic and opportunist pathogenic bacteria. At present, 40 staphylococcal species are known, nine of which with two subspecies, and one with three subspecies. However, the taxonomy of staphylococci is not yet complete, and several novel species have undergone a validation procedure only recently. Some taxa of the genus *Staphylococcus* are relevant to human medicine, while others are found only in animals or food. Based on their capacity to produce free coagulase, staphylococci are traditionally divided into two groups: coagulase-positive species, considered to be pathogenic, and the less dangerous coagulase-negative species (Table 1).

The most relevant of the coagulase-positive species is *Staphylococcus aureus* subsp. *aureus* (further *S. aureus*), pathogenic to both humans and animals. It is an extraordinary versatile pathogen and the major causative agent of numerous hospital- and community acquired infections. The spread, survival and prevalence of antibiotic resistant clones of *S. aureus* are immensely important problems for human health. Major antibiotic resistance problems are typically associated with methicillin (oxacillin) resistant *S. aureus* strains (MRSA) and, more recently, vancomycin-intermediate (VISA) and vancomycin-resistant (VRSA) strains. Up to 30% of the human population carries *S. aureus* without any symptoms. In most cases, however, the colonizing strain serves as an endogenous reservoir for clinical infections (von Eiff et al. 2004). The disease spectrum includes abscesses, bacteremia, central nervous

Table 1 Summary of validly published taxa of the genus *Staphylococcus* (modified according to Garrity et al. 2007; Euzéby 2009)

Species/subspecies of the genus <i>Staphylococcus</i>	Coagulase production	Host or source	Reference
<i>S. arlettae</i>	–	Goats, poultry	Schleifer et al. 1984
<i>S. aureus</i> subsp. <i>anaerobius</i>	+	Sheep	de la Fuente et al. 1985
<i>S. aureus</i> subsp. <i>aureus</i>	+	Humans, animals, environment	Rosenbach 1884
<i>S. auricularis</i>	–	Humans	Kloos and Schleifer 1983
<i>S. capitis</i> subsp. <i>capitis</i>	–	Humans	Kloos and Schleifer 1975
<i>S. capitis</i> subsp. <i>urealyticus</i>	–	Humans, primates	Bannerman and Kloos 1991
<i>S. caprae</i>	–	Humans, goats	Devriese et al. 1983
<i>S. carnosus</i> subsp. <i>carnosus</i>	–	Meat products	Schleifer and Fischer 1982
<i>S. carnosus</i> subsp. <i>utilis</i>	–	Foodstuffs	Probst et al. 1998
<i>S. chromogenes</i>	–	Animals, milk	Hájek et al. 1986
<i>S. cohnii</i> subsp. <i>cohnii</i>	–	Humans	Schleifer and Kloos 1975
<i>S. cohnii</i> subsp. <i>urealyticus</i>	–	Humans, animals	Kloos and Wolfshohl 1991
<i>S. condimenti</i>	–	Soy sauce	Probst et al. 1998
<i>S. delphini</i>	+	Dolphins	Varaldo et al. 1988
<i>S. epidermidis</i>	–	Humans, animals, environment	Winslow and Winslow 1908; Schleifer and Kloos 1975
<i>S. equorum</i> subsp. <i>equorum</i>	–	Horses, cattle	Schleifer et al. 1984
<i>S. equorum</i> subsp. <i>linens</i>	–	Semi-hard cheeses	Place et al. 2003
<i>S. felis</i>	–	Cats	Igimi et al. 1989
<i>S. fleurettii</i>	–	Goat's milk cheese	Vernozy-Rozand et al. 2000
<i>S. gallinarum</i>	–	Poultry, birds	Devriese et al. 1983
<i>S. haemolyticus</i>	–	Humans, domestic animals, environment	Schleifer and Kloos 1975
<i>S. hominis</i> subsp. <i>hominis</i>	–	Humans	Kloos and Schleifer 1975
<i>S. hominis</i> subsp. <i>novobiosepticus</i>	–	Humans	Kloos et al. 1998b
<i>S. hyicus</i>	+#	Animals, foodstuffs	Devriese et al. 1978
<i>S. intermedius</i>	+	Mammals, birds	Hájek 1976
<i>S. kloosii</i>	–	Wild animals	Schleifer et al. 1984
<i>S. lentus</i>	–	Animals, rarely humans	Kloos et al. 1976
<i>S. lugdunensis</i>	–	Humans	Freny et al. 1988
<i>S. lutrae</i>	+	Otters	Foster et al. 1997
<i>S. microti</i>	–	Voles	Nováková et al. 2010
<i>S. muscae</i>	–	Flies, pigs	Hájek et al. 1992
<i>S. nepalensis</i>	–	Goats, rarely environment	Spergser et al. 2003
<i>S. pasteurii</i>	–	Humans, animals, foodstuffs	Chesneau et al. 1993a
<i>S. pettenkoferi</i>	–	Humans	Trülsch et al. 2007

(continued)

Table 1 (continued)

Species/subspecies of the genus <i>Staphylococcus</i>	Coagulase production	Host or source	Reference
<i>S. piscifermentans</i>	–	Fermented fishes	Tanasupawat et al. 1992
<i>S. pseudintermedius</i>	+	Animals	Devriese et al. 2005
<i>S. saccharolyticus</i>	–	Humans	Kilpper-Bälz and Schleifer 1981
<i>S. saprophyticus</i> subsp. <i>bovis</i>	–	Animals	Hájek et al. 1996
<i>S. saprophyticus</i> subsp. <i>saprophyticus</i>	–	Humans, mammals	Shaw et al. 1951
<i>S. schleiferi</i> <i>coagulans</i>	+	Dogs	Igimi et al. 1990
<i>S. schleiferi</i> subsp. <i>schleiferi</i>	–	Humans	Freney et al. 1988
<i>S. sciuri</i> subsp. <i>carnaticus</i>	–	Meat products	Kloos et al. 1997
<i>S. sciuri</i> subsp. <i>rodentium</i>	–	Rodents	Kloos et al. 1997
<i>S. sciuri</i> subsp. <i>sciuri</i>	–	Humans, animal	Kloos et al. 1976
<i>S. simiae</i>	–	Squirrel monkeys	Pantůček et al. 2005
<i>S. simulans</i>	–	Humans, mammals	Kloos and Schleifer 1975
<i>S. succinus</i> subsp. <i>casei</i>	–	Surface ripened cheese	Place et al. 2002
<i>S. succinus</i> subsp. <i>succinus</i>	–	Amber	Lambert et al. 1998
<i>S. vitulinus</i>	–	Animals, foodstuffs	Webster et al. 1994
<i>S. warneri</i>	–	Humans, primates	Kloos and Schleifer 1975
<i>S. xylosus</i>	–	Humans, animals, environment	Schleifer and Kloos 1975

only some strains

system infections, endocarditis, osteomyelitis, pneumonia, urinary tract infections, and syndromes caused by exotoxins, including staphylococcal scaled skin syndrome, toxic shock syndrome and food poisoning.

S. aureus is a bacterium which has gained notoriety over recent years due to its ability to evolve new virulent and drug resistant strains (Chambers 2001). Strains of this species carry in their genomes genes encoding virulence factors such as enterotoxins, toxic shock syndrome toxin, exfoliative toxins and immune evasion factors. These genes are often carried on mobile genetic elements including bacteriophages. In recent years, increasingly virulent strains of *S. aureus* have emerged in the community, causing severe skin and soft tissue infections and a lethal form of hemolytic pneumonia in children (Gillet et al. 2002). These new strains are associated with a bi-component toxin called Panton-Valentine leukocidin (PVL). The increasing levels of antibiotic resistance and the emergence of epidemic strains of bacterial pathogens over the last decade highlight the adaptability of bacteria and the remarkable speed of bacterial evolution (Enright et al. 2002).

Besides *S. aureus*, coagulase-positive staphylococcal species also includes *Staphylococcus delphini*, *Staphylococcus hyicus*, *Staphylococcus intermedius*, *Staphylococcus lutrae*, *Staphylococcus pseudintermedius* and *Staphylococcus schleiferi* spp. *coagulans*. These species appear in different animal species where they may lead to skin, mucose and organ diseases. In veterinary microbiology, coagulase-positive *S. intermedius*, *S. hyicus* and *S. schleiferi* spp. *coagulans* have received attention. Contact with them may lead to transfer to human hosts and a possible infection process.

The most clinically important coagulase-negative staphylococci are *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus lugdunensis*, *Staphylococcus saprophyticus*, *Staphylococcus warneri*, *Staphylococcus simulans* and *Staphylococcus sciuri*. *S. epidermidis* is a typical opportunist pathogen known to cause a number of infections in weakened patients. Certain types of hospital-acquired infections (e.g. those associated with the use of catheters) are due to strains producing slime, i.e. an adherence factor. *S. haemolyticus* is the second most frequently isolated species of coagulase-negative staphylococci after *S. epidermidis*. It has been detected in patients with infected wounds, urinary tract infections, endocarditis, and bacteremia. Interestingly, vancomycin resistance was reported in this species. *S. hominis* is the third most commonly isolated species whose multi-resistant subspecies *S. hominis* spp. *novobiosepticus* (Kloos et al. 1998b), mostly isolated from hemocultures or catheters, causes severe infections of the bloodstream (Chaves et al. 2005; Palazzo et al. 2008). *S. saprophyticus* is the causative agent of urinary tract infections. The species *S. sciuri* may cause peritonitis and is considered to be the natural reservoir of the *mecA* gene – the methicillin resistance determinant (Marsou et al. 1999; Couto et al. 2003). *S. lugdunensis* is an opportunist pathogen, commonly associated with endocarditis, septicemia, and prosthetic device infections. Diseases caused by other species such as *Staphylococcus cohnii*, *S. simulans*, or *S. warneri* are less frequent (Iwantscheff et al. 1985; Waldon et al. 2002). Other coagulase-negative staphylococcal taxa are part of normal microflora in humans and animals, but may also play a role in some infections. Therefore, they are not to be automatically considered contaminants since possibly implicated in severe and sometimes fatal diseases in weakened patients.

The Staphylococcus aureus Genome

Our understanding of the diversity and evolution of *S. aureus* has been greatly enhanced by sequencing of the *S. aureus* genomes. Comparative genomic analysis allows identifying variation in the genomes and understanding the mechanisms by which this versatile bacterium has accumulated changes within its genome structure (Lindsay 2008). Adaptation via mutations tends to be a slow process, whereas horizontal gene transfer allows a bacterium to adapt to a new niche very rapidly. Currently, there are 19 finished *Staphylococcus* genomic sequences in nucleotide databases and additional sequences are underway (Table 2).

Table 2 Finished staphylococcal genomes

Species	Strain	Size (kb)	No. of plasmids	No. of prophages	GenBank accession no.	Reference
<i>S. aureus</i>	Mu50 (VISA)	2878	1	2	NC_002758	Kuroda et al. 2001
<i>S. aureus</i>	N315 (MRSA)	2813	1	1	NC_002745	Kuroda et al. 2001
<i>S. aureus</i>	MW2 (caMRSA)	2820	0	2	NC_003923	Baba et al. 2002
<i>S. aureus</i>	MRSA252	2902	0	2	NC_002952	Holden et al. 2004
<i>S. aureus</i>	MSSA476	2799	1	2	NC_002953	Holden et al. 2004
<i>S. aureus</i>	RF122 (bovine)	2742	0	1	NC_007622	Herron-Olson et al. 2007
<i>S. aureus</i>	COL (MRSA)	2809	1	1	NC_002951	Gill et al. 2005
<i>S. aureus</i>	USA300 (caMRSA)	2872	3	2	NC_007793	Diep et al. 2006
<i>S. aureus</i>	NCTC 8325	2969	0	3	NC_007795	Gillaspy et al. 2006
<i>S. aureus</i>	JH1 (VISA)	2906	1	4	NC_009632	Mwangi et al. 2007
<i>S. aureus</i>	JH9 (VISA)	2906	1	4	NC_009487	Mwangi et al. 2007
<i>S. aureus</i>	Mu3 (hetero-VISA)	2880	0	2	NC_009782	Neoh et al. 2008
<i>S. aureus</i>	Newman	2878	0	4	NC_009641	Baba et al. 2008
<i>S. aureus</i>	USA300_TCH1516	2872	1	2	NC_010079	Highlander et al. 2007
<i>S. carnosus</i>	TM300	2566	0	1	NC_012121	Rosenstein et al. 2009
<i>S. epidermidis</i>	ATCC 12228	2499	6	0	NC_004461	Zhang et al. 2003
<i>S. epidermidis</i>	RP62A	2616	1	1	NC_002976	Gill et al. 2005
<i>S. haemolyticus</i>	JCSC1435	2685	0	2	NC_007168	Takeuchi et al. 2005
<i>S. saprophyticus</i>	ATCC 15305	2516	2	1	NC_007350	Kuroda et al. 2005

Approximately 78% of *S. aureus* genomes comprise a core component consisting of genes present in all strains. The remaining 22% of the genes comprise an “accessory genome”, containing genes that encode a diverse array of non-essential functions, ranging from virulence, drug and metal resistance to substrate utilization and miscellaneous metabolism (Fitzgerald et al. 2001). Many of the regions that make up the accessory genome are mobile genetic elements (MGEs) that can be transferred horizontally between bacteria, including bacteriophages, *S. aureus* pathogenicity islands, staphylococcal cassette chromosomes (SCC), plasmids and transposons (Lindsay and Holden 2006). Most virulence genes tend to be found on phages and genomic islands, while resistance genes rely on SCC, plasmids and transposons for transfer. At the same time, clinical isolates of epidemic strains can differ significantly in their carriage of MGEs.

Mobile Genetic Elements of Staphylococcus aureus

Given the steadily varying content of MGEs and of their individual types in the *S. aureus* species, these elements are the source of genetic polymorphism used for strain typing. Therefore, MGEs are one of the most important targets of molecular typing methods in *S. aureus*.

Plasmids

One or more plasmids are usually found in clinical isolates of *S. aureus*. There are at least three families of *S. aureus* plasmids (Novick 1990), which are classified into fifteen incompatibility groups (Novick 1987). Most plasmids that have been described encode antimicrobial resistance determinants, and some have also been attributed other clinically significant properties, such as toxin production (e.g. enterotoxins SED and SEJ, and exfoliative toxin ETB), whereas some staphylococcal plasmids are phenotypically cryptic.

Class I plasmids are usually found at high copy number and encode only one or two resistance genes. Plasmids can be transferred between *S. aureus* by generalized transduction at approximately 100 times the frequency of chromosomal markers (Ubelaker and Rosenblum 1978). They can also be mobilized by some class III conjugative plasmids at low frequency (Projan and Archer 1989). Class II plasmids typically carry resistance to one or more of the β -lactams (*bla*), heavy metals (*ars*, *cad*, *mer*), antiseptics (*qac*) or aminoglycosides (*aacA*–*aphD*). The resistance genes are often on transposons that have integrated into the plasmid, and plasmid variants are often due to rearrangements of these elements. Class III plasmids encode transfer (*tra*) genes that allow conjugative transfer of the plasmid between bacterial isolates at low frequency on solid surfaces (Thomas and Archer 1989). Since some class III plasmids are up to 60 kb, they can be too large to be transferred by transduction. It is becoming increasingly apparent that much horizontal transfer of plasmids is dependent on generalized transduction by bacteriophage, but the distribution and frequency of such phages are not known.

Bacteriophages

Temperate staphylococcal bacteriophages of the *Siphoviridae* family are classified assuming their lytic activity, morphology and serological properties into the six tentative phage species that includes serologically and morphologically related phages (Ackermann and DuBow 1987). In *S. aureus*, phages integrated into the chromosome (prophages) are common, with most strains carrying between one and four prophages (Pantůček et al. 2004; Goerke et al. 2009). Temperate bacteriophages also have been widely used in phage typing, a routine diagnostic tool for the differentiation of clinical strains. Recently, some phages, by their very nature, are of greatest importance as potential antibacterial agents in medicine for treatment of serious staphylococcal infections (phage-based therapeutics) (Gorski et al. 2007).

At present, more than 80 complete staphylococcal phage and prophage genomes can be retrieved from the GenBank database, and knowledge about them has opened new possibilities for the understanding of their role in the pathogenicity of the staphylococcal strains (Kwan et al. 2005).

As prophages are incorporated in *S. aureus* genomes, they play an important role in the biology of this species, usually changing its phenotype as a result of lysogenic conversion associated with virulence factors. At least two *S. aureus* phage integration sites are within known virulence genes, beta-hemolysin (*hlyB*) and lipase (*geh*), and thus, phage negative conversion leads to loss of these gene products. Many *S. aureus* bacteriophage genomes contain additional virulence genes located between the phage lysin gene and the right attachment site, such as *sak* (staphylokinase, a thrombolytic enzyme), *chp* (chemotaxis inhibitory protein and inhibitor of leukocyte migration), *scn* (staphylococcal complement inhibitor), *sea* (enterotoxin A, a common cause of food poisoning), additional enterotoxin genes *see*, *seg*, *sek* and *sep* (enterotoxins E, G, K and P), *eta* (exfoliative toxin A, a cause of scalded skin syndrome), *lukS-PV* and *lukF-PV* (Panton-Valentine leukocidin implicated in hemolytic pneumonia and severe skin and soft tissue infections) (Brussow et al. 2004; van Wamel et al. 2006). Additional putative virulence factor genes with unknown function were revealed in some prophages integrated in chromosomes of genome sequenced strains.

An understanding of bacteriophage carriage has importance not only in the determination of a high genomic variability in *S. aureus* but also from an overall genomics standpoint concerning the evolution and virulence of the bacterial strains. As most of *S. aureus* strains are lysogenic or polylysogenic and because there are differences in prophage content in closely related strains, detection of prophages and their characterization is very valuable for differentiating the clinical strains in epidemiological studies (see Section [Methods Without DNA amplification](#), Prophage profiling).

Staphylococcus Aureus Pathogenicity Islands

S. aureus pathogenicity islands (SaPI) are approximately 15–16 kb mobile elements that encode many virulence genes, including the superantigens, toxic shock syndrome toxin-1 (*tst*) and enterotoxins B and C (*seb* and *sec*). In the sequenced genomes of

S. aureus strains, six SaPI types have been identified, four of which carry superantigen genes (Yarwood et al. 2002; Novick 2003; Baba et al. 2008). Many of the genes contained in SaPIs are homologous to the described phage genes, suggesting they are of bacteriophage origin.

Staphylococcal Cassette Chromosomes

Staphylococcal cassette chromosomes (SCC) are mobile elements 21–67 kb in size. They often encode antibiotic resistance genes, such as the *mec* operon for methicillin resistance (SCC*mec*) (Hanssen and Sollid 2006) and genes for fusidic acid resistance (SCC*far*) (Holden et al. 2004). SCC*mec* elements are rather divergent. In the structure of SCC*mec* elements, the *ccr* gene complex, *mec* gene complex and junkyard regions are used for the exact description of structural variants.

At least six different variants of SCC*mec* are found in *S. aureus* (Ito 2009). SCC frequently includes transposons and integrated plasmids. Genes responsible for a particularly mucoid version of the *S. aureus* capsule have also been reported on SCC*cap* (Luong et al. 2002). How the element replicates and then transfers horizontally between bacteria is not clear. Since the original SCCs are too large to fit into a generalized transducing phage head, this may account for why SCC*mec* spread relatively infrequently. However, the SCC*mec* type IV present usually in community acquired MRSA (caMRSA) appear to spread much more easily between isolates (Robinson and Enright 2003); this could be because of their smaller size, which allows them to be transduced.

Transposons

S. aureus transposons are small mobile elements that often encode resistance genes (β -lactamase, resistance to erythromycin and tetracycline). All the transposons encode a transposase gene, and the product of this gene catalyses excision and/or replication of the element, as well as integration. Horizontal transfer of transposons to other *S. aureus* cells is presumably mediated by another MGE that is transferred, most likely a plasmid transferred by transduction or conjugation. Conjugative transposons have also been described in *S. aureus*. However, it is not clear if native conjugative transposons are found in *S. aureus* (Novick 1990).

Horizontal Gene Transfer in *Staphylococcus aureus*

For some bacterial pathogens such as staphylococci or streptococci, strain diversity can be reliant on particular types of MGEs and the mechanism of genetic exchange. All horizontal gene transfer in bacteria is via three mechanisms: transformation, conjugation and transduction (Thomas and Nielsen 2005). However, *S. aureus* probably

lack typical genes necessary for transformation, and conjugative transfer of elements between *S. aureus* is very infrequent (Robinson and Enright 2004a). Therefore, most horizontal transfer by *S. aureus* is probably dependent on bacteriophages, which therefore play a pivotal role in the evolution of this pathogen. The horizontal transfer of virulence factors by bacteriophage occurs in two ways: first, by transfer and integration of a bacteriophage that encodes a virulence gene on its genome (lysogenic conversion); and second, by generalized transduction. SaPIs transfer horizontally by specialized transduction in the presence of a helper phage (Ruzin et al. 2001; Tormo et al. 2008; Chen and Novick 2009). A novel but poorly understood mechanism of genetic exchange, namely mixed-culture transfer or phage-mediated conjugation, has also been identified in staphylococci. Although phage, or components of phage, plays a role in a mixed-culture transfer, the process has been shown to be mechanistically distinct from transduction (Thompson and Pattee 1977).

Genetic exchanges inside *S. aureus* and between *S. aureus* and other staphylococcal species are one of the most important topics in the research of the evolution and pathogenesis of *S. aureus*. A scarcity of recombination contributes to the highly clonal structure of *S. aureus*; lateral transfer of MGEs is controlled by some cryptic rules, resulting in mutual exclusion of toxin genes and preference of specific toxin genes within certain lineages. For example, the community-acquired MRSA (positive for SCCmec) that also carry genes for PVL on a bacteriophage seem to spread, diversify and cause skin and soft tissue infection in otherwise healthy people (Kennedy et al. 2008). A better understanding of the underlying mechanism of genetic exchange would help to reconstruct the history of lineage formation and to make some interesting predictions, for example, whether those dangerous determinants, such as gene *vanA* corresponding to high level vancomycin resistance obtained from enterococci and the PVL locus, would disseminate to a larger extent (Feng et al. 2008).

Identification of Staphylococcal Species

Phenotypic Identification

Staphylococcus species can be identified on the basis of microscopy and colony morphology supplemented with a variety of conventional physiological and biochemical characters from which some could be considered key characteristics (Kloos and Bannerman 1999). Biochemical identification based on conventional tube and plate tests is still frequently used in many routine clinical laboratories. The most important and standard biochemical traits for species identification of staphylococci are the production of coagulase, clumping factor, hemolysins, catalase, oxidase, urease, alkaline phosphatase, nitrate reduction, arginin dihydrolase, the activity of pyrrolidonyl arylamidase, ornithine decarboxylase, β -galactosidase, acetoin production, and novobiocin, lysostaphin, lysozyme, furazolidone, bacitracin

and polymyxin B resistance, and acid production from a set of carbohydrates. Molecular phenotyping methods include estimations of cell wall composition; configuration of lactate produced in glucose fermenting; presence of NAD-dependent L- or D-lactate dehydrogenase; metabolic pathways of galactose (and lactose) degradation; fructose category 1,6-bisphosphatase; and cytochrome types (Frenay et al. 1999). Some of the tests described above may not be suitable for routine use in certain clinical laboratories because a number of specialized methods are required by the laboratory. A better manner of staphylococcal identification is the application of rapid manual, mechanized or automated identification systems representing a modification of classical methods (API Staph, ID 32 Staph, Vitek System, STAPHYtest 24, Biolog, etc.).

For species characterization, whole-cell and extracellular proteins (Clink and Pennington 1987; Berber et al. 2003) or cellular fatty acid analysis and/or combined with gas chromatography may be applied. In general, fatty acid profiles correlate with the data on DNA homology and phenotype features (Behme et al. 1996). Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was experimentally applied to intact bacterial cells or specimens prepared using extraction procedures. This chemotaxonomic method was used for identification of the staphylococcal species and MRSA strains (Du et al. 2002; Carbonnelle et al. 2007).

DNA Based Identification of Staphylococcal Species

For accurate and reliable identification of staphylococci, a molecular genetic method, based on DNA analysis, should be applied. Different methods can provide a different quality of information related to species evolution and their applicability is discussed widely in literature (Gurtler and Mayall 2001; van Belkum et al. 2001). As with other bacterial species, direct methods may be applied in staphylococcal taxonomy for the analysis of conservative operating gene sequences of 300–2500 bp, whose number is estimated around 30 (Palys et al. 1997; Zeigler 2003), and/or indirect methods using restriction fragment length polymorphism (RFLP) or amplified fragment length polymorphism (AFLP).

Among the direct methods utilizing DNA sequence analysis for the *Staphylococcus* species, besides the sequence analysis 16S rRNA (Takahashi et al. 1999; Becker et al. 2004), the suitable analyses also correlating well with the above method are those of highly conservative gene sequences for 60 kDa chaperonin (*cpn60*) (Goh et al. 1996; Kwok and Chow 2003), superoxid dismutase gene (*sodA*) (Poyart et al. 2001), RNA polymerase B gene (*rpoB*) (Drancourt and Raoult 2002; Mellmann et al. 2006) and *dnaJ* gene (Shah et al. 2007). For identification purposes, other conservative sequences were also tested in genetic studies, such as e.g. *femA* gene (Vannuffel et al. 1999), elongation factor Tu gene (*tuf*) (Martineau et al. 2001) and glyceraldehyde-3-phosphate dehydrogenase gene (*gap*) (Layer et al. 2007; Ghebremedhin et al. 2008).

Ribotyping and automated ribotyping are suitable for identification of species and subspecies within the genus *Staphylococcus* (see Section [Methods Without DNA Amplification](#), Ribotype Pattern Analysis). Less laborious and also less expensive are methods focused on identification of amplification fragment length polymorphisms, such as the internal transcribed spacer-PCR method based on the different distances separating conserved functional RNA genes that allows detection of a wide range of length and sequence polymorphisms at the genus- and species-levels. Analysis of these 16S–23S rRNA intergenic spacer sequences proved useful in differentiating between species (Jensen et al. 1993; Mendoza et al. 1998; Couto et al. 2001). Another internal transcribed spacer-PCR method is based on the different distances separating conserved tRNA genes in staphylococcal species (Welsh and McClelland 1992). Since tRNA genes contain sequence motifs that are highly conserved among the bacteria, consensus tRNA gene primers can be designed to explore intergenic length polymorphisms between species (Maes et al. 1997).

The detection of a number of genes and conserved genomic sequences has been used for identification of the staphylococcal species by hybridization, PCR or PCR-RFLP. PCR based identification of the clinically most important species *S. aureus* targets specific genes and sequences such as thermonuclease or heat-stable nuclease (*nuc*) (Brakstad et al. 1992, 1995; Chesneau et al. 1993b; Barski et al. 1996), Sa2052 fragment (Štěpán et al. 2001), Sa442 fragment (Martineau et al. 1998; Klaassen et al. 2003), 16S rRNA (Saruta et al. 1995; Schmitz et al. 1997; Mason et al. 2001), 23S rRNA (Straub et al. 1999; Akineden et al. 2001; Stephan et al. 2001) and 16S–23S spacer region (Saruta et al. 1997). Targeting loci whose intraspecies DNA sequence similarities are lower than 80% allowed the development of PCR-RFLP assays for species identification based on *dnaJ* (Hauschild and Stepanovic 2008), *gap* (Yugueros et al. 2001) and *tuf* genes (Kontos et al. 2003).

Recently, real-time PCR (quantitative PCR, qPCR) has been used for identification of *S. aureus* species (Du et al. 2002; Palomares et al. 2003) and the species of coagulase-negative staphylococci (Edwards et al. 2001).

Considerable acceleration in molecular biology assays has profited from DNA microarray technology. The microarray containing 16S rDNA probes can be used for the specific identification of bacteria (Wilson et al. 2002). The low-density microarrays containing tens of oligonucleotides were used for identification of five staphylococcal species and methicillin resistance using the probes *femA* and *mecA* (Hamels et al. 2001). The microarray containing 23S rDNA probes was used for the identification of pathogenic bacteria including *S. aureus* (Anthony et al. 2000). Microelectronic chip arrays enable anchored *in situ* amplification, discrimination and detection on the same platform simplifying the process of bacterial identification (Westin et al. 2001).

A novel technique that combines the unique performance characteristics of peptide nucleic acid probes (PNA) with fluorescence *in situ* hybridization (FISH) for identification of *S. aureus* directly from positive blood cultures without cultivation and biotyping has been described (Kempf et al. 2000) and has recently been applied to the rapid and specific identification of bacteria including *S. aureus* (Oliveira et al. 2003).

Typing Methods

Conventional Strain Typing Methods for Staphylococcus aureus

Commonly used conventional methods for the typing of staphylococcal strains include biotyping, phage typing, antibiotic susceptibility testing, and multilocus enzyme electrophoresis (MLEE). MLEE typing of *S. aureus* is not a widely used typing technique because this method might now be replaced by DNA-based methods targeted on genomic polymorphisms of housekeeping genes. Phage typing is performed with the phages of the International Typing Set. As more than 20% of the strains are insensitive to all the phages used, the discriminatory power of phage typing is rather limited. Antibiotic susceptibility testing also exhibits low discriminatory power but it may serve as a useful epidemiological marker in tracing specific antibiotic-resistant clones of *S. aureus*.

Genotyping Methods for Staphylococcus aureus

Genotypic methods focus on the characterization of chromosomal, plasmid, or total genomic DNA. The objectives of these methods are to assess relevant parameters of the staphylococcal genome and to detect polymorphism of DNA sequences by either direct or indirect methods. All the methods are aimed at the generation of DNA fingerprints specific for individual species and/or isolates of distinct clonal lineages. The indirect genotypic methods include techniques either without DNA amplification (restriction analysis of chromosomal or plasmid DNA and hybridization techniques), or with DNA amplification by PCR and those identifying conformation polymorphisms of nucleic acids (Versalovic et al. 1993; Tenover et al. 1994, Tenover et al. 1997; Vaneechoutte 1996; Struelens 1998; Andrei and Zervos 2006; Singh et al. 2006; Tenover 2007). The resulting patterns can be evaluated either visually or by means of computer image analysis.

Typing systems are generally rated based on seven criteria, namely typeability, reproducibility, stability, discriminatory power of typing systems, epidemiologic concordance, ease of interpretation, and ease of use (Maslow et al. 1993; Maslow and Mulligan 1996; Struelens 1996).

In this respect, the genotypic methods have the advantage over phenotypic methods of being independent of gene expression under laboratory assay conditions. Thus, the genotypic methods yield reproducible results regardless of variation in laboratory assay conditions (e.g. cultivation media used); they are rapid, do not require *in vitro* cultivation and allow identification of fastidious or nonculturable bacteria. Generally, the discriminatory ability of genotypic compared to phenotypic methods is higher (some of genotypic methods achieve typeability of up to 100%) and thus allow typing of *S. aureus* strains that cannot be differentiated by phenotypic techniques used for staphylococci (e.g. bacteriophage typing).

Table 3 Comparison of genotyping systems applied to *Staphylococcus aureus* (modified according to Witte et al. 2006)

Typing system	Reproducibility	Discriminatory power
Multilocus sequence typing (MLST)	High	Good
<i>spa</i> typing	High	Good
Restriction fragment length polymorphism		
<i>Sma</i> I macrorestriction pattern (PFGE)	High	Excellent
Ribotyping	Good	Moderate
Hybridization of <i>mecA</i> and Tn554 probes to genomic digests	High	Good
Plasmid profiles	High	Weak
PCR-detected polymorphisms		
rRNA gene spacer (PCR ribotyping)	High	Moderate
rep-PCR	Moderate	Good
Multiple-locus variable-number tandem repeat analysis (MLVA)	High	Excellent

The main typing systems used for epidemiological purposes in *S. aureus* are summarized in Table 3. The methods are compared in respect to their reproducibility and discriminatory power. Typing by means of PCR methods is rapid and inexpensive and provides results which are sufficient for quick analysis concerning the relatedness of nosocomial isolates. *Spa* sequence typing has the advantage of high reproducibility; the sequence data are portable and can be stored in databases. Moreover, *spa* typing has a good discriminatory power and can, therefore, replace *Sma*I macrorestriction analysis.

Cookson et al. (2007) evaluated three molecular typing methods used within the HARMONY group (Cookson and the HARMONY participants 2008) to examine their usefulness for multicenter MRSA surveillance networks that use these different methodologies. PFGE groupings were compared to those made by *spa* gene sequencing and multilocus sequence typing (MLST), combined with PCR analysis of *SCCmec*. A high level of discrimination was achieved using each of the three methodologies. The authors pointed out that the choice of typing method used to investigate the epidemiology of MRSA should be dictated by the environment and/or timescale being examined and the aims of the investigation. For a short-term outbreak in a single hospital, the PFGE method could be used. For more widespread epidemiological typing, the *SCCmec* and MLST could be used for clonal confirmation of isolates.

Methods Without DNA Amplification

Pulsed-Field Gel Electrophoresis (PFGE) of Restriction Endonuclease Fragments (Macrorestriction Analysis) In this method chromosomal DNA is isolated and digested with rare-cutting restriction enzyme, e.g. *Sma*I or *Csp*I in *S. aureus*. Usually 10–30 macrorestriction fragments in the size range 1 kb–1 Mb are generated and separated by PFGE. Strains with identical restriction endonuclease

patterns are assigned to the same macrorestriction type (PFGE type). Strains that differ by one or two band shifts corresponding to a single genetic event (a point mutation resulting in the loss or the gain of a restriction site, an insertion, a deletion, or an inversion) are assigned to a subtype, while isolates with three and more such differences are considered to be different types. According to these criteria, genetic relationships among isolates are determined for epidemiological purposes (Tenover et al. 1995). In comparison with other genotyping methods, macrorestriction analysis has shown equal or greater discriminatory power (Maslow and Mulligan 1996), and superior reproducibility (Tenover et al. 1997). Macrorestriction analysis was also used to determine interspecies and intraspecies genome variability in staphylococci (Snopková et al. 1994; Pantůček et al. 1996). This method is used for studies of outbreaks and local epidemiology (Richardson and Reith 1993; Dominguez et al. 1994; Cox et al. 1995; Chang et al. 2000; von Eiff et al. 2001), and it has also been applied to macroepidemiological studies (Aires de Sousa et al. 1998; Oliveira et al. 1998; van Belkum et al. 1998; Booth et al. 2001; McDougal et al. 2003). Recently, harmonized protocols for epidemiological typing of MRSA have been described (Murchan et al. 2003).

In long term epidemiologic studies, PFGE markers may be insufficient for the reliable application of this method (Blanc et al. 2002), therefore the combination of DNA sequence based typing techniques (*spa* typing and MLST) and PFGE represent high interlaboratory reproducibility (Aires de Sousa et al. 2006; Strommenger et al. 2006; Hallin et al. 2007).

Selective Restriction Fragment Hybridization (SRFH) This method is used for the detection of genomic DNA polymorphism by Southern blot hybridization. Total cellular DNA is digested with frequent-cutting restriction enzyme, e.g. *ClaI*. Restriction fragments are separated by agarose gel electrophoresis, transferred to nylon membrane and hybridized with radioactively or non-radioactively labeled probes that target various genes or sequences, e.g., *mecA*, *Tn554*, *agr* or *aacA-aphD* of *S. aureus* (Kreiwirth et al. 1993; Tenover et al. 1994). The important modifications of SRFH method used for molecular typing of staphylococci are ribotyping and prophage profiling.

Ribotype Pattern Analysis The ribosomal DNA gene restriction patterns determination (ribotyping) is a special modification of RFLP typing using probes targeting 16S rRNA and 23S rRNA genes. Ribotyping refers to a Southern blot analysis in which strains are characterized for their restriction fragment length polymorphism associated with the ribosomal operones. Since rRNA genes are highly conserved, ribotyping provides an extremely accurate means of identification. In addition, small variations in the fingerprint allow isolates to be compared and form the basis for the characterization of bacteria at the strain level.

The most often used restriction endonucleases for staphylococci are *HindIII*, *ClaI* (Tenover et al. 1994), *EcoRI* (Blumberg et al. 1992), *EcoRV* or *KpnI* (Blanc et al. 1994). The fragments containing specific sequences are then detected using a labelled piece of homologous DNA as a probe. The most often used is DNA complementary to 16S and 23S rRNA isolated from *Escherichia coli* and commercially available for ribotyping (Falkinham 1994; Pfaller and Hollis 2004).

The use of ribosomal RNA gene (rDNA) restriction patterns as a potential taxonomic tool has been applied to the analysis of taxonomic and/or epidemiological relationships among strains of different groups of organisms (Altwegg and Mayer 1989) including staphylococci (de Buyser et al. 1989; el Solh et al. 1990; Chesneau et al. 1992, 2000). The method is usable for both the identification and typing of staphylococci occurring in clinical material (Melter et al. 1999, 2003; Růžicková et al. 2003) as well as for strains isolated from food (Pantůček et al. 1999), or from different animals and environment (Hesselbarth and Schwarz 1995). Ribotyping generally is a robust method that exhibits good reproducibility and stability; however its discriminatory power is lower than that of chromosomal DNA macrorestriction analysis with *Sma*I (Prévost et al. 1992).

Moreover, ribotyping was used as basic method for the description of several new taxa among species and subspecies of family *Staphylococcaceae* during the last 2 decades (Webster et al. 1994; Kloos et al. 1998a; Mannerová et al. 2003; Pantůček et al. 1999) and is also one part of the “requested minimal standards techniques” for the description of novel staphylococci (Freney et al. 1999). Ribotyping provides an important means to subtype strains and to monitor the incidence of various clones of pathogenic *S. epidermidis* in hospitals (Malíková et al. 2007).

Ribotyping with other molecular techniques demonstrated the occurrence of several “non-human” staphylococci, *S. nepalensis*, *S. succinus* and *S. equorum*, in clinical material (Nováková et al. 2006a, b) and for *S. nepalensis* and *S. succinus* strains there has been the first incidence in human clinical sources, probably as a rare pathogens. Besides, ribotyping method was of cardinal importance for reclassification of “*Staphylococcus pulvereri*” as *S. vitulinus* (Švec et al. 2004) based on strains from human clinical material as well.

Automated Ribotyping Until recently, some genetic investigations, such as ribotyping, were limited to highly specialized laboratories due to the time and expertise required. An automatic performing of ribotyping now enables the new commercially available system RiboPrinter® Microbial Characterisation System (DuPont Qualicon, USA). This fully automated ribotyping technique offers high standardization and significantly accelerates the entire process of ribotyping. It provides much faster results, speeding up the ribotyping process from 5 or more days to only 8 h, without sacrificing accuracy. Automated ribotyping was used for rapid identification or for taxonomic diversity study of gram-positive cocci including staphylococci (Andollina et al. 2004; Carretto et al. 2005). From a comparison of manual and automatic ribotyping results, there is clear evidence of the lower discrimination power of the automatic system which is not able to distinguish similar heavy and weak fragments (Dalsgaard et al. 1999). The advantage of ribotyping, especially automated ribotyping with RiboPrinter®, is typeability, ease of use, reliability and cost; this method is ideal for epidemiological studies (Vaneechoutte 1996; van Belkum et al. 2001).

Plasmid Profiling Plasmids are accessory genetic elements representing an important part of the staphylococcal genome. The majority of *S. aureus* plasmids are responsible for the antibiotic resistance of the host strains (e.g. penicillinase, tetracycline, chloramphenicol, and erythromycin plasmids). For the molecular typing

of multiresistant *S. aureus* strains, the isolated intact plasmid DNA is separated by agarose gel electrophoresis and the numbers and sizes of plasmids are determined (Hall et al. 1989; Etienne et al. 1990; Tveten et al. 1991; Blumberg et al. 1992). Since the content of plasmid types (i.e. plasmid profile) harbored by clinical strains shows considerable variation, plasmid analysis is suitable for the study of relatively recent epidemiological relationships, whereas analysis of chromosomal DNA reflects relationships over a longer period of time.

The discriminatory power of plasmid profiling could be increased by restriction analysis of plasmids (REAP). In this variant, the isolated plasmid DNA is digested with restriction endonucleases; in *S. aureus* typing, combination of *Hind*III and *Eco*RI has been used (Tenover et al. 1994). The REAP method could be complemented with DNA hybridization analysis using probes targeting specific plasmid genes (e.g. genes encoding antibiotic resistance).

Prophage Profiling Lysogenization leads to changes in host macrorestriction maps due to the integration of phage genomes at specific *attB* sites on the bacterial chromosome (Borecká et al. 1996). Thus prophages contribute to interstrain genetic variability in *S. aureus*. In particular, bacteriophages play an important role in the pathogenicity of *S. aureus* by either carrying accessory virulence factors (*eta*, *lukSF*-PV, *sak*, *sea*) or interrupting chromosomal virulence genes (*hly* or *geh*) upon insertion. Phages increase the genome plasticity of *S. aureus* during infection facilitating the adaptation of the pathogen to various host conditions (Goerke et al. 2006a, b). An understanding of bacteriophage carriage has importance not only in the determination of a high genomic variability in *S. aureus* but also from an overall genomics standpoint concerning evolution and virulence of the bacterial strains. Therefore new molecular diagnostics techniques utilizing prophage profiling for the precise estimation of bacteriophage content and potential of virulence genes or occasion of horizontal transfer of MGEs are necessary in strain typing.

The prophage content of *S. aureus* strains can be determined by selective hybridization with phage-species specific probes followed by RFLP analysis (Doškař et al. 2000) or by the multiplex PCR assay targeting structural genes of head-tail modules (Pantůček et al. 2004) and phage integrase genes (Goerke et al. 2009). A rapid strain differentiation method of MRSA strains by detecting phage-derived ORFs has been developed recently (Suzuki et al. 2006).

Methods Based on DNA Amplification of Specific Genes and Sequences

Gene-Specific PCR As with other bacterial species, the PCR method is applied to identify specific gene sequences or variable genomic loci in the case of molecular diagnostics of staphylococci. When comparing PCR typing with other DNA fingerprinting techniques, their finer discriminating ability is noteworthy, but this ability may be raised significantly by optimizing reaction conditions and searching for new primers, derived from genes or DNA sequences characterizing specific strains. The introduction of the PCR method significantly speeds up and facilitates identification of staphylococci in clinical material. Simultaneous amplification of several

genes in one reaction mixture allows multiplex PCR assay. Genes and sequences frequently used for PCR-based molecular identification of *S. aureus*, for molecular typing of *S. aureus* strains and identification of genes coding for surface protein adhesins, staphylococcal toxins and those associated with antibiotic resistance and virulence are reviewed by Štěpán et al. (2004).

SCCmec Typing In MRSA strains, PCR analysis targeting staphylococcal cassette chromosome SCCmec is frequently combined with *spa* typing and clonal designations based on MLST. Recently, many multiplex PCR assays for identification of the SCCmec element structural types and their variants have been developed (Hanssen et al. 2004; Zhang et al. 2005; Oliveira et al. 2006a, b; Kondo et al. 2007; Milheirico et al. 2007a, b; Berglund et al. 2009). The nomenclature of SCCmec is not yet finished (Chongtrakool et al. 2006; Stephens et al. 2007; Ito 2009), staphylococcal genomes seem to change continuously and novel SCCmec types are described (Boyle-Vavra et al. 2005; Jansen et al. 2006; Heusser et al. 2007; Berglund et al. 2008; Shore et al. 2008; Takano et al. 2008; Zhang et al. 2009).

PCR-RFLP Typing In this method, a specific gene or genomic region is amplified and the amplicon is cut with restriction endonuclease resulting in specific RFLP pattern, e.g. the coagulase gene cleaved with *AluI* (Goh et al. 1992) or protein A gene cleaved with *RsaI* (Frenay et al. 1994).

Ribosome Spacer PCR (RS-PCR) or PCR Ribotyping This method is based on the polymorphic nature of the 16S–23S rRNA internal transcribed spacer sequences (Barry et al. 1991) and allows the detection of length and sequence polymorphisms at the genus- (see Section [DNA Based Identification of Staphylococcal Species](#)) and species-levels. It has been used in typing strains within the genus *Staphylococcus* (Gurtler and Barrie 1995; Kumari et al. 1997).

Multiple-Locus Variable Numbers of Tandem Repeat (VNTR) Analysis (MLVA) MLVA enables simultaneous PCR-RFLP analysis of several loci containing VNTR. The *sdr*, *clfA*, *clfB*, *ssp* and *spa* genes are used for *S. aureus*. For molecular typing of MRSA strains, MLVA showed equivalent discriminatory power and reproducibility to PFGE (Sabat et al. 2003). Recently it has been found that MLVA can even be used to identify unrelated strains with identical PFGE patterns or confirm a close genetic composition of linked isolates (Tenover et al. 2007; Moser et al. 2009).

Arbitrarily Primed PCR (AP-PCR, RAPD) AP-PCR is based on the use of short oligonucleotide primers with an arbitrary sequence at low-stringency PCR conditions (Welsh and McClelland 1990; Williams et al. 1990). A number of technical parameters need to be strictly standardized for optimal reproducibility (Grundmann et al. 1997). The PCR-patterns obtained are evaluated preferably visually rather than by using gel analysis software (Burr and Pepper 1997). The method is adequate for rapid comparative typing but less suitable for library typing in surveillance programs (van Belkum et al. 1995).

Repetitive PCR (rep-PCR) The PCR amplification of genomic regions between interspersed repeated DNA sequences (referred to as “rep”) is based on different positions

of DNA elements on the chromosome and various distances separating them, depending on the particular bacterial strain and species. Repetitive Extragenic Palindromic (REP) elements and Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences belong to these repetitive elements (Versalovic et al. 1991). Other repetitive elements used in rep-PCR are IS sequence of *S. aureus* (IS256) (Deplano et al. 1997), RepMP3 from *Mycoplasma pneumoniae* usable for gram-positive bacteria (van der Zee et al. 1999), (GTG)₅ repetitive motif (Nováková and Švec 2008), and BOX sequence from *Streptococcus pneumoniae*, which seems to be an excellent tool for the identification of *S. epidermidis* (Wieser and Busse 2000).

Opposing PCR primers matching these sequences yield various numbers of differently long fragments under high stringency amplification conditions depending on the bacterial strain, from which DNA originates; however, low-stringency PCR conditions and different primer combinations are used in practice (van Belkum et al. 1993; Te Witt et al. 2009). A method based on RFLP analysis of rep-PCR-amplicons derived from staphylococcal repetitive element STAR was developed for typing MRSA strains (Quelle et al. 2003). Compared to AP-PCR, rep-PCR produces fewer amplified DNA fragments, but can provide good discriminatory power and has the major advantage of higher reproducibility. Nevertheless, the rep-PCR methods produce an array of DNA fragments showing interlaboratory variability thus leading to the problem of data comparability. A particular improvement to this approach is fluorophore-enhanced rep-PCR that uses primers labeled at their 5' ends with a fluorescent compound, separation of PCR products in a nondenaturing polyacrylamide gel, which is scanned with a laser scanning unit enabling digital processing of DNA fingerprints (Del Vecchio et al. 1995; Versalovic et al. 1995). Another modification is automated laser fluorescence analysis (ALFA) also using 5' fluorescently labeled primers with PCR products being separated in a denaturing polyacrylamide gel and resolved in an automated DNA sequencer, which moderately improves the resolution and interlaboratory reproducibility of rep-PCR patterns compared to agarose gel analysis (Deplano et al. 2000).

Amplified Fragment Length Polymorphism (AFLP) and Infrequent-Restriction-Site Amplification (IRS-PCR) AFLP (Vos et al. 1995) and IRS-PCR (Mazurek et al. 1996) are innovative PCR-based strategies, which also appear to offer high resolution and good reproducibility. The techniques involve three steps: (i) total restriction of the genomic DNA and ligation of specially designed oligonucleotide adapters, (ii) selective amplification of sets of restriction fragments, and (iii) gel analysis of the amplified fragments. PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing. Using this method, sets of restriction fragments may be visualized by PCR without knowledge of their nucleotide sequence. The AFLP technique provides a novel and very powerful DNA fingerprinting tool for DNAs of any origin or complexity. This method has a high discriminatory power (Sloos et al. 1998, 2000; Melles et al. 2007) and enables species and strain identification of staphylococci (Velappan et al. 2001; van der Zee et al. 2005). The genetic background of *S. aureus* strains of veterinary or human origin and their population

structure was determined by means of AFLP analysis (Jarraud et al. 2002; Boerema et al. 2006; Melles et al. 2008). High-throughput AFLP (htAFLP) to characterize molecular markers associated with bacterial phenotypes was described recently (Savelkoul et al. 2007; Melles et al. 2009). Results of AFLP seem to be highly concordant with those of MLST in the delineation of genotypic clusters of closely related isolates (Sakwinska et al. 2009).

Methods Based on Direct DNA Sequence Determination

Nucleic acid sequencing of amplified genes is a direct method for the detection of DNA sequence polymorphism. It is the most sensitive method and has the highest discriminatory power.

***spa* Typing** DNA sequencing has been used to study staphylococcal protein A gene (*spaA*) polymorphism (Frenay et al. 1996). The number of 24 bp repeats designated as short sequence repeats (SSRs) in the region X of *spa* has been related to the dissemination potential of MRSA. Higher numbers of repeats associate with higher epidemic capability because a longer X region results in a better exposition of the Fc-binding region of protein A, thereby facilitating colonization of host surfaces. *Spa* typing, i.e. the determination of the organization of the repeat units in the X region, give reproducible, unambiguous, and easily interpreted results and seems to be adequate for outbreak investigations but should be complemented with other techniques in long-term surveillance or in studies comparing distant clonal lineages (Shopsin et al. 1999; Oliveira et al. 2001; Harmsen et al. 2003; Koreen et al. 2004; Kahl et al. 2005). Recent developments of sequence based typing techniques like *spa* typing and MLST have greatly improved reproducibility and exchangeability of typing results (Aires de Sousa et al. 2006). Moreover, distinctive *spa* types seem to be associated with particular MLST sequence types. Therefore, after the implementation of algorithms which allow the classification of related *spa* types into particular clonal lineages (Agius et al. 2007; Mellmann et al. 2007) *spa* typing is characterized as a frontline tool in epidemiological typing of *S. aureus* (Ruppitsch et al. 2006; Strommenger et al. 2008a).

Multilocus Sequence Typing (MLST) MLST provides an approach to molecular epidemiology that can identify and track the global spread of virulent or antibiotic-resistant isolates of bacterial pathogens using the Internet (Enright and Spratt 1999). MLST is a highly discriminatory method of characterizing bacterial isolates on the basis of the sequences of 450 bp internal fragments of seven housekeeping genes (Maiden et al. 1998): carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*).

The sequences obtained are assigned allele numbers following a comparison of the DNA sequence with the sequences of previously typed strains by using the MLST website (www.mlst.net). The allele numbers at each of the seven loci define the

allelic profile or sequence type (ST). Novel alleles and STs not found on the MLST website are confirmed by repeating both the PCR and sequencing.

MLST was utilized to identify the MRSA and MSSA clones among isolates from patients with serious community- and hospital-acquired infections (Enright et al. 2000). This method is suitable for studying both the evolution of pandemic MRSA clones and local epidemiology (Feil and Enright 2004; Robinson and Enright 2004b).

Currently MLST is used predominantly to study bacterial populations and clone evolution (Robinson and Enright 2004a; Robinson et al. 2005). Visualization of the MLST sequence database requires the development of new bioinformatics tools such as eBURST, the key program for evolution model design, providing detailed information about how methicillin-resistant and other clones emerge and spread (Feil et al. 2004). For studying minor genetic variations between *S. aureus* isolates, the MLST method should be accompanied by *spa* typing. In order to achieve an even distribution of the genes studied throughout the chromosome, the seven-gene sequence typing included in the MLST standard protocol may be extended by another seven genes named *sasA*, *sasB*, *sasD*, *sasE*, *sasF*, *sasH*, and *sasI* (Mazmanian et al. 2001; Robinson and Enright 2003).

MLST provides generally highly reproducible and comparable results. However, it has the disadvantage of being expensive and technically demanding. Therefore, there are attempts to detect sequence polymorphisms of the seven loci by other methods instead of DNA sequencing, e.g. PCR-RFLP (Diep et al. 2003), DGGE (Gurtler et al. 2002), and DNA microarrays (van Leeuwen et al. 2003).

Innovative High-Throughput Molecular Methods

PCR-Gene Single-Strand Conformation Polymorphism Typing (PCR-SSCP) This typing method is based on conformation polymorphism of single strands derived from polymorphic sequences. In *S. aureus*, the method was used for analysis of DNA gyrase A subunit gene (*gyrA*) (Tokue et al. 1994). This method has been used successfully for molecular identification of bacterial species based on 16S rRNA gene (Widjoatmodjo et al. 1995; Delbes and Montel 2005).

Denaturing Gradient Gel Electrophoresis (DGGE) The separation of DNA molecules by DGGE is based on the melting properties of DNA. In the DGGE system, DNA fragments which have the optimum size of 150–1,000 bp are electrophoresed through a polyacrylamide gel that contains a linear denaturing gradient. The molecules form branched structures that have retarded mobility in the gel matrix. If the gradient conditions are chosen properly, DGGE allows the sequence specific separation of a mixture of DNA PCR-products of the same length differing by a single base being branching. In staphylococci the DGGE method was used for detection of *mec* gene variants in MRSA isolates (Ahmadinejad et al. 1998), detection of mutations in VS2 region of the 16S–23S rRNA intergenic spacer region (Gurtler et al. 2001) and *Staphylococcus* species identification in meat products (Rantsiou et al. 2005; Iacumin et al. 2006).

DNA Microarrays DNA microarray technology considerably accelerates molecular biology assays. Oligonucleotides anchored on the membrane or on the glass slide hybridize with labeled PCR products or with cDNA. The result is evaluated by the apparatus – the scan is digitized and analyzed using appropriate software. The high-density microarrays containing thousands of oligonucleotides constituting approximately 90% of the *S. aureus* genome have been used in the study of deletions in open reading frames (Somerville et al. 2002) and genotyping and genetic composition analysis (Dunman et al. 2004).

The knowledge of the comprehensive genome sequence with a higher number of *S. aureus* strains currently enables the use of the microarray technique for comparative genomics studies to investigate gene variation in dominant lineages and identification of virulence genes of *S. aureus* (Lindsay et al. 2006), including the opportunity to investigate MGE variation and their exchange among *S. aureus* (Lindsay and Holden 2006; Sung et al. 2008). The method is also already used for strain characterization and genotyping, mostly MRSA (Palka-Santini et al. 2007; Strommenger et al. 2007; Monecke et al. 2008; Spence et al. 2008; Vautor et al. 2009).

Molecular Diagnostics of Toxigenic Staphylococcus aureus Strains

Nearly all *S. aureus* strains secrete a group of exoproteins including various enzymes and cytotoxins which contribute to their ability to colonize and cause disease in mammals. Traditionally, differentiation of toxigenic strains has involved analysis of phenotypic markers based on the detection of staphylococcal toxins. Some strains produce one or more additional exoproteins, which include toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins (SEs), the exfoliative toxins (ETs) and leukocidin. Each of these toxins is known to have potent effects on cells of the immune system, but many of them have other biological effects as well. TSST-1 and the staphylococcal enterotoxins are also known as pyrogenic toxin superantigens, the leading cause of several acute or chronic human diseases (Bohach et al. 1990; Prévost 2005). The enterotoxins SEA, SEB, SEC and SEE are potent emetic agents whereas SEG, SEI, SEJ and SEK are not (Bergdoll 1983; Jarraud et al. 2001; Kuroda et al. 2001).

The most frequently used methods for detection of staphylococcal toxins are ELISA, reverse passive latex agglutination (Igarashi et al. 1986; Park and Szabo 1986; Rose et al. 1989) and radioimmunological assays (Thompson et al. 1986). The methods which directly detect toxins are laborious and obviously vary with environmental parameters, affecting the expression of the gene responsible for the toxin production.

On the other hand, genotypic characterization is a more useful tool for molecular study of toxigenic *S. aureus* strains. Conventional DNA typing differentiates strains on basis of genetic variation at the level of chromosome, plasmids or genes. The genes for the toxins are carried by different MGEs (Lindsay 2008). *Sma*I macrorrestriction analysis using PFGE is a highly discriminatory method for detection of the

genetic relationships or differences among toxigenic isolates (Mackenzie et al. 1995; Hrstka et al. 2006). For epidemiological purposes, PFGE is combined with *spa* typing (Koreen et al. 2004; Růžicková et al. 2008).

PCR assays using one or several oligonucleotide primer pairs that flank the target DNA of genes encoding the toxins are necessary methods for primary detection of toxigenic strains (Becker et al. 1998; Jarraud et al. 1999; Monday and Bohach 1999; Mehrotra et al. 2000; Sharma et al. 2000; Løvseth et al. 2004; Růžicková et al. 2005). PCR amplifications of target toxin-coding sequences were used for the preparation of hybridization probes that enabled to locate the *eta*, *tst* and *seh* genes on the staphylococcal chromosome (Růžicková et al. 2003, 2008; Hrstka et al. 2006).

The most sensitive approach to detect polymorphisms in PCR products or cloned DNA fragments is direct sequencing. PCR product sequence analysis is highly sensitive and an extremely reproducible method for identifying and typing toxigenic staphylococci (Jones and Khan 1986). DNA sequence analysis enabled the identification of genes coding the new types of enterotoxins SEG, SEH, SEI, SEK, SEL, SEM, SEO, SEP, SEQ, SER, SEU and SEV (Jarraud et al. 2001; Kuroda et al. 2001; Baba et al. 2002; Letertre et al. 2003; Omoe et al. 2003; Fueyo et al. 2005; Jørgensen et al. 2005; Thomas et al. 2006).

Recent works identified *S. aureus* bacteriophages as important contributors to pathogenesis and evolution of staphylococcal genomes (Novick 2003; van Wamel et al. 2006). Prophage content detection (prophage profiling) is a useful method for diagnostics of the toxigenic strains that are responsible for outbreaks of pemphigus neonatorum (Růžicková et al. 2003) or staphylococcal food poisoning caused by enterotoxin A (Le Loir et al. 2003; Chen et al. 2004). PCR detection of genes coding the exfoliative toxin A or enterotoxin A, which are harbored by prophages, are also used to determine the genetic modification of strains lysogenized by ETA- or SEA- converting phages (Yamaguchi et al. 2000; Endo et al. 2003).

Staphylococcal Toxic Shock Syndrome (TSS)

TSS is an acute and potentially fatal illness characterized by high fever, diffuse erythematous rash, desquamation of the skin 1–2 weeks after onset, hypotension, and involvement of three or more organ systems (Todd et al. 1978; Davis et al. 1980). In the early 1980s, an epidemic of TSS occurred among young women in the United States. Nearly all of these TSS cases were associated with menstruation, and the presence of *S. aureus* localized to cervical or vaginal colonization (Shands et al. 1980). TSST-1 is encoded by *tstH* gene present on the bacterial chromosome within staphylococcal pathogenicity islands designated SaPI-1, SaPI-2 and SaPI-bov (Kreiswirth 1989; Lindsay et al. 1998; Ruzin et al. 2001). PCR is most frequently used for identification of the *tstH* positive *S. aureus* strains (Johnson et al. 1991; Becker et al. 1998). The TSST-1 producing strains of identical or similar genotype profile, based on PFGE and prophage content, could be found in distant localities (Hrstka et al. 2006).

Staphylococcal Food Poisoning

A form of gastroenteritis called staphylococcal food poisoning (SFP) results from ingestion of one or more preformed staphylococcal enterotoxins (SEs) on food that has been contaminated with enterotoxigenic *S. aureus* strains (Bergdoll 1983). To date, 19 types of SEs have been described. On the basis of their antigenicity and mode of action in the host, SEs have been divided into two groups. The members of group 1, classical emetic toxins designated SEA, SEB, SEC₁, SEC_{bov}, SED and SEE, are the cause of about 95% of SFP in humans. Group 2 includes toxins possibly involved in the remaining 5% of SFP outbreaks, i.e. other recently identified new enterotoxin types, the emetic activities of which are not currently fully understood (Thomas et al. 2007).

Molecular diagnostics of the SE-producing *S. aureus* strains are mostly directed on the genes coding the SEs located in various genome components. The prevailing *sea* gene, coding for the major classical enterotoxin type SEA and genes *see*, *seg*, *sek* and *sep* are carried by prophages (Betley and Mekalanos 1985; Couch et al. 1988; Kuroda et al. 2001; Brussow et al. 2004). The genes for SEC_{2,3}, SEC_{bov}, SEK, SEL, SEM and SEQ are located on SaPIs (Kuroda et al. 2001; Yarwood et al. 2002), while *seb*, *sec*, *sed*, *sej* and *ser* are the plasmid-born genes (Altboum et al. 1985; Zhang et al. 1998; Omoe et al. 2003). Enterotoxins SEB, SEG, SEH, SEI, SEN, SEO, SEU and SEV are encoded by chromosomal genes (Shafer and Iandolo 1978; Baba et al. 2002; Letertre et al. 2003; Thomas et al. 2006). The PCR assays targeting enterotoxin genes are the most commonly used detection methods.

Staphylococcal Epidermolysis Caused by the Exfoliative Toxins

Some *S. aureus* strains are producers of exfoliative toxin (ET) that cause a human epidermolytic disease. Clinical features of these skin disorders vary from localized blisters to severe staphylococcal scalded-skin syndrome (SSSS) occurring especially in neonates or young children (Ladhani et al. 1999). Serologically, this toxin isolated from human strains has been categorized into three isoforms, the most frequently occurring ETA and ETB toxins and much less frequently occurring ETD toxin. The *etd* gene was localized within 14.8-kb *etd* pathogenicity island (Yamaguchi et al. 2002). A fourth exfoliative toxin C (ETC) was isolated from a horse strain of *S. aureus* (Sato et al. 1994), however, it has not been associated with human disease and has not yet been genetically characterized. The *eta* gene encoding ETA is located on a prophage (Yamaguchi et al. 2000; Endo et al. 2003), whereas the *etb* gene encoding ETB is located on a large plasmid (Yamaguchi et al. 2001). All these ETs cause intraepidermal splitting of the epidermis (Ladhani 2003).

Accurate identification of exfoliative toxin producing *S. aureus* strains is crucial to hospital epidermolytic infection control and epidemiological surveillance. PCR-based methods utilizing specific PCR primers are better applicable to identification of the ET-producing *S. aureus* strains than a conditions-dependent phenotype

method. There are several reports in the literature which describe the use of multiplex PCR for the detection of various enterotoxins and exfoliative toxins (Becker et al. 1998; Mehrotra et al. 2000) and for the detection of *eta*, *etb* and *etd* genes in *S. aureus* strains (Růžicková et al. 2005).

Staphylococcal Cytotoxins

A number of *S. aureus* strains produce a several cytotoxic substances, including the hemolysins alpha, beta and delta.

Alpha-hemolysin, which is by far the most carefully examined of the *S. aureus* toxins, is toxic to a wide range of mammalian cells, particularly to rabbit erythrocytes (Freer and Arbuthnott 1982).

Beta-hemolysin (sphingomyelinase C) was also shown to be highly hemolytic for sheep but not rabbit erythrocytes. The gene, named *hly*, is chromosomally located (Projan et al. 1989). In diagnostic microbiology, a presumptive test for group B streptococci involves beta-hemolysin. This test (the CAMP test) is carried out by either dropping pure beta-hemolysin or culture fluid containing toxin on a lawn of presumptive group B streptococci and by seeking out enhanced hemolytic activity on blood agar (Dinges et al. 2000).

Delta-hemolysin made by 97% of *S. aureus* strains is capable of causing membrane damage in a variety of mammalian cells (Freer and Birkbeck 1982). The 514-nucleotide transcript is encoded by the *hly* gene (Peng et al. 1988). PCR for detection of *hly*, *hly* and *hly* genes in *S. aureus* strains is the mostly frequently used method for the diagnostics of hemolytic staphylococci (Jarraud et al. 2002).

The serious factor of *S. aureus* virulence belonging to cytotoxins is Panton-Valentine leukocidin (PVL). This is a bi-component toxin, often classified into the bi-component pore forming “gamma-hemolysin-like” group (Kaneko and Kamio 2004), and also including staphylococcal gamma-hemolysin, representing two leukotoxins HlgA-HlgB and HlgC-HlgB, the bovine LukM-LukF leukocidin and the Luke-LukD leukocidin. The components are encoded by genes *lukS-PV* and *lukF-PV* carried on a prophage genome and located between the lytic gene *ply* and right attachment site *attR* (Kaneko et al. 1998). PVL is a cytotoxin causing leukocyte and tissue necrosis and is implicated in severe wound infections, skin and soft tissue abscesses, furunculosis, osteomyelitis or necrotizing pneumonia (Lina et al. 1999; Mongkolrattanothai et al. 2003). While the gamma-hemolysin locus is encountered in almost all *S. aureus* strains, for PVL a mere 2% of the *S. aureus* strains are reported to produce this toxin (Prévost et al. 1995; Dinges et al. 2000), while for community-acquired methicillin-resistant *S. aureus* (caMRSA) the occurrence is more frequent. For diagnostics of the *lukS-PV* and *lukF-PV* genes in *S. aureus* strains different PCR assays have been applied (Lina et al. 1999; Bonnstedter et al. 2007; Strommenger et al. 2008b; Zhang et al. 2008). Allelic variation in genes coding for PVL that were not lineage-specific have been reported recently (Wolter et al. 2007).

Although Panton-Valentine leukocidin has been known since the 1930s, *S. aureus* strains carrying *lukS-PV* and *lukF-PV* genes have only become of concern in the

last decade. The recent emergence, successful spread, survival and prevalence of virulent and antibiotic resistant clones of caMRSA is an immensely important problem for human health (Vandenesch et al. 2003; Tristan et al. 2007). Most notably, the accumulation of MGEs in some isolates makes them increasingly virulent and able to supply new clinical challenges. The strains associated with lysogenic conversion of PVL and acquisition of the arginine catabolic mobile element (ACME) and additional virulence determinants can cause complicated infections with high mortality (Witte et al. 2005; Gillet et al. 2007; Diep et al. 2008).

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References

- Ackermann HW, DuBow MS (1987) Viruses of procaryotes, vol 2: natural groups of bacteriophages. CRC Press, Boca Raton, FL, pp 101–155
- Agius P, Kreiswirth B, Naidich S, Bennett K (2007) Typing *Staphylococcus aureus* using the *spa* gene and novel distance measures. IEEE/ACM Trans Comput Biol Bioinform 4:693–704
- Ahmadinejad M, Snyder JW, Perlin MH (1998) A combined molecular approach to screen for *mec* gene variants from methicillin-resistant *Staphylococcus aureus*. Diagn Microbiol Infect Dis 30:17–20
- Aires de Sousa M, Sanches IS, Ferro ML, Vaz MJ, Saraiva Z, Tendeiro T, Serra J, de Lencastre H (1998) Intercontinental spread of a multidrug-resistant methicillin-resistant *Staphylococcus aureus* clone. J Clin Microbiol 36:2590–2596
- Aires de Sousa M, Boye K, de Lencastre H, Deplano A, Enright MC, Etienne J, Friedrich A, Harmsen D, Holmes A, Huijsdens XW, Kearns AM, Mellmann A, Meugnier H, Rasheed JK, Spalburg E, Strommenger B, Struelens MJ, Tenover FC, Thomas J, Vogel U, Westh H, Xu J, Witte W (2006) High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. J Clin Microbiol 44:619–621
- Akineden O, Annemuller C, Hassan AA, Lammler C, Wolter W, Zschock M (2001) Toxin genes and other characteristics of *Staphylococcus aureus* isolates from milk of cows with mastitis. Clin Diagn Lab Immunol 8:959–964
- Altoum Z, Hertman I, Sarid S (1985) Penicillinase plasmid-linked genetic determinants for enterotoxins B and C1 production in *Staphylococcus aureus*. Infect Immun 47:514–521
- Altwegg M, Mayer LW (1989) Bacterial molecular epidemiology based on a nonradioactive probe complementary to ribosomal RNA. Res Microbiol 140:325–333
- Andrei A, Zervos MJ (2006) The application of molecular techniques to the study of hospital infection. Arch Pathol Lab Med 130:662–668
- Andollina A, De Cesare A, Berton G, Modelli L, Manfreda G (2004) Identification and genetic characterisation of orthopaedic *Staphylococcus* isolates collected in Italy by automated *EcoRI* ribotyping. FEMS Microbiol Lett 234:275–280
- Anthony RM, Brown TJ, French GL (2000) Rapid diagnosis of bacteremia by universal amplification of 23S ribosomal DNA followed by hybridization to an oligonucleotide array. J Clin Microbiol 38:781–788
- Baba T, Takeuchi F, Kuroda M, Yuzawa H, Aoki K, Oguchi A, Nagai Y, Iwama N, Asano K, Naimi T, Kuroda H, Cui L, Yamamoto K, Hiramatsu K (2002) Genome and virulence determinants of high virulence community-acquired MRSA. Lancet 359:1819–1827

- Baba T, Bae T, Schneewind O, Takeuchi F, Hiramatsu K (2008) Genome sequence of *Staphylococcus aureus* strain Newman and comparative analysis of staphylococcal genomes: polymorphism and evolution of two major pathogenicity islands. *J Bacteriol* 190:300–310
- Bannerman TL, Kloos WE (1991) *Staphylococcus capitis* subsp. *ureolyticus* subsp. nov. from human skin. *Int J Syst Bacteriol* 41:144–147
- Barry T, Collieran G, Glennon M, Dunican LK, Gannon F (1991) The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. *PCR Methods Appl* 1:51–56
- Barski P, Piechowicz L, Galinski J, Kur J (1996) Rapid assay for detection of methicillin-resistant *Staphylococcus aureus* using multiplex PCR. *Mol Cell Probes* 10:471–475
- Becker K, Roth R, Peters G (1998) Rapid and specific detection of toxigenic *Staphylococcus aureus*: use of two multiplex PCR enzyme immunoassays for amplification and hybridization of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin 1 gene. *J Clin Microbiol* 36:2548–2553
- Becker K, Harmsen D, Mellmann A, Meier C, Schumann P, Peters G, von Eiff C (2004) Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of *Staphylococcus* species. *J Clin Microbiol* 42:4988–4995
- Behme RJ, Shuttleworth R, McNabb A, Colby WD (1996) Identification of staphylococci with a self-educating system using fatty acid analysis and biochemical tests. *J Clin Microbiol* 34:3075–3084
- Berber I, Cokmus C, Atalan E (2003) Comparison of *Staphylococcus* spp. cellular and extracellular proteins by SDS-PAGE. *Mikrobiologija* 72:54–59
- Bergdoll MS (1983) Enterotoxins. In: Easmon CSF, Adlam C (eds) *Staphylococci and staphylococcal infections*. Academic Press, London, UK
- Berglund C, Ito T, Ikeda M, Ma XX, Soderquist B, Hiramatsu K (2008) Novel type of staphylococcal cassette chromosome *mec* in a methicillin-resistant *Staphylococcus aureus* strain isolated in Sweden. *Antimicrob Agents Chemother* 52:3512–3516
- Berglund C, Ito T, Ma XX, Ikeda M, Watanabe S, Soderquist B, Hiramatsu K (2009) Genetic diversity of methicillin-resistant *Staphylococcus aureus* carrying type IV SCC*mec* in Orebro County and the western region of Sweden. *J Antimicrob Chemother* 63:32–41
- Betley MJ, Mekalanos JJ (1985) Staphylococcal enterotoxin A is encoded by phage. *Science* 229:185–187
- Blanc DS, Lugeon C, Wenger A, Siegrist HH, Francioli P (1994) Quantitative antibiogram typing using inhibition zone diameters compared with ribotyping for epidemiological typing of methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol* 32:2505–2509
- Blanc DS, Francioli P, Hauser PM (2002) Poor value of pulsed-field gel electrophoresis to investigate long-term scale epidemiology of methicillin-resistant *Staphylococcus aureus*. *Infect Genet Evol* 2:145–148
- Blumberg HM, Rimland D, Kiehlbauch JA, Terry PM, Wachsmuth IK (1992) Epidemiologic typing of *Staphylococcus aureus* by DNA restriction fragment length polymorphisms of rRNA genes: elucidation of the clonal nature of a group of bacteriophage-nontypeable, ciprofloxacin-resistant, methicillin-susceptible *S. aureus* isolates. *J Clin Microbiol* 30:362–369
- Boerema JA, Clemens R, Brightwell G (2006) Evaluation of molecular methods to determine enterotoxigenic status and molecular genotype of bovine, ovine, human and food isolates of *Staphylococcus aureus*. *Int J Food Microbiol* 107:192–201
- Bohach GA, Fast DJ, Nelson RD, Schlievert PM (1990) Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. *Crit Rev Microbiol* 17:251–272
- Bonnstetter KK, Wolter DJ, Tenover FC, McDougal LK, Goering RV (2007) Rapid multiplex PCR assay for identification of USA300 community-associated methicillin-resistant *Staphylococcus aureus* isolates. *J Clin Microbiol* 45:141–146
- Booth MC, Pence LM, Mahasreshti P, Callegan MC, Gilmore MS (2001) Clonal associations among *Staphylococcus aureus* isolates from various sites of infection. *Infect Immun* 69:345–352

- Borecká P, Rosypal S, Pantůček R, Doškař J (1996) Localization of prophages of serological group B and F on restriction fragments defined in the restriction map of *Staphylococcus aureus* NCTC 8325. *FEMS Microbiol Lett* 143:203–210
- Boyle-Vavra S, Ereshefsky B, Wang CC, Daum RS (2005) Successful multiresistant community-associated methicillin-resistant *Staphylococcus aureus* lineage from Taipei, Taiwan, that carries either the novel staphylococcal chromosome cassette *mec* (SCC*mec*) type V_T or SCC*mec* type IV. *J Clin Microbiol* 43:4719–4730
- Brakstad OG, Aasbakk K, Maeland JA (1992) Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. *J Clin Microbiol* 30:1654–1660
- Brakstad OG, Maeland JA, Chesneau O (1995) Comparison of tests designed to identify *Staphylococcus aureus* thermostable nuclease. *APMIS* 103:219–224
- Brussow H, Canchaya C, Hardt WD (2004) Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol Mol Biol Rev* 68:560–602
- Burr MD, Pepper IL (1997) Variability in presence-absence scoring of AP PCR fingerprints affects computer matching of bacterial isolates. *J Microbiol Methods* 29:63–68
- Carbonnelle E, Beretti JL, Cottyn S, Quesne G, Berche P, Nassif X, Ferroni A (2007) Rapid identification of staphylococci isolated in clinical microbiology laboratories by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol* 45:2156–2161
- Carretto E, Barbarini D, Couto I, De Vitis D, Marone P, Verhoef J, De Lencastre H, Brisse S (2005) Identification of coagulase-negative staphylococci other than *Staphylococcus epidermidis* by automated ribotyping. *Clin Microbiol Infect* 11:177–184
- Chambers HF (2001) The changing epidemiology of *Staphylococcus aureus*? *Emerg Infect Dis* 7:178–182
- Chang HR, Lian JD, Shu KH, Cheng CH, Wu MJ, Chen CH, Lau YJ, Hu BS (2000) Use of pulsed-field gel electrophoresis in the analysis of recurrent *Staphylococcus aureus* infections in patients on continuous ambulatory peritoneal dialysis. *Am J Nephrol* 20:463–467
- Chaves F, Garcia-Alvarez M, Sanz F, Alba C, Otero JR (2005) Nosocomial spread of a *Staphylococcus hominis* subsp. *novobiosepticus* strain causing sepsis in a neonatal intensive care unit. *J Clin Microbiol* 43:4877–4879
- Chen J, Novick RP (2009) Phage-mediated intergeneric transfer of toxin genes. *Science* 323:139–141
- Chen TR, Chiou CS, Tsen HY (2004) Use of novel PCR primers specific to the genes of staphylococcal enterotoxin G, H, I for the survey of *Staphylococcus aureus* strains isolated from food-poisoning cases and food samples in Taiwan. *Int J Food Microbiol* 92:189–197
- Chesneau O, Aubert S, Morvan A, Guesdon JL, el Solh N (1992) Usefulness of the ID32 staph system and a method based on rRNA gene restriction site polymorphism analysis for species and subspecies identification of staphylococcal clinical isolates. *J Clin Microbiol* 30:2346–2352
- Chesneau O, Morvan A, Grimont F, Labischinski H, el Solh N (1993a) *Staphylococcus pasteurii* sp. nov., isolated from human, animal, and food specimens. *Int J Syst Bacteriol* 43:237–244
- Chesneau O, Allignet J, el Solh N (1993b) Theronuclease gene as a target nucleotide sequence for specific recognition of *Staphylococcus aureus*. *Mol Cell Probes* 7:301–310
- Chesneau O, Morvan A, Aubert S, el Solh N (2000) The value of rRNA gene restriction site polymorphism analysis for delineating taxa in the genus *Staphylococcus*. *Int J Syst Evol Microbiol* 50:689–697
- Chongtrakool P, Ito T, Ma XX, Kondo Y, Trakulsomboon S, Tiensasitorn C, Jamklang M, Chavalit T, Song JH, Hiramatsu K (2006) Staphylococcal cassette chromosome *mec* (SCC*mec*) typing of methicillin-resistant *Staphylococcus aureus* strains isolated in 11 Asian countries: a proposal for a new nomenclature for SCC*mec* elements. *Antimicrob Agents Chemother* 50:1001–1012
- Clink J, Pennington TH (1987) Staphylococcal whole-cell polypeptide analysis: evaluation as a taxonomic and typing tool. *J Med Microbiol* 23:41–44
- Cookson BD, Robinson DA, Monk AB, Murchan S, Deplano A, de Ryck R, Struelens MJ, Scheel C, Fussing V, Salmenlinna S, Vuopio-Varkila J, Cuny C, Witte W, Tassios PT, Legakis NJ, van Leeuwen W, van Belkum A, Vindel A, Garaizar J, Haeggman S, Olsson-Liljequist B, Ransjö

- U, Muller-Premru M, Hryniewicz W, Rossney A, O'Connell B, Short BD, Thomas J, O'Hanlon S, Enright MC (2007) Evaluation of molecular typing methods in characterizing a European collection of epidemic methicillin-resistant *Staphylococcus aureus* strains: the HARMONY collection. *J Clin Microbiol* 45:1830–1837
- Cookson B, and the HARMONY participants (2008) HARMONY – the International Union of Microbiology Societies' European Staphylococcal Typing Network. *Eurosurveillance* 13(4–6):1–5
- Couch JL, Soltis MT, Betley MJ (1988) Cloning and nucleotide sequence of the type E staphylococcal enterotoxin gene. *J Bacteriol* 170:2954–2960
- Couto I, Pereira S, Miragaia M, Sanches IS, de Lencastre H (2001) Identification of clinical staphylococcal isolates from humans by internal transcribed spacer PCR. *J Clin Microbiol* 39:3099–3103
- Couto I, Wu SW, Tomasz A, de Lencastre H (2003) Development of methicillin resistance in clinical isolates of *Staphylococcus sciuri* by transcriptional activation of the *mecA* homologue native to the species. *J Bacteriol* 185:645–653
- Cox RA, Conquest C, Mallaghan C, Marples RR (1995) A major outbreak of methicillin-resistant *Staphylococcus aureus* caused by a new phage-type (EMRSA-16). *J Hosp Infect* 29:87–106
- Dalsgaard A, Forslund A, Fussing V (1999) Traditional ribotyping shows a higher discrimination than the automated RiboPrinter system in typing *Vibrio cholerae* O1. *Lett Appl Microbiol* 28:327–333
- Davis JP, Chesney PJ, Wand PJ, LaVenture M (1980) Toxic-shock syndrome: epidemiologic features, recurrence, risk factors, and prevention. *N Engl J Med* 303:1429–1435
- de Buyser ML, Morvan A, Grimont F, el Solh N (1989) Characterization of *Staphylococcus* species by ribosomal RNA gene restriction patterns. *J Gen Microbiol* 135:989–999
- de la Fuente R, Suarez G, Schleifer KH (1985) *Staphylococcus aureus* subsp. *anaerobius* subsp. nov., the causal agent of abscess disease of sheep. *Int J Syst Bacteriol* 35:99–102
- Del Vecchio VG, Petroziello JM, Gress MJ, McCleskey FK, Melcher GP, Crouch HK, Lupski JR (1995) Molecular genotyping of methicillin-resistant *Staphylococcus aureus* via fluorophore-enhanced repetitive-sequence PCR. *J Clin Microbiol* 33:2141–2144
- Delbes C, Montel MC (2005) Design and application of a *Staphylococcus*-specific single strand conformation polymorphism-PCR analysis to monitor *Staphylococcus* populations diversity and dynamics during production of raw milk cheese. *Lett Appl Microbiol* 41:169–174
- Deplano A, Vaneechoutte M, Verschraegen G, Struelens MJ (1997) Typing of *Staphylococcus aureus* and *Staphylococcus epidermidis* strains by PCR analysis of inter-IS256 spacer length polymorphisms. *J Clin Microbiol* 35:2580–2587
- Deplano A, Schuermans A, Van Eldere J, Witte W, Meugnier H, Etienne J, Grundmann H, Jonas D, Noordhoek GT, Dijkstra J, van Belkum A, van Leeuwen W, Tassios PT, Legakis NJ, van der Zee A, Bergmans A, Blanc DS, Tenover FC, Cookson BC, O'Neil G, Struelens MJ (2000) Multicenter evaluation of epidemiological typing of methicillin-resistant *Staphylococcus aureus* strains by repetitive-element PCR analysis. *J Clin Microbiol* 38:3527–3533
- Devriese LA, Hájek V, Oeding P, Meyer SA, Schleifer KH (1978) *Staphylococcus hyicus* (Sompolinsky 1953) comb. nov. and *Staphylococcus hyicus* subsp. *chromogenes* subsp. nov. *Int J Syst Bacteriol* 28:482–490
- Devriese LA, Poutrel B, Kilpper-Bälz R, Schleifer KH (1983) *Staphylococcus gallinarum* and *Staphylococcus caprae*, 2 new species from animals. *Int J Syst Bacteriol* 33:480–486
- Devriese LA, Vancanneyt M, Baele M, Vaneechoutte M, De Graef E, Snauwaert C, Cleenwerck I, Dawyndt P, Swings J, Decostere A, Haesebrouck F (2005) *Staphylococcus pseudintermedius* sp. nov., a coagulase-positive species from animals. *Int J Syst Evol Microbiol* 55:1569–1573
- Diep BA, Perdreau-Remington F, Sensabaugh GF (2003) Clonal characterization of *Staphylococcus aureus* by multilocus restriction fragment typing, a rapid screening approach for molecular epidemiology. *J Clin Microbiol* 41:4559–4564
- Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, Lin F, Lin J, Carleton HA, Mongodin EF, Sensabaugh GF, Perdreau-Remington F (2006) Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* 367:731–739

- Diep BA, Palazzolo-Ballance AM, Tattévin P, Basuino L, Braughton KR, Whitney AR, Chen L, Kreiswirth BN, Otto M, DeLeo FR, Chambers HF (2008) Contribution of Pantone-Valentine leukocidin in community-associated methicillin-resistant *Staphylococcus aureus* pathogenesis. *PLoS ONE* 3:e3198
- Dinges MM, Orwin PM, Schlievert PM (2000) Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Rev* 13:16–34
- Dominguez MA, de Lencastre H, Linares J, Tomasz A (1994) Spread and maintenance of a dominant methicillin-resistant *Staphylococcus aureus* (MRSA) clone during an outbreak of MRSA disease in a Spanish hospital. *J Clin Microbiol* 32:2081–2087
- Doškař J, Pallová P, Pantůček R, Rosypal S, Růžicková V, Pantůčková P, Kailarová J, Klepárník K, Malá Z, Boček P (2000) Genomic relatedness of *Staphylococcus aureus* phages of the International Typing Set and detection of serogroup A, B, and F prophages in lysogenic strains. *Can J Microbiol* 46:1066–1076
- Drancourt M, Raoult D (2002) *rpoB* gene sequence-based identification of *Staphylococcus* species. *J Clin Microbiol* 40:1333–1338
- Du XM, Yang RF, Guo ZB, Song YJ, Wang J (2002) Identification of *Staphylococcus aureus* and determination of its methicillin resistance by matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry. *Anal Chem* 74:5487–5491
- Dunman PM, Mounts W, McAleese F, Immermann F, Macapagal D, Marsilio E, McDougal L, Tenover FC, Bradford PA, Petersen PJ, Projan SJ, Murphy E (2004) Uses of *Staphylococcus aureus* GeneChips in genotyping and genetic composition analysis. *J Clin Microbiol* 42:4275–4283
- Edwards KJ, Kaufmann ME, Saunders NA (2001) Rapid and accurate identification of coagulase-negative staphylococci by real-time PCR. *J Clin Microbiol* 39:3047–351
- el Solh N, de Buyser ML, Morvan A, Grimont F, Salesse-Walcher S, Aubert S, Monzon-Monreo C, Chesneau O, Allignet J (1990) Use of *Bacillus subtilis* 16S rRNA genes as a probe to identify species, subspecies and types in the genus *Staphylococcus*. In: Novick RP (ed) *Molecular biology of the Staphylococci*. VCH, New York, pp 585–593
- Endo Y, Yamada T, Matsunaga K, Hayakawa Y, Kaidoh T, Takeuchi S (2003) Phage conversion of exfoliative toxin A in *Staphylococcus aureus* isolated from cows with mastitis. *Vet Microbiol* 96:81–90
- Enright MC, Spratt BG (1999) Multilocus sequence typing. *Trends Microbiol* 7:482–487
- Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG (2000) Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol* 38:1008–1015
- Enright MC, Robinson DA, Randle G, Feil EJ, Grundmann H, Spratt BG (2002) The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc Natl Acad Sci U S A* 99:7687–7692
- Etienne J, Poitevin-Later F, Renaud F, Fleurette J (1990) Plasmid profiles and genomic DNA restriction endonuclease patterns of 30 independent *Staphylococcus lugdunensis* strains. *FEMS Microbiol Lett* 55:93–97
- Euzéby JP (2009) LPSN (List of Prokaryotic Names with Standing in Nomenclature). <http://www.bacterio.net/>. Accessed 1 Jan 2009
- Falkinham JO 3rd (1994) Nucleic acid probes. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds) *Methods for general and molecular bacteriology*. ASM, Washington, DC, pp 701–710
- Feil EJ, Enright MC (2004) Analyses of clonality and the evolution of bacterial pathogens. *Curr Opin Microbiol* 7:308–313
- Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG (2004) eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol* 186:1518–1530
- Feng Y, Chen CJ, Su LH, Hu S, Yu J, Chiu CH (2008) Evolution and pathogenesis of *Staphylococcus aureus*: lessons learned from genotyping and comparative genomics. *FEMS Microbiol Rev* 32:23–37

- Fitzgerald JR, Sturdevant DE, Mackie SM, Gill SR, Musser JM (2001) Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. *Proc Natl Acad Sci USA* 98:8821–8826
- Foster G, Ross HM, Hutson RA, Collins MD (1997) *Staphylococcus lutrae* sp. nov., a new coagulase-positive species isolated from otters. *Int J Syst Bacteriol* 47:724–726
- Freer JH, Birkbeck TH (1982) Possible conformation of delta-lysin, a membrane-damaging peptide of *Staphylococcus aureus*. *J Theor Biol* 94:535–540
- Freer JH, Arbutnott JP (1982) Toxins of *Staphylococcus aureus*. *Pharmacol Ther* 19:55–106
- Frenay HM, Bunschoten AE, Schouls LM, van Leeuwen WJ, Vandenbroucke-Grauls CM, Verhoef J, Mooi FR (1996) Molecular typing of methicillin-resistant *Staphylococcus aureus* on the basis of protein A gene polymorphism. *Eur J Clin Microbiol Infect Dis* 15:60–64
- Frenay HM, Theelen JP, Schouls LM, Vandenbroucke-Grauls CM, Verhoef J, van Leeuwen WJ, Mooi FR (1994) Discrimination of epidemic and nonepidemic methicillin-resistant *Staphylococcus aureus* strains on the basis of protein A gene polymorphism. *J Clin Microbiol* 32:846–847
- Frenay J, Brun Y, Bes M, Meugnier H, Grimont F, Grimont PAD, Nervi C, Fleurette J (1988) *Staphylococcus lugdunensis* sp. nov. and *Staphylococcus schleiferi* sp. nov., 2 species from human clinical specimens. *Int J Syst Bacteriol* 38:168–172
- Frenay J, Kloos WE, Hájek V, Webster JA, Bes M, Brun Y, Vernozy-Rozand C (1999) Recommended minimal standards for description of new staphylococcal species. Subcommittee on the taxonomy of staphylococci and streptococci of the International Committee on Systematic Bacteriology. *Int J Syst Bacteriol* 49:489–502
- Fueyo JM, Mendoza MC, Alvarez MA, Martin MC (2005) Relationships between toxin gene content and genetic background in nasal carried isolates of *Staphylococcus aureus* from Asturias, Spain. *FEMS Microbiol Lett* 243:447–454
- Garrity GM, Lilburn TG, Cole JR, Harrison SH, Euzéby JP, Tindall BJ (2007) The bacteria: Phylum Firmicutes: Class “Bacilli”. In: TOBA (The Taxonomic Outline of Bacteria and Archaea), Release 7.7, part 9, pp 333–398. <http://www.taxonomicoutline.org/>
- Ghebremedhin B, Layer F, König W, König B (2008) Genetic classification and distinguishing of *Staphylococcus* species based on different partial *gap*, 16S rRNA, *hsp60*, *rpoB*, *sodA*, and *tuf* gene sequences. *J Clin Microbiol* 46:1019–1025
- Gill SR, Fouts DE, Archer GL, Mongodin EF, Deboy RT, Ravel J, Paulsen IT, Kolonay JF, Brinkac L, Beanan M, Dodson RJ, Daugherty SC, Madupu R, Angiuoli SV, Durkin AS, Haft DH, Vamathevan J, Khouri H, Utterback T, Lee C, Dimitrov G, Jiang L, Qin H, Weidman J, Tran K, Kang K, Hance IR, Nelson KE, Fraser CM (2005) Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol* 187:2426–2438
- Gillaspay AF, Worrell V, Orvis J, Roe BA, Dyer DW, Iandolo JJ (2006) The *Staphylococcus aureus* NCTC 8325 genome. In: Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JJ (eds) Gram-positive pathogens, 2nd edn. ASM, Washington, DC, pp 381–412
- Gillet Y, Issartel B, Vanhems P, Fournet JC, Lina G, Bes M, Vandenesch F, Piemont Y, Brousse N, Floret D, Etienne J (2002) Association between *Staphylococcus aureus* strains carrying gene for Pantone-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. *Lancet* 359:753–759
- Gillet Y, Vanhems P, Lina G, Bes M, Vandenesch F, Floret D, Etienne J (2007) Factors predicting mortality in necrotizing community-acquired pneumonia caused by *Staphylococcus aureus* containing Pantone-Valentine leukocidin. *Clin Infect Dis* 45:315–321
- Goerke C, Koller J, Wolz C (2006a) Ciprofloxacin and trimethoprim cause phage induction and virulence modulation in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 50:171–177
- Goerke C, Wirtz C, Fluckiger U, Wolz C (2006b) Extensive phage dynamics in *Staphylococcus aureus* contributes to adaptation to the human host during infection. *Mol Microbiol* 61:1673–1685
- Goerke C, Pantůček R, Holtfrete S, Schulte BDG, Bröker B, Doškař J, Wolz C (2009) Diversity of prophages in dominant *Staphylococcus aureus* clonal lineages. *J Bacteriol* 191:3462–3468

- Goh SH, Byrne SK, Zhang JL, Chow AW (1992) Molecular typing of *Staphylococcus aureus* on the basis of coagulase gene polymorphisms. *J Clin Microbiol* 30:1642–1645
- Goh SH, Potter S, Wood JO, Hemmingsen SM, Reynolds RP, Chow AW (1996) HSP60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. *J Clin Microbiol* 34:818–823
- Gorski A, Borysowski J, Miedzybrodzki R, Weber-Dabrowska B (2007) Bacteriophages in medicine. In: Mc Grath S, van Sinderen D (eds) *Bacteriophage genetics and molecular biology*. Caister Academic Press, Norfolk, UK, pp 125–158
- Grundmann HJ, Towner KJ, Dijkshoorn L, Gerner-Smidt P, Maher M, Seifert H, Vaneechoutte M (1997) Multicenter study using standardized protocols and reagents for evaluation of reproducibility of PCR-based fingerprinting of *Acinetobacter* spp. *J Clin Microbiol* 35:3071–3077
- Gurtler V, Barrie HD (1995) Typing of *Staphylococcus aureus* strains by PCR-amplification of variable-length 16S-23S rDNA spacer regions: characterization of spacer sequences. *Microbiology* 141:1255–1265
- Gurtler V, Barrie HD, Mayall BC (2001) Use of denaturing gradient gel electrophoresis to detect mutation in VS2 of the 16S-23S rDNA spacer amplified from *Staphylococcus aureus* isolates. *Electrophoresis* 22:1920–1924
- Gurtler V, Mayall BC (2001) Genetic transfer and genome evolution in MRSA. *Microbiology* 147:3195–3197
- Gurtler V, Barrie HD, Mayall BC (2002) Denaturing gradient gel electrophoretic multilocus sequence typing of *Staphylococcus aureus* isolates. *Electrophoresis* 23:3310–3320
- Hájek V (1976) *Staphylococcus intermedius*, a new species isolated from animals. *Int J Syst Bacteriol* 26:401–408
- Hájek V, Devriese LA, Mordarski M, Goodfellow M, Pulverer G, Varaldo PE (1986) Elevation of *Staphylococcus hyicus* subsp. *chromogenes* (Devriese et al., 1978) to species status – *Staphylococcus chromogenes* (Devriese et al., 1978) comb. nov. *Syst Appl Microbiol* 8:169–173
- Hájek V, Ludwig W, Schleifer KH, Springer N, Zitzelsberger W, Kroppenstedt RM, Kocur M (1992) *Staphylococcus muscae*, a new species isolated from flies. *Int J Syst Bacteriol* 42:97–101
- Hájek V, Meugnier H, Bes M, Brun Y, Fiedler F, Chmela Z, Lasne Y, Fleurette J, Frenay J (1996) *Staphylococcus saprophyticus* subsp. *bovis* subsp. nov., isolated from bovine nostrils. *Int J Syst Bacteriol* 46:792–796
- Hall LM, Jordens JZ, Wang F (1989) Methicillin-resistant *Staphylococcus aureus* from China characterized by digestion of total DNA with restriction enzymes. *Epidemiol Infect* 103:183–192
- Hallin M, Deplano A, Denis O, De Mendonca R, De Ryck R, Struelens MJ (2007) Validation of pulsed-field gel electrophoresis and *spa* typing for long-term, nationwide epidemiological surveillance studies of *Staphylococcus aureus* infections. *J Clin Microbiol* 45:127–133
- Hamels S, Gala JL, Dufour S, Vannuffel P, Zammattéo N, Remacle J (2001) Consensus PCR and microarray for diagnosis of the genus *Staphylococcus*, species, and methicillin resistance. *Biotechniques* 31:1364–1366, 1368, 1370–1362
- Hanssen AM, Kjeldsen G, Sollid JU (2004) Local variants of staphylococcal cassette chromosome *mec* in sporadic methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative staphylococci: evidence of horizontal gene transfer? *Antimicrob Agents Chemother* 48:285–296
- Hanssen AM, Sollid JUE (2006) SCC*mec* in staphylococci: genes on the move. *FEMS Immunol Med Microbiol* 46:8–20
- Harmsen D, Claus H, Witte W, Rothganger J, Turnwald D, Vogel U (2003) Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. *J Clin Microbiol* 41:5442–5448
- Hauschild T, Stepanovic S (2008) Identification of *Staphylococcus* spp. by PCR-restriction fragment length polymorphism analysis of *dnaJ* gene. *J Clin Microbiol* 46:3875–3879
- Herron-Olson L, Fitzgerald JR, Musser JM, Kapur V (2007) Molecular correlates of host specialization in *Staphylococcus aureus*. *PLoS ONE* 2:e1120

- Hesselbarth J, Schwarz S (1995) Comparative ribotyping of *Staphylococcus intermedius* from dogs, pigeons, horses and mink. *Vet Microbiol* 45:11–17
- Heusser R, Ender M, Berger-Bachi B, McCallum N (2007) Mosaic staphylococcal cassette chromosome *mec* containing two recombinase loci and a new *mec* complex, B2. *Antimicrob Agents Chemother* 51:390–393
- Highlander SK, Hulten KG, Qin X, Jiang H, Yerrapragada S, Mason EO Jr, Shang Y, Williams TM, Fortunov RM, Liu Y, Igboeli O, Petrosino J, Tirumalai M, Uzman A, Fox GE, Cardenas AM, Muzny DM, Hemphill L, Ding Y, Dugan S, Blyth PR, Buhay CJ, Dinh HH, Hawes AC, Holder M, Kovar CL, Lee SL, Liu W, Nazareth LV, Wang Q, Zhou J, Kaplan SL, Weinstock GM (2007) Subtle genetic changes enhance virulence of methicillin resistant and sensitive *Staphylococcus aureus*. *BMC Microbiol* 7:99
- Holden MT, Feil EJ, Lindsay JA, Peacock SJ, Day NP, Enright MC, Foster TJ, Moore CE, Hurst L, Atkin R, Barron A, Bason N, Bentley SD, Chillingworth C, Chillingworth T, Churcher C, Clark L, Corton C, Cronin A, Doggett J, Dowd L, Feltwell T, Hance Z, Harris B, Hauser H, Holroyd S, Jagels K, James KD, Lennard N, Line A, Mayes R, Moule S, Mungall K, Ormond D, Quail MA, Rabinowitz E, Rutherford K, Sanders M, Sharp S, Simmonds M, Stevens K, Whitehead S, Barrell BG, Spratt BG, Parkhill J (2004) Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proc Natl Acad Sci U S A* 101:9786–9791
- Hrstka R, Růžicková V, Petráš P, Pantůček R, Rosypal S, Doškař J (2006) Genotypic characterization of toxic shock syndrome toxin-1-producing strains of *Staphylococcus aureus* isolated in the Czech Republic. *Int J Med Microbiol* 296:49–54
- Iacumin L, Comi G, Cantoni C, Coccolin L (2006) Ecology and dynamics of coagulase-negative cocci isolated from naturally fermented Italian sausages. *Syst Appl Microbiol* 29:480–486
- Igarashi H, Fujikawa H, Shingaki M, Bergdoll MS (1986) Latex agglutination test for staphylococcal toxic shock syndrome toxin 1. *J Clin Microbiol* 23:509–512
- Igimi S, Kawamura S, Takahashi E, Mitsuoka T (1989) *Staphylococcus felis*, a new species from clinical specimens from cats. *Int J Syst Bacteriol* 39:373–377
- Igimi S, Takahashi E, Mitsuoka T (1990) *Staphylococcus schleiferi* subsp. *coagulans* subsp. nov., isolated from the external auditory meatus of dogs with external ear otitis. *Int J Syst Bacteriol* 40:409–411
- Ito T (2009) SCCmec. <http://www.staphylococcus.net/>. Accessed 1 March 2009
- Iwantscheff A, Kuhnen E, Brandis H (1985) Species distribution of coagulase-negative staphylococci isolated from clinical sources. *Zentralbl Bakteriol Mikrobiol Hyg [A]* 260:41–50
- Jansen WT, Beitsma MM, Koeman CJ, van Wamel WJ, Verhoef J, Fluit AC (2006) Novel mobile variants of staphylococcal cassette chromosome *mec* in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 50:2072–2078
- Jarraud S, Cozon G, Vandenesch F, Bes M, Etienne J, Lina G (1999) Involvement of enterotoxins G and I in staphylococcal toxic shock syndrome and staphylococcal scarlet fever. *J Clin Microbiol* 37:2446–2449
- Jarraud S, Peyrat MA, Lim A, Tristan A, Bes M, Mougé C, Etienne J, Vandenesch F, Bonneville M, Lina G (2001) *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J Immunol* 166:669–677
- Jarraud S, Mougé C, Thioulouse J, Lina G, Meugnier H, Forey F, Nesme X, Etienne J, Vandenesch F (2002) Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. *Infect Immun* 70:631–641
- Jensen MA, Webster JA, Straus N (1993) Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl Environ Microbiol* 59:945–952
- Johnson WM, Tyler SD, Ewan EP, Ashton FE, Pollard DR, Rozee KR (1991) Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. *J Clin Microbiol* 29:426–430

- Jones CL, Khan SA (1986) Nucleotide sequence of the enterotoxin B gene from *Staphylococcus aureus*. J Bacteriol 166:29–33
- Jørgensen HJ, Mork T, Caugant DA, Kearns A, Rorvik LM (2005) Genetic variation among *Staphylococcus aureus* strains from Norwegian bulk milk. Appl Environ Microbiol 71:8352–8361
- Kahl BC, Mellmann A, Deiwick S, Peters G, Harmsen D (2005) Variation of the polymorphic region X of the protein A gene during persistent airway infection of cystic fibrosis patients reflects two independent mechanisms of genetic change in *Staphylococcus aureus*. J Clin Microbiol 43:502–505
- Kaneko J, Kimura T, Narita S, Tomita T, Kamio Y (1998) Complete nucleotide sequence and molecular characterization of the temperate staphylococcal bacteriophage phiPVL carrying Panton-Valentine leukocidin genes. Gene 215:57–67
- Kaneko J, Kamio Y (2004) Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. Biosci Biotechnol Biochem 68:981–1003
- Kempf VA, Trebesius K, Autenrieth IB (2000) Fluorescent *In situ* hybridization allows rapid identification of microorganisms in blood cultures. J Clin Microbiol 38:830–838
- Kennedy AD, Otto M, Braughton KR, Whitney AR, Chen L, Mathema B, Mediavilla JR, Byrne KA, Parkins LD, Tenover FC, Kreiswirth BN, Musser JM, DeLeo FR (2008) Epidemic community-associated methicillin-resistant *Staphylococcus aureus*: recent clonal expansion and diversification. Proc Natl Acad Sci U S A 105:1327–1332
- Kilpper-Bälz R, Schleifer KH (1981) Transfer of *Peptococcus saccharolyticus* Foubert and Douglas to the genus *Staphylococcus* – *Staphylococcus saccharolyticus* (Foubert and Douglas) comb. nov. Zentralbl Bakteriol Mikrobiol Hyg [C] 2:324–331
- Klaassen CH, de Valk HA, Horrevorts AM (2003) Clinical *Staphylococcus aureus* isolate negative for the Sa442 fragment. J Clin Microbiol 41:4493
- Kloos WE, Schleifer KH (1975) Isolation and characterization of staphylococci from human skin. 2. Descriptions of 4 new species – *Staphylococcus warneri*, *Staphylococcus capitis*, *Staphylococcus hominis*, and *Staphylococcus simulans*. Int J Syst Bacteriol 25:62–79
- Kloos WE, Schleifer KH, Smith RF (1976) Characterization of *Staphylococcus sciuri* sp. nov. and its subspecies. Int J Syst Bacteriol 26:22–37
- Kloos WE, Schleifer KH (1983) *Staphylococcus auricularis* sp. nov. – an inhabitant of the human external ear. Int J Syst Bacteriol 33:9–14
- Kloos WE, Wolfshohl JF (1991) *Staphylococcus cohnii* subspecies: *Staphylococcus cohnii* subsp. *cohnii* subsp. nov. and *Staphylococcus cohnii* subsp. *urealyticum* subsp. nov. Int J Syst Bacteriol 41:284–289
- Kloos WE, Ballard DN, Webster JA, Hubner RJ, Tomasz A, Couto I, Sloan GL, Dehart HP, Fiedler F, Schubert K, de Lencastre H, Sanches IS, Heath HE, Leblanc PA, Ljungh A (1997) Ribotype delineation and description of *Staphylococcus sciuri* subspecies and their potential as reservoirs of methicillin resistance and staphylolytic enzyme genes. Int J Syst Bacteriol 47:313–323
- Kloos WE, Ballard DN, George CG, Webster JA, Hubner RJ, Ludwig W, Schleifer KH, Fiedler F, Schubert K (1998a) Delimiting the genus *Staphylococcus* through description of *Macrococcus caseolyticus* gen. nov., comb. nov. and *Macrococcus equiperficus* sp. nov., and *Macrococcus bovicus* sp. nov. and *Macrococcus carouelicus* sp. nov. Int J Syst Bacteriol 48:859–877
- Kloos WE, George CG, Olgiate JS, Van Pelt L, McKinnon ML, Zimmer BL, Muller E, Weinstein MP, Mirrett S (1998b) *Staphylococcus hominis* subsp. *novobioceticus* subsp. nov., a novel trehalose- and N-acetyl-D-glucosamine-negative, novobiocin- and multiple-antibiotic-resistant subspecies isolated from human blood cultures. Int J Syst Bacteriol 48:799–812
- Kloos WE, Bannerman TL (1999) *Staphylococcus* and *Micrococcus*. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Tenover RH (eds) Manual of clinical microbiology, 7th edn. ASM Press, Washington, DC, pp 264–282
- Kondo Y, Ito T, Ma XX, Watanabe S, Kreiswirth BN, Etienne J, Hiramatsu K (2007) Combination of multiplex PCRs for staphylococcal cassette chromosome *mec* type assignment: rapid identifi-

- cation system for *mec*, *ccr*, and major differences in junkyard regions. *Antimicrob Agents Chemother* 51:264–274
- Kontos F, Petinaki E, Spiliopoulou I, Maniati M, Maniatis AN (2003) Evaluation of a novel method based on PCR restriction fragment length polymorphism analysis of the *tuf* gene for the identification of *Staphylococcus species*. *J Microbiol Methods* 55:465–469
- Koreen L, Ramaswamy SV, Graviss EA, Naidich S, Musser JM, Kreiswirth BN (2004) *spa* typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro- and macrovariation. *J Clin Microbiol* 42:792–799
- Kreiswirth BN (1989) Genetics and expression of toxic shock syndrome toxin 1: overview. *Rev Infect Dis* 11(Suppl 1):S97–S100
- Kreiswirth B, Kornblum J, Arbeit RD, Eisner W, Maslow JN, McGeer A, Low DE, Novick RP (1993) Evidence for a clonal origin of methicillin resistance in *Staphylococcus aureus*. *Science* 259:227–230
- Kumari DN, Keer V, Hawkey PM, Parnell P, Joseph N, Richardson JF, Cookson B (1997) Comparison and application of ribosome spacer DNA amplicon polymorphisms and pulsed-field gel electrophoresis for differentiation of methicillin-resistant *Staphylococcus aureus* strains. *J Clin Microbiol* 35:881–885
- Kuroda M, Ohta T, Uchiyama I, Baba T, Yuzawa H, Kobayashi I, Cui L, Oguchi A, Aoki K, Nagai Y, Lian J, Ito T, Kanamori M, Matsumaru H, Maruyama A, Murakami H, Hosoyama A, Mizutani-Ui Y, Takahashi NK, Sawano T, Inoue R, Kaito C, Sekimizu K, Hirakawa H, Kuhara S, Goto S, Yabuzaki J, Kanehisa M, Yamashita A, Oshima K, Furuya K, Yoshino C, Shiba T, Hattori M, Ogasawara N, Hayashi H, Hiramatsu K (2001) Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* 357:1225–1240
- Kuroda M, Yamashita A, Hirakawa H, Kumano M, Morikawa K, Higashide M, Maruyama A, Inose Y, Matoba K, Toh H, Kuhara S, Hattori M, Ohta T (2005) Whole genome sequence of *Staphylococcus saprophyticus* reveals the pathogenesis of uncomplicated urinary tract infection. *Proc Natl Acad Sci U S A* 102:13272–13277
- Kwan T, Liu J, DuBow M, Gros P, Pelletier J (2005) The complete genomes and proteomes of 27 *Staphylococcus aureus* bacteriophages. *Proc Natl Acad Sci U S A* 102:5174–5179
- Kwok AY, Chow AW (2003) Phylogenetic study of *Staphylococcus* and *Micrococcus* species based on partial *hsp60* gene sequences. *Int J Syst Evol Microbiol* 53:87–92
- Ladhani S, Joannou CL, Lochrie DP, Evans RW, Poston SM (1999) Clinical, microbial, and biochemical aspects of the exfoliative toxins causing staphylococcal scalded-skin syndrome. *Clin Microbiol Rev* 12:224–242
- Ladhani S (2003) Understanding the mechanism of action of the exfoliative toxins of *Staphylococcus aureus*. *FEMS Immunol Med Microbiol* 39:181–189
- Lambert LH, Cox T, Mitchell K, Rossello-Mora RA, Del Cueto C, Dodge DE, Orkand P, Cano RJ (1998) *Staphylococcus succinus* sp. nov., isolated from Dominican amber. *Int J Syst Bacteriol* 48:511–518
- Lay F, Ghebremedhin B, König W, König B (2007) Differentiation of *Staphylococcus* spp. by terminal-restriction fragment length polymorphism analysis of glyceraldehyde-3-phosphate dehydrogenase-encoding gene. *J Microbiol Methods* 70:542–549
- Le Loir Y, Baron F, Gautier M (2003) *Staphylococcus aureus* and food poisoning. *Genet Mol Res* 2:63–76
- Letertre C, Perelle S, Dilasser F, Fach P (2003) Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus*. *J Appl Microbiol* 95:38–43
- Lina G, Piemont Y, Godail-Gamot F, Bes M, Peter MO, Gauduchon V, Vandenesch F, Etienne J (1999) Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clin Infect Dis* 29:1128–1132
- Lindsay JA, Ruzin A, Ross HF, Kurepina N, Novick RP (1998) The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. *Mol Microbiol* 29:527–543
- Lindsay JA, Holden MT (2006) Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. *Funct Integr Genomics* 6:186–201

- Lindsay JA, Moore CE, Day NP, Peacock SJ, Witney AA, Stabler RA, Husain SE, Butcher PD, Hinds J (2006) Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. *J Bacteriol* 188:669–676
- Lindsay JA (ed) (2008) *Staphylococcus Molecular Genetics*. Caister Academic Press, Norfolk, UK, pp 1–278
- Løvseth A, Loncarevic S, Berdal KG (2004) Modified multiplex PCR method for detection of pyrogenic exotoxin genes in staphylococcal isolates. *J Clin Microbiol* 42:3869–3872
- Luong TT, Ouyang S, Bush K, Lee CY (2002) Type 1 capsule genes of *Staphylococcus aureus* are carried in a staphylococcal cassette chromosome genetic element. *J Bacteriol* 184:3623–3629
- Mackenzie A, Johnson W, Heyes B, Norrish B, Jamieson F (1995) A prolonged outbreak of exfoliative toxin A-producing *Staphylococcus aureus* in a newborn nursery. *Diagn Microbiol Infect Dis* 21:69–75
- Maes N, De Gheldre Y, De Ryck R, Vanechoutte M, Meugnier H, Etienne J, Struelens MJ (1997) Rapid and accurate identification of *Staphylococcus* species by tRNA intergenic spacer length polymorphism analysis. *J Clin Microbiol* 35:2477–2481
- Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* 95:3140–3145
- Malíková L, Sedláček I, Nováková D, Němec M (2007) Ribotyping and biotyping of *Staphylococcus epidermidis* isolated from hospital environment. *Folia Microbiol* 52:375–380
- Mannerová S, Pantůček R, Doškař J, Švec P, Snauwaert C, Vancanneyt M, Swings J, Sedláček I (2003) *Macroccoccus brunensis* sp. nov., *Macroccoccus hajekii* sp. nov. and *Macroccoccus lamae* sp. nov., from the skin of llamas. *Int J Syst Evol Microbiol* 53:1647–1654
- Marsou R, Bes M, Boudouma M, Brun Y, Meugnier H, Frenay J, Vandenesch F, Etienne J (1999) Distribution of *Staphylococcus sciuri* subspecies among human clinical specimens, and profile of antibiotic resistance. *Res Microbiol* 150:531–541
- Martineau F, Picard FJ, Roy PH, Ouellette M, Bergeron MG (1998) Species-specific and ubiquitous-DNA-based assays for rapid identification of *Staphylococcus aureus*. *J Clin Microbiol* 36:618–623
- Martineau F, Picard FJ, Ke D, Paradis S, Roy PH, Ouellette M, Bergeron MG (2001) Development of a PCR assay for identification of staphylococci at genus and species levels. *J Clin Microbiol* 39:2541–2547
- Maslow J, Mulligan ME (1996) Epidemiologic typing systems. *Infect Control Hosp Epidemiol* 17:595–604
- Maslow JN, Mulligan ME, Arbeit RD (1993) Molecular epidemiology: application of contemporary techniques to the typing of microorganisms. *Clin Infect Dis* 17:153–162
- Mason WJ, Blevins JS, Beenken K, Wibowo N, Ojha N, Smeltzer MS (2001) Multiplex PCR protocol for the diagnosis of staphylococcal infection. *J Clin Microbiol* 39:3332–3338
- Mazmanian SK, Ton-That H, Schneewind O (2001) Sortase-catalysed anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. *Mol Microbiol* 40:1049–1057
- Mazurek GH, Reddy V, Marston BJ, Haas WH, Crawford JT (1996) DNA fingerprinting by infrequent-restriction-site amplification. *J Clin Microbiol* 34:2386–2390
- McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC (2003) Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. *J Clin Microbiol* 41:5113–5120
- Mehrotra M, Wang G, Johnson WM (2000) Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. *J Clin Microbiol* 38:1032–1035
- Melles DC, van Leeuwen WB, Snijders SV, Horst-Kreft D, Peeters JK, Verbrugh HA, van Belkum A (2007) Comparison of multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) for genetic typing of *Staphylococcus aureus*. *J Microbiol Methods* 69:371–375

- Melles DC, Tenover FC, Kuehnert MJ, Witsenboer H, Peeters JK, Verbrugh HA, van Belkum A (2008) Overlapping population structures of nasal isolates of *Staphylococcus aureus* from healthy Dutch and American individuals. *J Clin Microbiol* 46:235–241
- Melles DC, Schouls L, Francois P, Herzig S, Verbrugh HA, van Belkum A, Schrenzel J (2009) High-throughput typing of *Staphylococcus aureus* by amplified fragment length polymorphism (AFLP) or multi-locus variable number of tandem repeat analysis (MLVA) reveals consistent strain relatedness. *Eur J Clin Microbiol Infect Dis* 28:39–45
- Mellmann A, Becker K, von Eiff C, Keckevoet U, Schumann P, Harmsen D (2006) Sequencing and staphylococci identification. *Emerg Infect Dis* 12:333–336
- Mellmann A, Weniger T, Berssenbrugge C, Rothganger J, Sammeth M, Stoye J, Harmsen D (2007) Based Upon Repeat Pattern (BURP): an algorithm to characterize the long-term evolution of *Staphylococcus aureus* populations based on spa polymorphisms. *BMC Microbiol* 7:98
- Melter O, Santos Sanches I, Schindler J, Aires de Sousa M, Mato R, Kovarova V, Zemlickova H, de Lencastre H (1999) Methicillin-resistant *Staphylococcus aureus* clonal types in the Czech Republic. *J Clin Microbiol* 37:2798–2803
- Melter O, Aires de Sousa M, Urbaskova P, Jakubu V, Zemlickova H, de Lencastre H (2003) Update on the major clonal types of methicillin-resistant *Staphylococcus aureus* in the Czech Republic. *J Clin Microbiol* 41:4998–5005
- Mendoza M, Meugnier H, Bes M, Etienne J, Freney J (1998) Identification of *Staphylococcus* species by 16S-23S rDNA intergenic spacer PCR analysis. *Int J Syst Bacteriol* 48:1049–1055
- Milheirico C, Oliveira DC, de Lencastre H (2007a) Multiplex PCR strategy for subtyping the staphylococcal cassette chromosome *mec* type IV in methicillin-resistant *Staphylococcus aureus*: 'SCCmec IV multiplex'. *J Antimicrob Chemother* 60:42–48
- Milheirico C, Oliveira DC, de Lencastre H (2007b) Update to the multiplex PCR strategy for assignment of *mec* element types in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 51:3374–3377
- Monday SR, Bohach GA (1999) Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. *J Clin Microbiol* 37:3411–3414
- Monecke S, Slickers P, Ehrlich R (2008) Assignment of *Staphylococcus aureus* isolates to clonal complexes based on microarray analysis and pattern recognition. *FEMS Immunol Med Microbiol* 53:237–251
- Mongkolrattanothai K, Boyle S, Kahana MD, Daum RS (2003) Severe *Staphylococcus aureus* infections caused by clonally related community-acquired methicillin-susceptible and methicillin-resistant isolates. *Clin Infect Dis* 37:1050–1058
- Moser SA, Box MJ, Patel M, Amaya M, Schelonka R, Waites KB (2009) Multiple-locus variable-number tandem-repeat analysis of methicillin-resistant *Staphylococcus aureus* discriminates within USA pulsed-field gel electrophoresis types. *J Hosp Infect* 71:333–339
- Murchan S, Kaufmann ME, Deplano A, de Ryck R, Struelens M, Zinn CE, Fussing V, Salmenlinna S, Vuopio-Varkila J, El Solh N, Cuny C, Witte W, Tassios PT, Legakis N, van Leeuwen W, van Belkum A, Vindel A, Laconcha I, Garaizar J, Haeggman S, Olsson-Liljequist B, Ransjo U, Coombes G, Cookson B (2003) Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. *J Clin Microbiol* 41:1574–1585
- Mwangi MM, Wu SW, Zhou Y, Sieradzki K, de Lencastre H, Richardson P, Bruce D, Rubin E, Myers E, Siggia ED, Tomasz A (2007) Tracking the in vivo evolution of multidrug resistance in *Staphylococcus aureus* by whole-genome sequencing. *Proc Natl Acad Sci U S A* 104:9451–9456
- Neoh HM, Cui L, Yuzawa H, Takeuchi F, Matsuo M, Hiramatsu K (2008) Mutated response regulator *graR* is responsible for phenotypic conversion of *Staphylococcus aureus* from heterogeneous vancomycin-intermediate resistance to vancomycin-intermediate resistance. *Antimicrob Agents Chemother* 52:45–53
- Nováková D, Pantůček R, Petráš P, Koukalová D, Sedláček I (2006a) Occurrence of *Staphylococcus nepalensis* strains in different sources including human clinical material. *FEMS Microbiol Lett* 263:163–168

- Nováková D, Sedláček I, Pantůček R, Štětina V, Švec P, Petráš P (2006b) *Staphylococcus equorum* and *Staphylococcus succinus* isolated from human clinical specimens. J Med Microbiol 55:523–528
- Nováková D, Švec P (2008) Application of the (GTG)₅-PCR fingerprinting for rapid identification of staphylococcal species occurring in humans. Clin Microbiol Infect 14(Suppl 7):S249
- Nováková D, Pantůček R, Hubálek Z, Falsen E, Büsse H-J, Schumann P, Sedláček I (2010) *Staphylococcus microti* sp. nov. isolated from the *Microtus arvalis*. Int J Syst Evol Microbiol 60:566–573
- Novick RP (1987) Plasmid incompatibility. Microbiol Rev 51:381–395
- Novick RP (1990) Molecular Biology of the Staphylococci. VCH Publishers, New York
- Novick RP (2003) Mobile genetic elements and bacterial toxinoses: the superantigen-encoding pathogenicity islands of *Staphylococcus aureus*. Plasmid 49:93–105
- Oliveira DC, Santos-Sanches I, Mato R, Tamayo M, Ribeiro G, Costa D, de Lencastre H (1998) Virtually all methicillin-resistant *Staphylococcus aureus* (MRSA) infections in the largest Portuguese teaching hospital are caused by two internationally spread multiresistant strains: the ‘Iberian’ and the ‘Brazilian’ clones of MRSA. Clin Microbiol Infect 4:373–384
- Oliveira DC, Crisostomo I, Santos-Sanches I, Major P, Alves CR, Aires-de-Sousa M, Thege MK, de Lencastre H (2001) Comparison of DNA sequencing of the protein A gene polymorphic region with other molecular typing techniques for typing two epidemiologically diverse collections of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 39:574–580
- Oliveira DC, Milheirico C, Vinga S, de Lencastre H (2006a) Assessment of allelic variation in the *ccrAB* locus in methicillin-resistant *Staphylococcus aureus* clones. J Antimicrob Chemother 58:23–30
- Oliveira DC, Milheirico C, de Lencastre H (2006b) Redefining a structural variant of staphylococcal cassette chromosome *mec*, SCCmec type VI. Antimicrob Agents Chemother 50:3457–3459
- Oliveira K, Brecher SM, Durbin A, Shapiro DS, Schwartz DR, De Girolami PC, Dakos J, Procop GW, Wilson D, Hanna CS, Haase G, Peltroche-Llacsahuanga H, Chapin KC, Musgnug MC, Levi MH, Shoemaker C, Stender H (2003) Direct identification of *Staphylococcus aureus* from positive blood culture bottles. J Clin Microbiol 41:889–891
- Omoe K, Hu DL, Takahashi-Omoe H, Nakane A, Shinagawa K (2003) Identification and characterization of a new staphylococcal enterotoxin-related putative toxin encoded by two kinds of plasmids. Infect Immun 71:6088–6094
- Palazzo IC, d’Azevedo PA, Secchi C, Pignatari AC, Darini AL (2008) *Staphylococcus hominis* subsp. *novobiosepticus* strains causing nosocomial bloodstream infection in Brazil. J Antimicrob Chemother 62:1222–1226
- Palka-Santini M, Putzfeld S, Cleven BE, Kronke M, Krut O (2007) Rapid identification, virulence analysis and resistance profiling of *Staphylococcus aureus* by gene segment-based DNA microarrays: application to blood culture post-processing. J Microbiol Methods 68:468–477
- Palomares C, Torres MJ, Torres A, Aznar J, Palomares JC (2003) Rapid detection and identification of *Staphylococcus aureus* from blood culture specimens using real-time fluorescence PCR. Diagn Microbiol Infect Dis 45:183–189
- Palys T, Nakamura LK, Cohan FM (1997) Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. Int J Syst Bacteriol 47:1145–1156
- Pantůček R, Götz F, Doškař J, Rosypal S (1996) Genomic variability of *Staphylococcus aureus* and the other coagulase-positive *Staphylococcus* species estimated by macrorestriction analysis using pulsed-field gel electrophoresis. Int J Syst Bacteriol 46:216–222
- Pantůček R, Sedláček I, Doškař J, Rosypal S (1999) Complex genomic and phenotypic characterization of the related species *Staphylococcus carnosus* and *Staphylococcus piscifermentans*. Int J Syst Bacteriol 49:941–951
- Pantůček R, Doškař J, Růžicková V, Kašpárek P, Oráčová E, Kvardová V, Rosypal S (2004) Identification of bacteriophage types and their carriage in *Staphylococcus aureus*. Arch Virol 149:1689–1703

- Pantůček R, Sedláček I, Petráš P, Koukalová D, Švec P, Štetina V, Vancanneyt M, Chraštinová L, Vokurková J, Růžicková V, Doškař J, Swings J, Hájek V (2005) *Staphylococcus simiae* sp. nov., isolated from South American squirrel monkeys. *Int J Syst Evol Microbiol* 55: 1953–1958
- Park CE, Szabo R (1986) Evaluation of the reversed passive latex agglutination (RPLA) test kits for detection of staphylococcal enterotoxins A, B, C, and D in foods. *Can J Microbiol* 32:723–727
- Peng HL, Novick RP, Kreiswirth B, Kornblum J, Schlievert P (1988) Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. *J Bacteriol* 170:4365–4372
- Pfäller MA, Hollis RJ (2004) Automated ribotyping. In: Pershing DH, Tenover FC, Versalovic J, Tang YW, Unger ER, Relman DA, White TJ (eds) *Molecular microbiology: Diagnostic principles and practice*. ASM Press, Washington, DC, pp 245–258
- Place RB, Hiestand D, Burri S, Teuber M (2002) *Staphylococcus succinus* subsp. *casei* subsp. nov., a dominant isolate from a surface ripened cheese. *Syst Appl Microbiol* 25:353–359
- Place RB, Hiestand D, Gallmann HR, Teuber M (2003) *Staphylococcus equorum* subsp. *linens*, subsp. nov., a starter culture component for surface ripened semi-hard cheeses. *Syst Appl Microbiol* 26:30–37
- Poyart C, Quesne G, Boumaila C, Trieu-Cuot P (2001) Rapid and accurate species-level identification of coagulase-negative staphylococci by using the *sodA* gene as a target. *J Clin Microbiol* 39:4296–4301
- Prévost G, Jaulhac B, Piemont Y (1992) DNA fingerprinting by pulsed-field gel electrophoresis is more effective than ribotyping in distinguishing among methicillin-resistant *Staphylococcus aureus* isolates. *J Clin Microbiol* 30:967–973
- Prévost G, Cribier B, Couppie P, Petiau P, Supersac G, Finck-Barbançon V, Monteil H, Piemont Y (1995) Panton-Valentine leucocidin and gamma-hemolysin from *Staphylococcus aureus* ATCC 49775 are encoded by distinct genetic loci and have different biological activities. *Infect Immun* 63:4121–4129
- Prévost G (2005) Toxins in *Staphylococcus aureus* pathogenesis. In: Proft T (ed) *Microbial toxins, molecular and cellular biology*. Horizon Bioscience. Norfolk, UK, pp 243–284
- Probst AJ, Hertel C, Richter L, Wassill L, Ludwig W, Hammes WP (1998) *Staphylococcus condimenti* sp. nov., from soy sauce mash, and *Staphylococcus carnosus* (Schleifer and Fischer 1982) subsp. *utilis* subsp. nov. *Int J Syst Bacteriol* 48:651–658
- Projan SJ, Archer GL (1989) Mobilization of the relaxable *Staphylococcus aureus* plasmid pC221 by the conjugative plasmid pGO1 involves three pC221 loci. *J Bacteriol* 171:1841–1845
- Projan SJ, Kornblum J, Kreiswirth B, Moghazeh SL, Eisner W, Novick RP (1989) Nucleotide sequence: the beta-hemolysin gene of *Staphylococcus aureus*. *Nucleic Acids Res* 17:3305
- Quelle LS, Corso A, Galas M, Sordelli DO (2003) STAR gene restriction profile analysis in epidemiological typing of methicillin-resistant *Staphylococcus aureus*: description of the new method and comparison with other polymerase chain reaction (PCR)-based methods. *Diagn Microbiol Infect Dis* 47:455–464
- Rantsiou K, Iacumin L, Cantoni C, Comi G, Cocolin L (2005) Ecology and characterization by molecular methods of *Staphylococcus* species isolated from fresh sausages. *Int J Food Microbiol* 97:277–284
- Richardson JF, Reith S (1993) Characterization of a strain of methicillin-resistant *Staphylococcus aureus* (EMRSA-15) by conventional and molecular methods. *J Hosp Infect* 25:45–52
- Robinson DA, Enright MC (2003) Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 47:3926–3934
- Robinson DA, Enright MC (2004a) Evolution of *Staphylococcus aureus* by large chromosomal replacements. *J Bacteriol* 186:1060–1064
- Robinson DA, Enright MC (2004b) Multilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus*. *Clin Microbiol Infect* 10:92–97
- Robinson DA, Monk AB, Cooper JE, Feil EJ, Enright MC (2005) Evolutionary genetics of the accessory gene regulator (*agr*) locus in *Staphylococcus aureus*. *J Bacteriol* 187:8312–8321

- Rose SA, Bankes P, Stringer MF (1989) Detection of staphylococcal enterotoxins in dairy products by the reversed passive latex agglutination (SET-RPLA) kit. *Int J Food Microbiol* 8:65–72
- Rosenbach EF (1884) Mikroorganismen bei den Wundinfektionskrankheiten des Menschen. Bergmann, Wiesbaden
- Rosenstein R, Nerz C, Biswas L, Resch A, Raddatz G, Schuster SC, Gotz F (2009) Genome analysis of the meat starter culture bacterium *Staphylococcus carnosus* TM300. *Appl Environ Microbiol* 75:811–822
- Ruppitsch W, Indra A, Stoger A, Mayer B, Stadlbauer S, Wewalka G, Allerberger F (2006) Classifying *spa* types in complexes improves interpretation of typing results for methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol* 44:2442–2448
- Růžicková V, Pantůček R, Petráš P, Doškař J, Sedláček I, Rosypal S (2003) Molecular typing of exfoliative toxin-producing *Staphylococcus aureus* strains involved in epidermolytic infections. *Int J Med Microbiol* 292:541–545
- Růžicková V, Voller J, Pantůček R, Petráš P, Doškař J (2005) Multiplex PCR for detection of three exfoliative toxin serotype genes in *Staphylococcus aureus*. *Folia Microbiol* 50:499–502
- Růžicková V, Karpíšková R, Pantůček R, Pospíšilová M, Černíková P, Doškař J (2008) Genotype analysis of enterotoxin H-positive *Staphylococcus aureus* strains isolated from food samples in the Czech Republic. *Int J Food Microbiol* 121:60–65
- Ruzin A, Lindsay J, Novick RP (2001) Molecular genetics of SaPII - a mobile pathogenicity island in *Staphylococcus aureus*. *Mol Microbiol* 41:365–377
- Sabat A, Krzyszton-Russjan J, Strzalka W, Filipek R, Kosowska K, Hryniewicz W, Travis J, Potempa J (2003) New method for typing *Staphylococcus aureus* strains: multiple-locus variable-number tandem repeat analysis of polymorphism and genetic relationships of clinical isolates. *J Clin Microbiol* 41:1801–1804
- Sakwinska O, Kuhn G, Balmelli C, Francioli P, Giddey M, Perreten V, Riesen A, Zysset F, Blanc DS, Moreillon P (2009) Genetic diversity and ecological success of *Staphylococcus aureus* strains colonizing humans. *Appl Environ Microbiol* 75:175–183
- Saruta K, Hoshina S, Machida K (1995) Genetic identification of *Staphylococcus aureus* by polymerase chain reaction using single-base-pair mismatch in 16S ribosomal RNA gene. *Microbiol Immunol* 39:839–844
- Saruta K, Matsunaga T, Kono M, Hoshina S, Ikawa S, Sakai O, Machida K (1997) Rapid identification and typing of *Staphylococcus aureus* by nested PCR amplified ribosomal DNA spacer region. *FEMS Microbiol Lett* 146:271–278
- Sato H, Matsumori Y, Tanabe T, Saito H, Shimizu A, Kawano J (1994) A new type of staphylococcal exfoliative toxin from a *Staphylococcus aureus* strain isolated from a horse with phlegmon. *Infect Immun* 62:3780–3785
- Savelkoul PH, Melles DC, Buffing N, Gorkink R, Simons G, van Belkum A (2007) High density whole genome fingerprinting of methicillin-resistant and -susceptible strains of *Staphylococcus aureus* in search of phenotype-specific molecular determinants. *J Microbiol Methods* 71:44–54
- Schleifer KH, Kloos WE (1975) Isolation and characterization of staphylococci from human skin. I. Amended descriptions of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* and descriptions of 3 new species – *Staphylococcus cohnii*, *Staphylococcus haemolyticus*, and *Staphylococcus xylosus*. *Int J Syst Bacteriol* 25:50–61
- Schleifer KH, Fischer U (1982) Description of a new species of the genus *Staphylococcus* – *Staphylococcus carnosus*. *Int J Syst Bacteriol* 32:153–156
- Schleifer KH, Kilpper-Bälz R, Devriese LA (1984) *Staphylococcus arlettiae* sp. nov., *Staphylococcus equorum* sp. nov. and *Staphylococcus kloosii* sp. nov. – 3 new coagulase-negative, novobiocin-resistant species from animals. *Syst Appl Microbiol* 5:501–509
- Schmitz FJ, Mackenzie CR, Hofmann B, Verhoef J, Finken-Eigen M, Heinz HP, Kohrer K (1997) Specific information concerning taxonomy, pathogenicity and methicillin resistance of staphylococci obtained by a multiplex PCR. *J Med Microbiol* 46:773–778
- Shafer WM, Iandolo JJ (1978) Chromosomal locus for staphylococcal enterotoxin B. *Infect Immun* 20:273–278

- Shah MM, Iihara H, Noda M, Song SX, Nhung PH, Ohkusu K, Kawamura Y, Ezaki T (2007) *dnaJ* gene sequence-based assay for species identification and phylogenetic grouping in the genus *Staphylococcus*. *Int J Syst Evol Microbiol* 57:25–30
- Shands KN, Schmid GP, Dan BB, Blum D, Guidotti RJ, Hargrett NT, Anderson RL, Hill DL, Broome CV, Band JD, Fraser DW (1980) Toxic-shock syndrome in menstruating women: association with tampon use and *Staphylococcus aureus* and clinical features in 52 cases. *N Engl J Med* 303:1436–1442
- Sharma NK, Rees CE, Dodd CE (2000) Development of a single-reaction multiplex PCR toxin typing assay for *Staphylococcus aureus* strains. *Appl Environ Microbiol* 66:1347–1353
- Shaw C, Stitt JM, Cowan ST (1951) Staphylococci and their classification. *J Gen Microbiol* 5:1010–1023
- Shopsin B, Gomez M, Montgomery SO, Smith DH, Waddington M, Dodge DE, Bost DA, Riehman M, Naidich S, Kreiswirth BN (1999) Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *J Clin Microbiol* 37:3556–3563
- Shore AC, Rossney AS, O'Connell B, Herra CM, Sullivan DJ, Humphreys H, Coleman DC (2008) Detection of staphylococcal cassette chromosome *mec*-associated DNA segments in multiresistant methicillin-susceptible *Staphylococcus aureus* (MSSA) and identification of *Staphylococcus epidermidis ccrAB4* in both methicillin-resistant *S. aureus* and MSSA. *Antimicrob Agents Chemother* 52:4407–4419
- Singh A, Goering RV, Simjee S, Foley SL, Zervos MJ (2006) Application of molecular techniques to the study of hospital infection. *Clin Microbiol Rev* 19:512–530
- Sloos JH, Horrevorts AM, Van Boven CP, Dijkshoorn L (1998) Identification of multiresistant *Staphylococcus epidermidis* in neonates of a secondary care hospital using pulsed field gel electrophoresis and quantitative antibiogram typing. *J Clin Pathol* 51:62–67
- Sloos JH, Dijkshoorn L, Vogel L, van Boven CP (2000) Performance of phenotypic and genotypic methods to determine the clinical relevance of serial blood isolates of *Staphylococcus epidermidis* in patients with septicemia. *J Clin Microbiol* 38:2488–2493
- Snopková S, Götz F, Doškař J, Rosypal S (1994) Pulsed-field gel electrophoresis of the genomic restriction fragments of coagulase-negative staphylococci. *FEMS Microbiol Lett* 124:131–139
- Somerville GA, Beres SB, Fitzgerald JR, DeLeo FR, Cole RL, Hoff JS, Musser JM (2002) In vitro serial passage of *Staphylococcus aureus*: changes in physiology, virulence factor production, and agr nucleotide sequence. *J Bacteriol* 184:1430–1437
- Spence RP, Wright V, Ala-Aldeen DA, Turner DP, Wooldridge KG, James R (2008) Validation of virulence and epidemiology DNA microarray for identification and characterization of *Staphylococcus aureus* isolates. *J Clin Microbiol* 46:1620–1627
- Spersger J, Wieser M, Taubel N, Rossello-Mora RA, Rosengarten R, Busse HJ (2003) *Staphylococcus nepalensis* sp. nov., isolated from goats of the Himalayan region. *Int J Syst Evol Microbiol* 53:2007–2011
- Štěpán J, Pantůček R, Růžicková V, Rosypal S, Hájek V, Doškař J (2001) Identification of *Staphylococcus aureus* based on PCR amplification of species specific genomic 826 bp sequence derived from a common 44-kb *SmaI* restriction fragment. *Mol Cell Probes* 15:249–257
- Štěpán J, Pantůček R, Doškař J (2004) Molecular diagnostics of clinically important staphylococci. *Folia Microbiol* 49:353–386
- Stephan R, Annemuller C, Hassan AA, Lammler C (2001) Characterization of enterotoxigenic *Staphylococcus aureus* strains isolated from bovine mastitis in north-east Switzerland. *Vet Microbiol* 78:373–382
- Stephens AJ, Huygens F, Giffard PM (2007) Systematic derivation of marker sets for staphylococcal cassette chromosome *mec* typing. *Antimicrob Agents Chemother* 51:2954–2964
- Straub JA, Hertel C, Hammes WP (1999) A 23S rDNA-targeted polymerase chain reaction-based system for detection of *Staphylococcus aureus* in meat starter cultures and dairy products. *J Food Prot* 62:1150–1156

- Strommenger B, Kettlitz C, Weniger T, Harmsen D, Friedrich AW, Witte W (2006) Assignment of *Staphylococcus* isolates to groups by *spa* typing, *Sma*I macrorestriction analysis, and multilocus sequence typing. *J Clin Microbiol* 44:2533–2540
- Strommenger B, Schmidt C, Werner G, Roessle-Lorch B, Bachmann TT, Witte W (2007) DNA microarray for the detection of therapeutically relevant antibiotic resistance determinants in clinical isolates of *Staphylococcus aureus*. *Mol Cell Probes* 21:161–170
- Strommenger B, Bräulke C, Heuck D, Schmidt C, Pasemann B, Nübel U, Witte W (2008a) *spa* Typing of *Staphylococcus aureus* as a frontline tool in epidemiological typing. *J Clin Microbiol* 46:574–581
- Strommenger B, Bräulke C, Pasemann B, Schmidt C, Witte W (2008b) Multiplex PCR for rapid detection of *Staphylococcus aureus* isolates suspected to represent community-acquired strains. *J Clin Microbiol* 46:582–587
- Struelens MJ (1996) Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. *Clin Microbiol Infect* 2:2–11
- Struelens MJ (1998) Tracking the epidemiology of antimicrobial drug resistance in hospitals: time to deploy molecular typing. *J Med Microbiol* 47:1035–1036
- Sung JM, Lloyd DH, Lindsay JA (2008) *Staphylococcus aureus* host specificity: comparative genomics of human versus animal isolates by multi-strain microarray. *Microbiology* 154:1949–1959
- Suzuki M, Tawada Y, Kato M, Hori H, Mamiya N, Hayashi Y, Nakano M, Fukushima R, Katai A, Tanaka T, Hata M, Matsumoto M, Takahashi M, Sakae K (2006) Development of a rapid strain differentiation method for methicillin-resistant *Staphylococcus aureus* isolated in Japan by detecting phage-derived open-reading frames. *J Appl Microbiol* 101:938–947
- Švec P, Vancanneyt M, Sedláček I, Engelbeen K, Štětina V, Swings J, Petráš P (2004) Reclassification of *Staphylococcus pulvereri* Zakrzewska-Czerwinska et al. 1995 as a later synonym of *Staphylococcus vitulinus* Webster et al. 1994. *Int J Syst Evol Microbiol* 54:2213–2215
- Takahashi T, Satoh I, Kikuchi N (1999) Phylogenetic relationships of 38 taxa of the genus *Staphylococcus* based on 16S rRNA gene sequence analysis. *Int J Syst Bacteriol* 49:725–728
- Takano T, Higuchi W, Otsuka T, Baranovich T, Enany S, Saito K, Isobe H, Dohmae S, Ozaki K, Takano M, Iwao Y, Shibuya M, Okubo T, Yabe S, Shi D, Reva I, Teng LJ, Yamamoto T (2008) Novel characteristics of community-acquired methicillin-resistant *Staphylococcus aureus* strains belonging to multilocus sequence type 59 in Taiwan. *Antimicrob Agents Chemother* 52:837–845
- Takeuchi F, Watanabe S, Baba T, Yuzawa H, Ito T, Morimoto Y, Kuroda M, Cui L, Takahashi M, Ankaï A, Baba S, Fukui S, Lee JC, Hiramatsu K (2005) Whole-genome sequencing of *Staphylococcus haemolyticus* uncovers the extreme plasticity of its genome and the evolution of human-colonizing staphylococcal species. *J Bacteriol* 187:7292–7308
- Tanasupawat S, Hashimoto Y, Ezaki T, Kozaki M, Komagata K (1992) *Staphylococcus piscifermentans* sp. nov., from fermented fish in Thailand. *Int J Syst Bacteriol* 42:577–581
- Te Witt R, Kanhai V, van Leeuwen WB (2009) Comparison of the DiversiLab system, pulsed field gel electrophoresis and multi locus sequence typing for the characterization of epidemic reference MRSA strains. *J Microbiol Methods* 77:130–133
- Tenover FC, Arbeit R, Archer G, Biddle J, Byrne S, Goering R, Hancock G, Hebert GA, Hill B, Hollis R et al (1994) Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus*. *J Clin Microbiol* 32:407–415
- Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 33:2233–2239
- Tenover FC, Arbeit RD, Goering RV (1997) How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. Molecular Typing Working Group of the Society for Healthcare Epidemiology of America. *Infect Control Hosp Epidemiol* 18:426–439

- Tenover FC (2007) Rapid detection and identification of bacterial pathogens using novel molecular technologies: infection control and beyond. *Clin Infect Dis* 44:418–423
- Tenover FC, Vaughn RR, McDougal LK, Fosheim GE, McGowan JE Jr (2007) Multiple-locus variable-number tandem-repeat assay analysis of methicillin-resistant *Staphylococcus aureus* strains. *J Clin Microbiol* 45:2215–2219
- Thomas CM, Nielsen KM (2005) Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat Rev Microbiol* 3:711–721
- Thomas WD Jr, Archer GL (1989) Identification and cloning of the conjugative transfer region of *Staphylococcus aureus* plasmid pGO1. *J Bacteriol* 171:684–691
- Thomas DY, Jarraud S, Lemerrier B, Cozon G, Echasserieau K, Etienne J, Gougeon ML, Lina G, Vandenesch F (2006) Staphylococcal enterotoxin-like toxins U2 and V, two new staphylococcal superantigens arising from recombination within the enterotoxin gene cluster. *Infect Immun* 74:4724–4734
- Thomas D, Chou S, Dauwalder O, Lina G (2007) Diversity in *Staphylococcus aureus* enterotoxins. *Chem Immunol Allergy* 93:24–41
- Thompson NE, Pattee PA (1977) Transformation in *Staphylococcus aureus*: role of bacteriophage and incidence of competence among strains. *J Bacteriol* 129:778–788
- Thompson NE, Razdan M, Kuntsmann G, Aschenbach JM, Evenson ML, Bergdoll MS (1986) Detection of staphylococcal enterotoxins by enzyme-linked immunosorbent assays and radioimmunoassays: comparison of monoclonal and polyclonal antibody systems. *Appl Environ Microbiol* 51:885–890
- Todd J, Fishaut M, Kapral F, Welch T (1978) Toxic-shock syndrome associated with phage-group-I staphylococci. *Lancet* 2:1116–1118
- Tokue Y, Sugano K, Saito D, Noda T, Ohkura H, Shimosato Y, Sekiya T (1994) Detection of novel mutations in the *gyrA* gene of *Staphylococcus aureus* by nonradioisotopic single-strand conformation polymorphism analysis and direct DNA sequencing. *Antimicrob Agents Chemother* 38:428–431
- Tormo MA, Ferrer MD, Maiques E, Ubeda C, Selva L, Lasa I, Calvete JJ, Novick RP, Penades JR (2008) *Staphylococcus aureus* pathogenicity island DNA is packaged in particles composed of phage proteins. *J Bacteriol* 190:2434–2440
- Tristan A, Bes M, Meugnier H, Lina G, Bozdogan B, Courvalin P, Reverdy ME, Enright MC, Vandenesch F, Etienne J (2007) Global distribution of Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus*. *Emerg Infect Dis* 13:594–600
- Trülsch K, Grabein B, Schumann P, Mellmann A, Antonenka U, Heesemann J, Becker K (2007) *Staphylococcus pettenkoferi* sp. nov., a novel coagulase-negative staphylococcal species isolated from human clinical specimens. *Int J Syst Evol Microbiol* 57:1543–1548
- Tveten Y, Kristiansen BE, Ask E, Jenkins A, Hofstad T (1991) DNA fingerprinting of isolates of *Staphylococcus aureus* from newborns and their contacts. *J Clin Microbiol* 29:1100–1105
- Ubelaker MH, Rosenblum ED (1978) Transduction of plasmid determinants in *Staphylococcus aureus* and *Escherichia coli*. *J Bacteriol* 133:699–707
- van Belkum A, Bax R, Peerbooms P, Goessens WH, van Leeuwen N, Quint WG (1993) Comparison of phage typing and DNA fingerprinting by polymerase chain reaction for discrimination of methicillin-resistant *Staphylococcus aureus* strains. *J Clin Microbiol* 31:798–803
- van Belkum A, Kluytmans J, van Leeuwen W, Bax R, Quint W, Peters E, Fluit A, Vandenbroucke-Grauls C, van den Brule A, Koeleman H et al (1995) Multicenter evaluation of arbitrarily primed PCR for typing of *Staphylococcus aureus* strains. *J Clin Microbiol* 33:1537–1547
- van Belkum A, van Leeuwen W, Kaufmann ME, Cookson B, Forey F, Etienne J, Goering R, Tenover F, Steward C, O'Brien F, Grubb W, Tassios P, Legakis N, Morvan A, El Solh N, de Ryck R, Struelens M, Salmenlinna S, Vuopio-Varkila J, Kooistra M, Talens A, Witte W, Verbrugh H (1998) Assessment of resolution and intercenter reproducibility of results of genotyping *Staphylococcus aureus* by pulsed-field gel electrophoresis of *SmaI* macrorestriction fragments: a multicenter study. *J Clin Microbiol* 36:1653–1659
- van Belkum A, Struelens M, de Visser A, Verbrugh H, Tibayrenc M (2001) Role of genomic typing in taxonomy, evolutionary genetics, and microbial epidemiology. *Clin Microbiol Rev* 14:547–560

- van der Zee A, Verbakel H, van Zon JC, Frenay I, van Belkum A, Peeters M, Buiting A, Bergmans A (1999) Molecular genotyping of *Staphylococcus aureus* strains: comparison of repetitive element sequence-based PCR with various typing methods and isolation of a novel epidemicity marker. *J Clin Microbiol* 37:342–349
- van der Zee A, Heck M, Sterks M, Harpal A, Spalburg E, Kazobagora L, Wannet W (2005) Recognition of SCCmec types according to typing pattern determined by multienzyme multiplex PCR-amplified fragment length polymorphism analysis of methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol* 43:6042–6047
- van Leeuwen WB, Jay C, Snijders S, Durin N, Lacroix B, Verbrugh HA, Enright MC, Troesch A, van Belkum A (2003) Multilocus sequence typing of *Staphylococcus aureus* with DNA array technology. *J Clin Microbiol* 41:3323–3326
- van Wamel WJ, Rooijackers SH, Ruyken M, van Kessel KP, van Strijp JA (2006) The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on beta-hemolysin-converting bacteriophages. *J Bacteriol* 188:1310–1315
- Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, Heffernan H, Liassine N, Bes M, Greenland T, Reverdy ME, Etienne J (2003) Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerg Infect Dis* 9:978–984
- Vanechoutte M (1996) DNA fingerprinting techniques for microorganisms: A proposal for classification and nomenclature. *Mol Biotechnol* 6:115–142
- Vannuffel P, Heusterspreute M, Bouyer M, Vandercam B, Philippe M, Gala JL (1999) Molecular characterization of *femA* from *Staphylococcus hominis* and *Staphylococcus saprophyticus*, and *femA*-based discrimination of staphylococcal species. *Res Microbiol* 150:129–141
- Vautor E, Magnone V, Rios G, Le Brigand K, Bergonier D, Lina G, Meugnier H, Barbry P, Thiery R, Pepin M (2009) Genetic differences among *Staphylococcus aureus* isolates from dairy ruminant species: a single-dye DNA microarray approach. *Vet Microbiol* 133:105–114
- Valardo PE, Kilpper-Bälz R, Biavasco F, Satta G, Schleifer KH (1988) *Staphylococcus delphini* sp. nov., a coagulase-positive species isolated from dolphins. *Int J Syst Bacteriol* 38:436–439
- Velappan N, Snodgrass JL, Hakovirta JR, Marrone BL, Burde S (2001) Rapid identification of pathogenic bacteria by single-enzyme amplified fragment length polymorphism analysis. *Diagn Microbiol Infect Dis* 39:77–83
- Vernozy-Rozand C, Mazuy C, Meugnier H, Bes M, Lasne Y, Fiedler F, Etienne J, Frenay J (2000) *Staphylococcus fleurettii* sp. nov., isolated from goat's milk cheeses. *Int J Syst Evol Microbiol* 50:1521–1527
- Versalovic J, Koeuth T, Lupski JR (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* 19:6823–6831
- Versalovic J, Woods CR Jr, Georgioui PR, Hamill RJ, Lupski JR (1993) DNA-based identification and epidemiologic typing of bacterial pathogens. *Arch Pathol Lab Med* 117:1088–1098
- Versalovic J, Kapur V, Koeuth T, Mazurek GH, Whittam TS, Musser JM, Lupski JR (1995) DNA fingerprinting of pathogenic bacteria by fluorophore-enhanced repetitive sequence-based polymerase chain reaction. *Arch Pathol Lab Med* 119:23–29
- von Eiff C, Becker K, Machka K, Stammer H, Peters G (2001) Nasal carriage as a source of *Staphylococcus aureus* bacteremia. *N Engl J Med* 344:11–16
- von Eiff C, Friedrich AW, Peters G, Becker K (2004) Prevalence of genes encoding for members of the staphylococcal leukotoxin family among clinical isolates of *Staphylococcus aureus*. *Diagn Microbiol Infect Dis* 49:157–162
- Vos P, Hogers R, Bleeker M, Reijans M, Vandelee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* 23:4407–4414
- Waldon E, Sobis-Glinkowska M, Szewczyk EM (2002) Evaluation of selected features of *Staphylococcus cohnii* enabling colonization of humans. *Folia Microbiol* 47:565–571
- Webster JA, Bannerman TL, Hubner RJ, Ballard DN, Cole EM, Bruce JL, Fiedler F, Schubert K, Kloos WE (1994) Identification of the *Staphylococcus sciuri* species group with *EcoRI*

- fragments containing rRNA sequences and description of *Staphylococcus vitulus* sp. nov. *Int J Syst Bacteriol* 44:454–460
- Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* 18:7213–7218
- Welsh J, McClelland M (1992) PCR-amplified length polymorphisms in tRNA intergenic spacers for categorizing staphylococci. *Mol Microbiol* 6:1673–1680
- Westin L, Miller C, Vollmer D, Canter D, Radtkey R, Nerenberg M, O'Connell JP (2001) Antimicrobial resistance and bacterial identification utilizing a microelectronic chip array. *J Clin Microbiol* 39:1097–1104
- Widjojoatmodjo MN, Fluit AC, Verhoef J (1995) Molecular identification of bacteria by fluorescence-based PCR-single-strand conformation polymorphism analysis of the 16S rRNA gene. *J Clin Microbiol* 33:2601–2606
- Wieser M, Busse HJ (2000) Rapid identification of *Staphylococcus epidermidis*. *Int J Syst Evol Microbiol* 50:1087–1093
- Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 18:6531–6535
- Wilson KH, Wilson WJ, Radosevich JL, DeSantis TZ, Viswanathan VS, Kuczmarski TA, Andersen GL (2002) High-density microarray of small-subunit ribosomal DNA probes. *Appl Environ Microbiol* 68:2535–2541
- Winslow CE, Winslow A (1908) *The systematic Relationships of the Coccaceae*. Wiley, New York
- Witte W, Braulke C, Cuny C, Strommenger B, Werner G, Heuck D, Jappe U, Wendt C, Linde HJ, Harmsen D (2005) Emergence of methicillin-resistant *Staphylococcus aureus* with Pantón-Valentine leukocidin genes in central Europe. *Eur J Clin Microbiol Infect Dis* 24:1–5
- Witte W, Strommenger B, Werner G (2006) The *Staphylococcus*: Diagnostics, typing and taxonomy. In: Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JJ (eds) *Gram-positive pathogens*, 2nd edn. ASM Press, Washington, DC, pp 371–380
- Wolter DJ, Tenover FC, Goering RV (2007) Allelic variation in genes encoding Pantón-Valentine leukocidin from community-associated *Staphylococcus aureus*. *Clin Microbiol Infect* 13:827–830
- Yamaguchi T, Hayashi T, Takami H, Nakasone K, Ohnishi M, Nakayama K, Yamada S, Komatsuzawa H, Sugai M (2000) Phage conversion of exfoliative toxin A production in *Staphylococcus aureus*. *Mol Microbiol* 38:694–705
- Yamaguchi T, Hayashi T, Takami H, Ohnishi M, Murata T, Nakayama K, Asakawa K, Ohara M, Komatsuzawa H, Sugai M (2001) Complete nucleotide sequence of a *Staphylococcus aureus* exfoliative toxin B plasmid and identification of a novel ADP-ribosyltransferase, EDIN-C. *Infect Immun* 69:7760–7771
- Yamaguchi T, Nishifuji K, Sasaki M, Fudaba Y, Aepfelbacher M, Takata T, Ohara M, Komatsuzawa H, Amagai M, Sugai M (2002) Identification of the *Staphylococcus aureus* *etd* pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. *Infect Immun* 70:5835–5845
- Yarwood JM, McCormick JK, Paustian ML, Orwin PM, Kapur V, Schlievert PM (2002) Characterization and expression analysis of *Staphylococcus aureus* pathogenicity island 3. Implications for the evolution of staphylococcal pathogenicity islands. *J Biol Chem* 277:13138–13147
- Yugueros J, Temprano A, Sanchez M, Luengo JM, Naharro G (2001) Identification of *Staphylococcus* spp. by PCR-restriction fragment length polymorphism of *gap* gene. *J Clin Microbiol* 39:3693–3695
- Zeigler DR (2003) Gene sequences useful for predicting relatedness of whole genomes in bacteria. *Int J Syst Evol Microbiol* 53:1893–1900
- Zhang S, Iandolo JJ, Stewart GC (1998) The enterotoxin D plasmid of *Staphylococcus aureus* encodes a second enterotoxin determinant (*sej*). *FEMS Microbiol Lett* 168:227–233
- Zhang YQ, Ren SX, Li HL, Wang YX, Fu G, Yang J, Qin ZQ, Miao YG, Wang WY, Chen RS, Shen Y, Chen Z, Yuan ZH, Zhao GP, Qu D, Danchin A, Wen YM (2003) Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228). *Mol Microbiol* 49:1577–1593

- Zhang K, McClure JA, Elsayed S, Louie T, Conly JM (2005) Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 43:5026–5033
- Zhang K, McClure JA, Elsayed S, Louie T, Conly JM (2008) Novel multiplex PCR assay for simultaneous identification of community-associated methicillin-resistant *Staphylococcus aureus* strains USA300 and USA400 and detection of *mecA* and Panton-Valentine leukocidin genes, with discrimination of *Staphylococcus aureus* from coagulase-negative staphylococci. J Clin Microbiol 46:1118–1122
- Zhang K, McClure JA, Elsayed S, Conly JM (2009) Novel staphylococcal cassette chromosome *mec* type, tentatively designated type VIII, harboring class A *mec* and type 4 *ccr* gene complexes in a Canadian epidemic strain of methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 53:531–540

Molecular Methods to Detect *Bacillus cereus* and *Bacillus thuringiensis* in Foods

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Abstract Numerous types of foods have been associated with food poisoning and a lot of cases have been linked to heat treated foods. *Bacillus cereus* group is involved in many outbreaks due to the consumption of cooked food. *B. cereus* is present in most raw foods and spores have been found also in packaging materials representing a contamination source for treated foods. Storage temperature is a critical point for processed foods in relation with spore germination. While vegetative cells die during heat treatment, spores can survive and germinate under not limiting conditions. *B. cereus* food poisoning is principally associated with temperature abuse during the storage of cooked foods. *B. cereus* may cause illness through the production of a high variety of toxins and enzymes, including a necrotizing enterotoxin, an emetic toxin, phospholipases, proteases and haemolysin inducing also non-gastro-intestinal (e.g. systemic or pulmonary) infections. Enterotoxins could also be produced from *B. circulans*, *B. lentus* and *B. mycoides*, *B. thuringiensis* and *B. anthracis* closely related to *B. cereus*. Many cases are confused with those caused by other pathogens. An accurate evaluation of potentially contaminated foods lead to the utilization of fast and sensitive methods in food monitoring to satisfy safety requirements. Classical methods which use enrichment methods due to the usual small number of *B. cereus* present in food, are time consuming. Moreover, recent data showed proteases and chitinases contribute to virulence leading to the need for suitable means of differentiating members of the *B. cereus* group during the monitoring of potentially contaminated foods. Some authors experienced difficulties distinguishing strains considered to be members of the “*B. cereus* group” such as *B. mycoides*, *B. thuringiensis* and *B. anthracis* either using classical or molecular methods. Identification by classical microbiological methods fails due to horizontal gene transfer from *B. cereus* group members. Characters responsible for some phenotypic characteristics can be lost from one species and can be acquired from

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another one causing confusion in taxonomy based upon phenotypic characters evaluation. Different molecular approaches were able to differentiate *B. anthracis* from *B. cereus* but failed to differentiate *B. cereus* from *B. thuringiensis*. Even with recent improvements in the molecular methods used for microbial phylogeny, *B. cereus*, *B. mycoides* and *B. thuringiensis* were referred to as *B. cereus* when 16S rRNA sequences were used for the differentiation. Genetic similarity between *B. cereus*, *B. thuringiensis* and *B. anthracis* has been investigated by means of DNA-DNA reassociation, multilocus enzyme electrophoresis (MEE) which compare the allozyme patterns of 10–20 genes, Pulsed Field Gel Electrophoresis (PFGE), Randomly Amplified Polymorphic DNA (RAPD)-PCR, repetitive extragenic palindromic (rep)-PCR, Real Time PCR, Microarrays. Genotypic approaches tend to be less dependent on bacterial growth variables, less time consuming and useful for determining phylogenetic relationships between microbial isolates and for assigning strains in specific groups. Molecular methods using PCR are useful in specific detection of *B. cereus* from plate isolates and for PCR application on microbial DNA extracted from food. Species identification can be achieved by specific couple of primers annealing *gyrB* gene sequence. To decrease the detection limit of *B. cereus* in food the type of treatment applied before DNA extraction is fundamental, as it allows the increase of the amplification reaction efficiency. Detection limit for artificially contaminated foods was 50 cells g⁻¹ for boiled rice, 5 × 10² cells g⁻¹ for minced meat, 30 cells g⁻¹ for salad, 20 cells mL⁻¹ for pasteurized milk and <10 cells mL⁻¹ for concentrated coffee. RE technique applied onto amplification products allowed differentiation between *B. cereus* and *B. thuringiensis* for the 81 sample tested using *Hinf*I endonuclease. RAPD-PCR and rep-PCR used for microbial typing gave different results. Cluster analyses for rep-PCR was more effective in discriminating between *B. cereus* and *B. thuringiensis* strains than RAPD-PCR. Strains were grouped in 14 clusters, from A to P using 70% similarity by RAPD-PCR, and in ten clusters from A to L by rep-PCR using 80% similarity in fingerprinting analysis.

PCR-TTGE and PCR-DGGE are methods used for single point mutations detection among DNA sequences. Amplicons obtained for *B. cereus* and *B. thuringiensis* strains were analyzed by this molecular technique to differentiate strains among the two species identified previously by the PCR-RE method. *B. cereus* strains were divided in seven levels whereas *B. thuringiensis* strains in two levels. The distribution of PCR products in each gel indicate high variability either within *B. thuringiensis* or *B. cereus*. No clear differentiation was obtained by TTGE between the two species considered. Horizontal gene transfer between strains of these different “species” makes it impossible to delineate discrete levels. A desirable goal is to differentiate the organisms detected in food products and patients to understand the real contribution of *B. cereus* and *B. thuringiensis* to human infections, either due to food consumption or to hospitalization, using fast and sensitive protocols.

Keywords *Bacillus cereus* • *Bacillus thuringiensis* • food • PCR • DGGE/TTGE • RE

Introduction

Food can be considered an ecosystem with many interactions among microorganisms and matrix. The study of the microorganisms which create or contaminate foods is of great importance. Good bacteria are important for industrial production of foods such as cheese, yoghurt, other fermented foods, bread, beer and wine, whereas the study of microorganisms causing food spoilage is of importance for human health. Pathogens are cause of illness and death in some countries and cause of infectious or gastrointestinal disease in other countries causing economic outly. Pathogen evolution due either to new human habits or to new industrial practices need reliable methods for pathogen detection. Microorganism enumeration, species and strain determination in food products is important to meet safety requirements. Microbial taxonomy range from genus to strain level depending upon the technique used: phenotypic or genotypic. Right now advances obtained in molecular methods allow their utilization in food microbiology. Molecular biology methods to study microbial populations without cultivation and for identification of bacteria have been developed (Giraffa and Neviani 2001). Numerous molecular methods providing good results in detection, identification and characterization of bacteria are PCR based methods which are of fast and simple application to food analysyes. Among the pathogens involved in human health *Bacillus cereus* group is interesting for the ability to survive in various habitats. To understand the real distribution of *Bacillus cereus* and *Bacillus thuringiensis* in different outbreak cases the possibility of differentiating between *B. cereus* and *B. thuringiensis* is a real need in monitoring potentially contaminated foods. PCR technology developed methods useful in many applications, in molecular epidemiology, in human diagnosis of pathogens, in quality control, in quality assurance of typing strains (Versalovic et al. 1994; Cherif et al. 2002; Cherif et al. 2003b; Manzano et al. 2008).

Bacillus cereus Group

General Characteristics

In the genus *Bacillus*, aerobic or facultatively anaerobic bacteria, is possible to distinguish the closely related species *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides* and *B. weihenstephanensis* forming the *B. cereus* group. *B. cereus* group is formed by Gram-positive spore forming rod-shaped bacteria, characterized by two morphological forms, the vegetative cell (from 1.0 to 1.2 μm in width and from 3.0 to 5.0 μm in length), straight or nearly so, motile or non-motile, and the endospore (non-swelling the sporangium) (Table 1).

The genus is characterized by the presence of endospores, not more than 1 per cell, very resistant to many adverse conditions (heat, cold, radiation, desiccation and disinfectants) and produced either in the presence or in the absence of air.

Table 1 *Bacillus anthracis*, *B. cereus*, *B. thuringiensis* characteristics

	Cell diameter (µm)	Sporangium swollen	Motility	Parasporal crystals	Catalase	Growth in 7% NaCl	Capsule
<i>B. anthracis</i>	>1	–	–	–	+	+	+
<i>B. cereus</i>	>1	–	+	–	+	+	–
<i>B. thuringiensis</i>	>1	–	+	+	+	+	–

–: ≥90% strains negative; +: ≥90% strains positive

Cell diameter, sporangium and catalase test do not allow differentiation, whereas parasporal crystals and capsule presence are considered important in differentiation among *B. anthracis*, *B. cereus* and *B. thuringiensis*, even if last reports by Cachat et al. (2008) showed the presence of a *B. thuringiensis* strain capable to produce a capsule resembling that of *B. anthracis*. Pannucci et al. (2002) reported many phenotypic differences among *B. cereus*, *B. thuringiensis* and *B. anthracis* isolates are conferred by plasmid encoded genes. The many species of the genus exhibit a great variety in physiologic characteristics: degradation of cellulose, starch, pectin, agar, hydrocarbons, production of enzymes and antibiotics, and other characteristics such as acidophily, alkaliphily, psychrophily and thermophily that allow adaptation to various environmental conditions (Clavel et al. 2004). Most species are widely distributed in nature and their presence in different habitat is due to passive distribution and persistence of spores (Setlow and Setlow 1998, Pirttijarvi et al. 2000; Hill et al. 2004). Differentiation between species of the genus was difficult at early attempts when endospore formation and aerobic growth were the main characters used for classification. As reported by many authors, the differentiation between *B. thuringiensis* and *B. cereus* is also very difficult in the case of molecular methods.

***Bacillus cereus* Group and Foods**

Bacillus species can be isolated from soil, growing plants, raw and cooked foods. The formation of heat-resistant endospores is of considerable importance in the spoilage of heat-treated foods (Kramer and Gilbert 1989). Foods incriminated in past outbreaks include cooked meat and vegetables, boiled or fried rice, vanilla sauce, custards, soups, and raw vegetable sprouts (Table 2). *Bacillus* food poisoning occurs when spores survive pasteurization or cooking and germinate and multiply producing toxins in not adequately stored food. *B. cereus*, responsible for the majority of foodborne illness attributed to *Bacillus*, produces a wide variety of toxins and enzymes active on different cell tissues associated with the capability of this microorganism to induce infections (Ghelardi et al. 2000). The role of *Bacillus cereus* as food-poisoning organism is known since 1950 (Hauge 1955), whereas *Bacillus thuringiensis*, *Bacillus circulans* and *Bacillus lentus* have been

Table 2 Foods contaminated by *B. cereus* group

Food
Pasteurized milk
Powder milk
Powdered infant formulae
Ricotta
Cheese
Liquid pasteurized egg yolk
Gnocchi
Tortellini
Fresh vegetables
Garlic
Cooked vegetables
Carrot broth
Vegetable cream
Spices
Flour
Bakery products
Fermented cereal
Meat
Boiler rice
Pastas
Sauces
Russian salad
Desserts
Wine
Beer
Potatoes croquettes

associated to food poisoning only in recent years (van Netten et al. 1990). *B. cereus*, *B. thuringiensis* and *B. anthracis* are important species for human health as they carry genes encoding virulence factors affecting pathogenesis (Sergeev et al. 2006). As some *B. thuringiensis* strains have been reported to possess genes known to be involved in *B. cereus* pathogenesis (Hansen and Hendriksen 2001) a differentiation of *B. cereus* and *B. thuringiensis* detected in food products could be a real contribution to the collection of epidemiological data. *B. cereus*, *B. thuringiensis* and *B. anthracis* have been subjected to several genomic studies to understand the taxonomic relationships. Data reported by Helgason et al. (2000a) reveal that *B. cereus*, *B. thuringiensis* and *B. anthracis* are indistinguishable using MEE or sequence analyses of nine genes, according with data obtained using DNA-DNA hybridization analysis by Kaneko et al. (1978) and Priest et al. (1994). Genetic evidence indicates that *B. cereus*, *B. thuringiensis* and *B. anthracis* are one species (Helgason et al. 2000b). Cherif et al. (2003a) grouped *B. anthracis* in a separate branch from *B. cereus* and *B. thuringiensis* on the basis of the phylogenetic relationship between the species of the *B. cereus* group using the long ITS containing tDNA. Data on ITS sequences confirm the difficulty in distinguishing *B. cereus* and *B. thuringiensis* apart from

the entomocidal genotype and phenotype (Cherif et al. 2003a). Also molecular methods using specific probes in the variable region V1 of 16S rDNA, *gyrB* genes, 16S rRNA sequences have been used for a specific differentiation from *B. cereus* and *B. thuringiensis* (Ash et al. 1991; Chen and Tsen 2002).

***B. cereus* Characteristics**

B. cereus is able to grow between 4°C and 55°C. Mesophilic strains grow between 10°C and 42°C, some psychrotrophic strains can grow at 4°C whereas other strains are able to grow at 52–55°C. *B. cereus* vegetative cells grow at pH values comprises between 4.5 and 9.3, but a slight growth was reported at pH 4.1 by Clavel et al. (2004). Spores are tolerant to acidity at pH between 1.0 and 5.2, heat resistant, can germinate and multiply in humid low acid foods in a range of temperature from 5–52°C. Survival of *B. cereus* spores at 95°C decreases when pH substrate decreases from 6.2 to 4.7 (Fernandez et al. 2002). *B. cereus* is able to grow in the presence of salt concentrations up to 7.5% depending on the pH values associated.

Toxin Production

Bacillus species are capable to produce different types of toxins and can be involved in gastrointestinal diseases and non-gastrointestinal diseases as eye infections, cutaneous infections, orthopaedic wounds, meningitis, bacteraemia and pulmonary infections. *B. cereus* produces many extracellular toxins including lecithinase, proteases, β -lactamase, sphingomyelinase, cereolysin, and hemolysin BL and can be considered responsible for many infections. Enterotoxins could also be produced by other species of the genera *Bacillus* including *B. thuringiensis* which vegetative cells may also produce soluble toxins, the heat sensitive α -exotoxin and a heat-resistant β -exotoxin, which is a nucleotide produced only by special strains of these same bacteria. A wide variety of toxins produced by *B. cereus* and *B. thuringiensis* have been associated with illness. Two principal types of food poisoning caused by *B. cereus* diarrheagenic and the emetic toxins have been described. The diarrheal type toxin, causing watery diarrhea and abdominal pain, is a 50,000–60,000 molecular weight complex protein inactivated by a heat treatment at 56°C for 5 min (heat-labile toxin) (Gilbert and Kramer 1984) usually produced in the intestine after bacterial cell ingestion (Granum 2001). The diarrheagenic syndrome is produced by a tripartite complex composed by B, L₁ and L₂ components named hemolysin BL (HBL). Colony immunoblot assay with polyclonal antibodies specific for L₁ (coded by *hblD* gene) and L₂ (coded by *hblC*) gene components has been employed by Ryan et al. (1997). Monoclonal antibodies against hemolysin BL enterotoxin complex was used by Dietrich et al. (1999).

Table 3 *Bacillus* species producing haemolysin BL responsible for diarrheal syndrome

<i>Bacillus amyloliquefaciens</i>
<i>Bacillus cereus</i>
<i>Bacillus coagulans</i>
<i>Bacillus lentimorbis</i>
<i>Bacillus lentus</i>
<i>Bacillus licheniformis</i>
<i>Bacillus megaterium</i>
<i>Bacillus mycoides</i>
<i>Bacillus pasteurii</i>
<i>Bacillus pseudomycoides</i>
<i>Bacillus subtilis</i>
<i>Bacillus thuringiensis</i>
<i>Bacillus weihenstephanensis</i>

The antibodies enabled the specific and sensitive detection of the hemolysin components in supernatants. Specific primer set was used by Hansen and Hendriksen (2001)) for *hbl* gene detection. This toxin complex is associated to other *Bacillus* species (Table 3).

The emetic type toxin is a 1.2 kDa molecular weight cereulide (ionosphoric water-soluble peptide) resistant to a treatment at 120°C for 1 h (heat-stable toxin) (Agata et al. 2002). This toxin may persist in foods after vegetative cells death. It causes an acute attack of vomiting after ingestion of contaminated food. For diarrheal syndrome food containing more than 10⁴ cells/spore per g has been considered risky, whereas for the emetic syndrome the number of *B. cereus* varies from 10⁵ to 10⁹ per g. Cereulide production begins at the end of the log growth phase. Outbreaks caused by *B. cereus* and/or the related species are often confused with outbreaks caused by other etiological agents. The symptoms of the diarrheal syndrome due to the presence of *B. cereus* are similar to the *C. perfringens* syndrome symptoms. Symptoms due to *B. cereus* emetic toxins are similar to *Staphylococcus* intoxication symptoms. For this reason it is necessary an accurate laboratory investigation. Moreover *B. cereus* and *B. thuringiensis* are very closely related species belonging to the *B. cereus* group which main difference is the presence on *B. thuringiensis* plasmids of genes coding for the insecticidal toxins (Chen and Tsen 2002; Rasko et al. 2005). Plasmids loss and horizontal spreading of plasmids cause many difficulties in distinguishing *B. thuringiensis* from *B. cereus* affecting taxonomy and pathogenicity (Ward and Ellard 1983; Wilcks et al. 1998). Gonzales et al. (1982) used the high-frequency transfer of plasmids between strains of *B. thuringiensis* to study the genetic relationship between plasmid and toxin production. Schnepf et al. (1998) worked on pesticidal proteins. Molecular methods have been developed to detect microbial toxins in foods. Radhika et al. (2002) utilized a probe to detect the phospholipase C. Virulence factors (enterotoxins, phospholipases and exotoxins) important for pathogenesis, were explored by the microarray-based detection by Sergeev et al. (2006).

***B. cereus* Detection**

B. cereus can be detected utilizing biochemical tests or molecular methods. As *B. cereus* is present in small numbers in many foods, enrichment methods and selective media are recommended. Surface plating procedures are usually employed for viable cell enumeration. Conventionally, *B. cereus* is detected by its ability to grow on selective plating media containing egg yolk, mannitol and the antibiotic polymyxin B. *B. cereus* colonies will normally be evident (about 5 mm diameter) after 24–48 h incubation at 30°C. Members of *Bacillus* species not fermenting mannitol, usually produce lecithinase, and exhibit β - haemolysis on blood agar are considered presumptively *B. cereus*. Confirmation analyses usually are done on five colonies picked from agar plates and tested for nitrate reduction, hemolysis on blood agar, endotoxin crystals presence, motility (Health Protection Agency 2005). Results with atypical *B. cereus* strains may not be sufficient for an adequate identification. In Table 4 are reported test results for some species of the *B. cereus* group.

Although new media have improved their selectivity, an immediate differentiation between *B. cereus* and *B. thuringiensis*, the species most frequently detected in foods, on the agar plates is not possible. The morphology of the colonies is the same and the main difference is the presence of intracellular crystal bodies. The capability to form crystals (plasmid coded) may be lost by laboratory cultures. Moreover, the possibility of having lipidic inclusions in *B. cereus* cells which could be confused with crystals, as they may be indefinite in shape, may difficult a clear differentiation. Biochemical and physiological test usually applied in *B. cereus* identification are the API 50CH/20E (Biomerieux sa, Marcy l'Etoile, France) combined with motility test, oxidase test, etc. (Valero et al. 2002). The change or loss of the phenotypic characteristics has been observed by many authors (Jakson et al. 1995; Daggaard et al. 1997) and the high level of lateral gene transfer do not allow the employment of the virulence associated gene-based detection to obtain species identification (Jensen et al. 2003). Cultural and biochemical characteristics needed for strains identification are obtained by the utilization of time-consuming techniques now replaceable with faster molecular methods (Henderson et al. 1995; Mantynen and Lindstrom 1998). Moreover, the International Committee on Bacterial Systematics recommended the use of DNA:DNA hybridization for the differentiation of bacterial species to avoid ambiguities due to phenotypic characterization (Wayne et al. 1987). As this method is complicated and not always reproducible, La Duc et al. (2004) proposed the sequence analysis of the *gyrB* gene, coding for the β -subunit of the

Table 4 Characteristics used for differentiating *Bacillus cereus* group species

	<i>B. anthracis</i>	<i>B. cereus</i>	<i>B. thuringiensis</i>	<i>B. mycoides</i>
Motility	–	+/-	+/-	–
Reduction Nitrate	+	+	+/-	+
Hemolysis	–	+	+	+
Body crystals	–	–	+	–

+: 90–100% positive; – : 90–100% positive; +/-: 50–50% positive

DNA gyrase, highly conserved, single copied housekeeping gene and evolving with a single base pair change every 50 million years (Yamamoto and Harayama 1995; Yamada et al. 1999). *gyrB* gene analysis is a simple method requiring PCR, sequencing and alignment to allow results. Moreover, single-copied housekeeping genes are more useful than multicopied functional genes in phylotipic discrimination when lateral gene transfer is common such as within *B. cereus* group.

A desirable approach is to detect organisms directly in the food as they are less dependent on bacterial growth variables and less time consuming. Genetic similarity between *B. cereus* and *B. thuringiensis* has been investigated by means of genotypic approaches which are the answers to the need of rapid, specific and safe control systems for food products used in industrial formulations. PCR based methods coupled with electrophoresis techniques, statistic and bioinformatic procedures modified the approach in microbiological laboratories. Two basic approaches can be used: (a) specific PCR techniques using specific primers annealing a known DNA region and the utilization of short random sequences (primer) annealing genome regions to obtain DNA fingerprinting for differentiation at species or strain level (Table 5).

These molecular techniques can be used either on isolates from agar plate medium or on microbial DNA directly extracted from food. The need to obtain fast methods in food analyses stimulate research to optimize molecular protocols. Pulsed field gel electrophoresis (PFGE) and multilocus enzymes electrophoresis are methods useful to analyze the genetic diversity of *B. cereus* and *B. thuringiensis* isolates (Helgason et al. 2000b). Fricker et al. (2007) proposed Real-Time PCR assays for the detection of emetic *Bacillus cereus* strains in foods. The advantage obtained in using Real-time PCR is the highly specificity, sensitivity and the quantification (Mackay 2004). The assay targeted a specific part of the cereulide synthetase (*ces*) gene allowing the detection of the microorganism responsible for the emetic food-poisoning outbreaks. The developed method was able to detect 10^0 CFUg⁻¹ after an enrichment time of 4–6 h, whereas the detection limit was 10^1 – 10^3 CFUg⁻¹ directly form food (Fricker et al. 2007). Also a PCR method using colony hybridization has been developed to detect *B. cereus* in foods by Radhika et al. (2002).

Table 5 PCR based techniques used in the last years to identify food interesting microorganisms

Technique	Product obtained	Result
PCR	Specific amplicon	Species identification
PCR-DGGE/TTGE ^a	Specific/unspecific amplicon	Species/strain identification
PCR-SSCP ^b	Specific amplicon	Strain identification
RAPD ^c -PCR	DNA Fingerprinting	Genomic DNA polymorphism
rep ^d -PCR		
RE ⁺ -PCR	Specific RE products	Species/strain identification

^aDGGE: Denaturing Gradient Gel Electrophoresis, TTGE: Temporal Temperature Gradient Electrophoresis

^bSSCP: Single Stand Conformation Polymorphism

^cRAPD: Randomly Amplified Polymorphic DNA

^drep-PCR: RE: Restriction Endonuclease

The detection of *B. cereus* from food was obtained using a PCR-generate labelled (with DIG-dUTP) probe (PL-1). A fluorogenic probe-based PCR assay has been used by Kim et al. (2000). The method, able to distinguish *B. cereus* from *B. thuringiensis*, based upon two single-base differences within cereolysin AB gene was applied on nonfat dry milk products (NFDM) due to their high percentage of contamination. Data reported by Kim et al. (2000) indicated an approximate sensitivity of 25 CFU g⁻¹ of *B. cereus*.

PCR Based Method for B. cereus Detection

PCR Optimization for Selective Detection of *B. cereus*

Microorganisms reported in Table 5 were used to optimize PCR protocol. They were grown overnight at 30°C on BHI agar (Oxoid). A single colony was selected from the plate and subjected to DNA extraction as described by Manzano et al. (2003a) for PCR applications. DNA concentration was standardized at about 50 ng/μL before being used in the PCR assay. The primers BCFW1 (5'-gtttctggtggttgcatgg-3') and BCREWNEW (5'-ttttgagcgatttaatgc-3') were designed utilizing DNA sequences retrieved from GenBank (Yamada et al. 1999) and anneal to the *gyrB* sequence. PCR assays were performed as reported by Manzano et al. (2003b).

Amplification products were obtained only for *Bacillus cereus* out of the microorganisms reported in Table 6, allowing their application in the selective detection of *B. cereus* strains directly in foods.

Artificial Food Contamination

B. cereus DSMZ 2301 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) was grown overnight onto PCA (Oxoid) plates, and then suspended in sterile distilled water. Different *B. cereus* concentrations ranging from 1 × 10⁶ to 1–10 cells mL⁻¹ were prepared using the Bürker camera. The real estimation of the inocula was made by plating 0.1 mL of each sample obtained by boiled rice, minced meat, salad and pasteurized milk inoculated with different concentrations of *B. cereus* onto *Bacillus cereus* Selective Agar base (BCSAB, Oxoid) immediately after the inoculation.

DNA Extraction from Food Samples

Extracting microbial DNA directly from food, *Taq* DNA polymerase inhibitory substances present in the sample could decrease the efficiency of the amplification reaction. Pre-extraction methods for different foods must be applied to obtain DNA useful for the amplification by PCR technique. To obtain reliable results (error-prone

Table 6 Microorganisms used to check primers BCFW1 and BCREVNEW

Strain	Source
<i>Bacillus cereus</i> 4282	DSMZ ^a
<i>Bacillus cereus</i> 2301	DSMZ ^a
<i>Bacillus cereus</i> 4222	DSMZ ^a
<i>Bacillus cereus</i> RB41	LB ^b
<i>Bacillus mycoides</i> 299	DSMZ ^a
<i>Bacillus mycoides</i> 2048	DSMZ ^a
<i>Bacillus thuringiensis</i> 2046	DSMZ ^a
<i>Bacillus thuringiensis</i> 350	DSMZ ^a
<i>Bacillus thuringiensis</i> 6080	DSMZ ^a
<i>Bacillus thuringiensis</i> 001	LB ^b
<i>Bacillus thuringiensis</i> 005	LB ^b
<i>Bacillus thuringiensis</i> BT	LB ^b
<i>Bacillus thuringiensis</i> 1	LB ^b
<i>Bacillus thuringiensis</i>	Pesticides ^c
<i>Bacillus cereus</i>	Foods ^d
<i>Bacillus cereus</i>	Humans ^e
<i>Bacillus pumilis</i>	Food ^d
<i>Bacillus subtilis</i>	Food ^d
<i>Bacillus laterosporus</i>	Food ^d
<i>Bacillus brevis</i>	Food ^d
<i>Bacillus coagulans</i>	Food ^d
<i>Bacillus lentus</i>	Food ^d
<i>Bacillus globisporius</i>	Food ^d
<i>Bacillus badius</i>	Food ^d
<i>Bacillus circulans</i>	Food ^d
<i>Salmonella enteritidis</i>	ISS ^g
<i>Salmonella scott</i>	ISS ^g
<i>Listeria monocytogenes</i> 7644	ATCC
<i>Listeria innocua</i>	ILC ^f
<i>Listeria ivanovii</i>	ILC ^f
<i>Lactobacillus plantarum</i> 200174	DSMZ
<i>Lactobacillus brevis</i> 20054	DSMZ
<i>Kokuria</i> spp	DSA ^h
<i>Kocuria kristinae</i>	DSA ^h
<i>Yersinia enterocolitica</i> 23715	ATCC
<i>Escherichia coli</i>	ISS ^g
<i>Proteus vulgaris</i>	DSA ^h
<i>Citrobacter freundii</i>	DSA ^h
<i>Staphylococcus carneus</i>	DSA ^h
<i>Staphylococcus aureus</i>	DSA ^h

^aDSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany

^bInstitute Pasteur Paris, Paris, France

^cStrains isolated from humans in the Orthopaedic and Traumatology Department of Toulouse Hospital (France)

^dStrains isolated from food and identified as described by Tiecco (2000), in the Department of Food Science, Udine, Italy

^eStrains isolated from pesticide in the Laboratoire de Bacteriologie (Toulouse, France)

^fIstituto lattiero caseario, Lodi Italia

^gIstituto Superiore della sanità, Roma, Italia

^hDipartimento di Scienze degli Alimenti, Udine, Italia

due to complexity of food matrices and its PCR inhibitory properties), experiments were repeated at least three times each (Manzano et al. 2003c).

10 grams of rice were used for DNA extraction. The pellet was resuspended in 1.5 mL solution of α -amylase from *Aspergillus oryzae* E.C. 3.2.1.1 (Sigma A-6211) and after 10 min was centrifuged at 14,000 rpm at 10°C for 10 min and washed two times in 1.5 mL sterile distilled water. Boiled rice treated with α -amylase enables the detection of *B. cereus* for the minimum level of 50 cells g⁻¹ (Manzano et al. 2003c).

Ten grams of minced meat inoculated with the *B. cereus* solution were centrifuged and the pellet was resuspended in EDTA 0.25 M, centrifuged and then washed two times in distilled water before being used for DNA extraction. The EDTA solution used to wash the pellet enabled the detection of *B. cereus* at the minimum level of 5×10^2 cell g⁻¹. 1 mL of milk was added to 8 mL 0.8% NaCl 0.1% peptone and inoculated with 1 mL of the *B. cereus* solution. The pellet was resuspended in EDTA 0.25 M and then washed two times in sterile distilled water and used for DNA extraction. Pellet from pasteurized milk washed in an EDTA solution enabled the detection of *B. cereus* at the minimum level of 20 cells g⁻¹.

Ten grams of salad were homogenized in 9 mL 0.8% NaCl 0.1% peptone after the addition of the *B. cereus* solution. The homogenate obtained was centrifuged and the pellet resuspended in EDTA 0.25 M and then washed two times in sterile distilled water. The pellet obtained was used for PCR protocol as reported by Manzano et al. (2003c). The minimum level of 30 cells g⁻¹ of *B. cereus*, was achieved for salads.

A coffee concentrate solution was artificially inoculated with solutions containing diluted culture of vegetative cells of *B. cereus* (DSMZ 2301). Different microbial concentrations ranging from 1×10^6 to ca 1×10^1 cells mL⁻¹ were tested to assess the lowest microbial concentration detectable using the PCR technique in association with the pre-extraction procedure developed. Inoculated coffee concentrate samples were centrifuged at 20,000 rpm at 4°C for 5 min. The pellet was resuspended in EDTA 0.25 M and washed twice in sterile distilled water before DNA extraction by using the DNeasy Plant Mini Kit (Qiagen, Milan, Italy). Eight not inoculated coffee concentrate samples were subjected to the same procedure described before. One to ten cells mL⁻¹ *B. cereus* was the lower limit for coffee concentrate after washing the pellet with EDTA 0.25 M and sterile distilled water before the DNA extraction by the DNeasy Plant Mini Kit (Qiagen) (Fig. 1).

Food matrix affected the detection capability of the microbial cells using the PCR technique, whereas the presence of natural contaminating microflora (as detected for salads) did not hinder with the amplification of the selected specific DNA target. The DNA pre-extraction protocol developed allow the obtainment of PCR products also in the presence of inhibiting substances and/or contaminating microflora. The positive results for inoculated samples and the negative results obtained for control samples demonstrated the effectiveness of the technique. The possibility to check food samples for the presence of *B. cereus* by PCR technique without the application of classical microbiological analyses is important for food industries and in catering. Various paper reported different results related to the usefulness of the *gyrB* gene sequence for differentiation at species level.

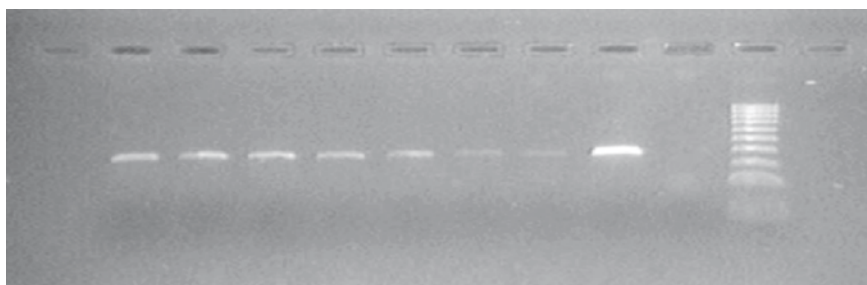


Fig. 1 Amplicons obtained using different inocula of *Bacillus cereus* in the coffee concentrate samples (Manzano et al. 2003b). Lanes 1–7, samples artificially contaminated; lane 1, 1×10^6 cells mL^{-1} ; lane 2, 1×10^5 cells mL^{-1} ; lane 3, 1×10^4 cells mL^{-1} ; lane 4, 1×10^3 cells mL^{-1} ; lane 5, 1×10^2 cells mL^{-1} ; lane 6, 10 cells mL^{-1} ; lane 7, 1–10 cells mL^{-1} ; lane 8, *B. cereus* DSMZ 2301 (positive control); lane 9, negative control; lane 10, (MWM) molecular weight marker (100 bp; Sigma, Germany)

RE-PCR Optimization for B. cereus/B. thuringiensis Differentiation

DNA digestion by Restriction Endonuclease (RE) is a simple method to utilize single point mutations present at restriction site to differentiate DNA sequences. This method is useful for strain typing (Peerbooms et al. 1987) or for differentiation at species level (Comi et al. 2000). The target DNA can be represented by the whole genome or by defined sequences i.e. PCR products. Presence or absence of a restriction site can be used to obtain species (Manzano et al. 1998) or strain differentiation. PCR products obtained according to the protocol reported by Manzano et al. (2003a) were subjected to endonuclease restriction with *Hinf* I to obtain differentiation between *B. cereus* and *B. thuringiensis*. Collection strains used to optimize protocol are reported in Table 7.

From 2.1 to 4.2 μg of the amplified DNA was digested using *Hinf* I enzyme according to the instructions of the manufacturer (Roche Diagnostics, Milan, Italy). The method was applied also to the isolates from humans, pesticides and food as reported in Table 8.

As reported by Manzano et al. (2008) all the strains from pesticides (A, B, C, D, E and I) were identified as *B. cereus* by RE. Seven strains isolated from food samples were identified as *B. cereus*, but the isolated from coffee that was identified as *B. thuringiensis* (Fig. 2).

Eleven out of the 62 strains from humans were identified as *B. thuringiensis* as reported in Table 9.

Results obtained by RE digestion of the PCR product obtained for pesticides samples were different as the expected. In fact usually it is assumed the *Bacillus* species present in the preparations is *B. thuringiensis* instead of *B. cereus*.

Table 7 *Bacillus* strains used for PCR optimization in RE-PCR protocol

Number of strains	Strain	Source
1	<i>Bacillus cereus</i>	DSMZ ^a 4282
1	<i>Bacillus cereus</i>	DSMZ ^a 2301
1	<i>Bacillus cereus</i>	DSMZ ^a 4222
1	<i>Bacillus cereus</i>	RB41
1	<i>Bacillus thuringiensis</i>	DSMZ ^a 2046
1	<i>Bacillus thuringiensis</i>	DSMZ ^a 350
1	<i>Bacillus thuringiensis</i>	DSMZ ^a 6080
1	<i>Bacillus thuringiensis</i>	001 ^b
1	<i>Bacillus thuringiensis</i>	005 ^b
1	<i>Bacillus thuringiensis</i>	BT ^b
1	<i>Bacillus thuringiensis</i>	1 ^b
1	<i>Bacillus mycoides</i>	DSMZ ^a 2048
1	<i>Bacillus mycoides</i>	DSMZ ^a 299

^aDSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany

^bInstitute Pasteur Paris, Paris, France

Table 8 *B. cereus/thuringiensis*, tested

6	<i>Bacillus cereus/thuringiensis</i>	Pesticides A, B, C, D, E, I ^a
8	<i>Bacillus</i>	Food ^c
62	<i>Bacillus</i> spp.	Humans ^b

^aStrains isolated from commercial products (supposed to containing *Bacillus thuringiensis* and used as organic pesticides by farmers) in the Laboratory of Bacteriology (Toulouse, France), and identified on the basis of parasporal crystal cell inclusions presence isolates from commercial preparations of biopesticides

^bLaboratoire de Bactériologie (Toulouse, France), strains isolated from patients in the Orthopaedic and Traumatology Department of Toulouse Hospital (France). Identification made by the API20E system (BioMérieux, Mercy, France)

^cStrains isolated from food in the Department of Food Science, Udine, Italy

Microbial Identification and Typing by Molecular Methods

Molecular biology techniques yielded numerous methods useful in typing microorganisms. Differentiation at strain level can be useful in the understanding of biodiversity within species. Techniques used to compare strains can act at genome or at gene level. Methods based on DNA Restriction Fragment Length Polymorphism (RFLP) can produce discrimination at strain level. Digestion on rRNA genes (ribotyping) can differentiate strains (Giraffa and Neviani 2000, 2001; Domig et al. 2003). A useful and simple method to evaluate DNA polymorphism is represented by PCR based techniques. Randomly amplified polymorphic DNA PCR (RAPD-PCR) and repetitive extragenic palindromic PCR (rep-PCR) are often applied to food strain characterization (Maukonen et al. 2003; Forney et al. 2004; Rossetti

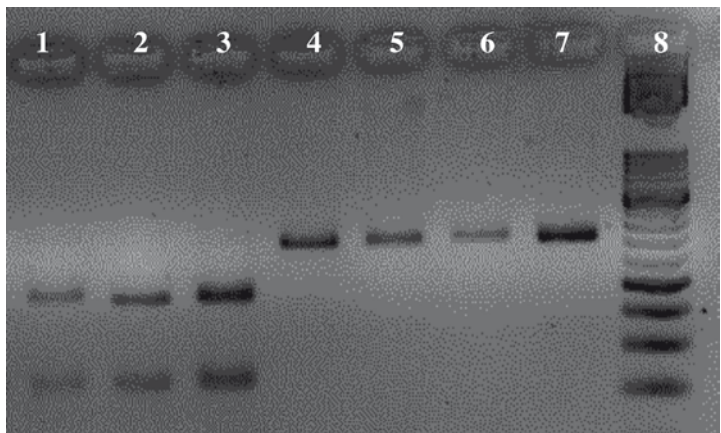


Fig. 2 PCR products digested by *Hinf* I endonuclease. Lane 1: *B. thuringiensis* DSMZ 2046; Lane 2: *B. thuringiensis* from coffee; Lane 3: *Bacillus thuringiensis* DSMZ 350; Lane 4: *Bacillus cereus* DSMZ 4282; Lane 5: *B. cereus* from ricotta ; Lane 6: *B. cereus* DSMZ 2301; Lane 7: uncut PCR product *B. cereus* DSMZ 2301 ; Lane 8: Molecular Weight Marker, (Sigma, Milan, Italy)

Table 9 Identification results by the RE method (*Hinf* I) on PCR product obtained by BCFW1 and BCRW1

Number of Strains	Source	Identification
62	^a Human samples	51 <i>B. cereus</i> 11 <i>B. thuringiensis</i>
13	^b Food samples	12 <i>B. cereus</i> 1 <i>B. thuringiensis</i>
6	^c Pesticide samples	6 <i>B. cereus</i>

^a62 strains isolated from hospitalized patients showing infections in the Orthopaedic and Traumatology Department of Toulouse Hospital (France)
^bStrains isolated from food in the Department of Food Science, Udine, Italy
^cStrains isolated from pesticide in the Laboratoire de Bacteriologie (Toulouse France)

and Giraffa 2005; Giusto et al. 2006). Short random DNA sequences (primers from eight to ten bases) are used to obtain RAPD-PCR fingerprinting usually from plate isolated microorganisms. Data obtained can be analyzed by informatic methods to facilitate fingerprinting interpretation. rep-PCR primers anneal repetitive chromosomal elements randomly distributed in chromosome producing fingerprints. To evaluate strain diversity also other molecular techniques based on PCR can be used.

A classic method to trace genetic relationships among strains is the utilization of the ribosomal operon. The region comprises between 16S and 23S ribosomal DNA (Internal Transcribed Spacers, ITS) which is often present in multiple copies in bacterial genome, and is hypervariable may differentiate between operons in the

cell (Gurtler and Stanisich 1996; Gurtler 1999). Using agarose gel ITS-PCR fingerprinting patterns is not possible to identify species, whereas using ITS homoduplex–heteroduplex polymorphisms (ITS-HHP) it is possible to detect intra- and interspecific differences (Jensen and Hubner 1996). Daffonchio et al. (2000) tested 141 strains belonging to six species of the *B. cereus* group and shown the ITS-HHP approach is suitable for strain typing. Numerous PCR methods has been developed for application in microbial differentiation: AFLP-PCR (Ticknor et al. 2001; Radnedge et al. 2003), Sau-PCR (Corich et al. 2005), AP-PCR (Welsh and McClelland 1990). Daffonchio et al. (2006) used a restriction site insertion-PCR (RSI-PCR) to identify specific single nucleotide polymorphism (SNP) present in the ITS regions used in species differentiation. It is possible also to utilize PCR associated to an electrophoretic method to obtain strain differentiation. The difference is the utilization of specific primers annealing the desired sequence (a specific gene or a conserved region) and the evaluation of point mutations present in the region analyzed to differentiate strains. This method could be used also to identify strains as reported by Samarzija et al. (2002). Multiple-Locus Sequence Typing (MLST) Analysis based on the sequences of the *clpC*, *dinB*, *gdpD*, *panC*, *purF*, and *yhjL* loci was used by Sorokin et al (2006) to differentiate psychrotrophic *B. cereus* and *B. thuringiensis* strains. Last years a comparative analysis using two-component signal transduction systems of *B. cereus*, *B. thuringiensis* and *B. anthracis* has been used to predict differences between members of the *B. cereus* group (de Been et al. 2006). This *in silico* comparative analysis of the signal transduction systems (TCS) revealed differences within the *B. cereus* group able to explain the higher number of environments in which *B. cereus* and *B. thuringiensis* can survive in respect with *B. anthracis*.

RAPD-PCR and rep-PCR Analyses on B. cereus and B. thuringiensis Strains

From a single colony selected DNA extraction was performed according to the method proposed by Andrighetto et al. (2001) for the rep-PCR and RAPD-PCR methods. RAPD was performed with primer M13 (5'-gagggtggcggctct-3') (Ehling-Schulz et al. 2005). Results obtained by RAPD-PCR using a similarity of 70% by the analyses of the RAPD-PCR fingerprints indicate the method is not useful for intra specific discrimination among *B. cereus* and *B. thuringiensis* strains isolated from food, pesticides and humans. The presence of 14 clusters (from A to P) (Fig. 3) confirms a high variability among *B. cereus* and *B. thuringiensis* species. Cluster N is the only one containing *B. cereus* from the same source (foods). No clusters formed by strains belonging to the same species were observed (Table 10) (Manzano et al. 2009).

The rep-technology has many applications in molecular epidemiology, in human diagnosis of infectious pathogens, quality control and assurance of typing strains for industrial applications and in food diagnostics (Versalovic et al. 1994).

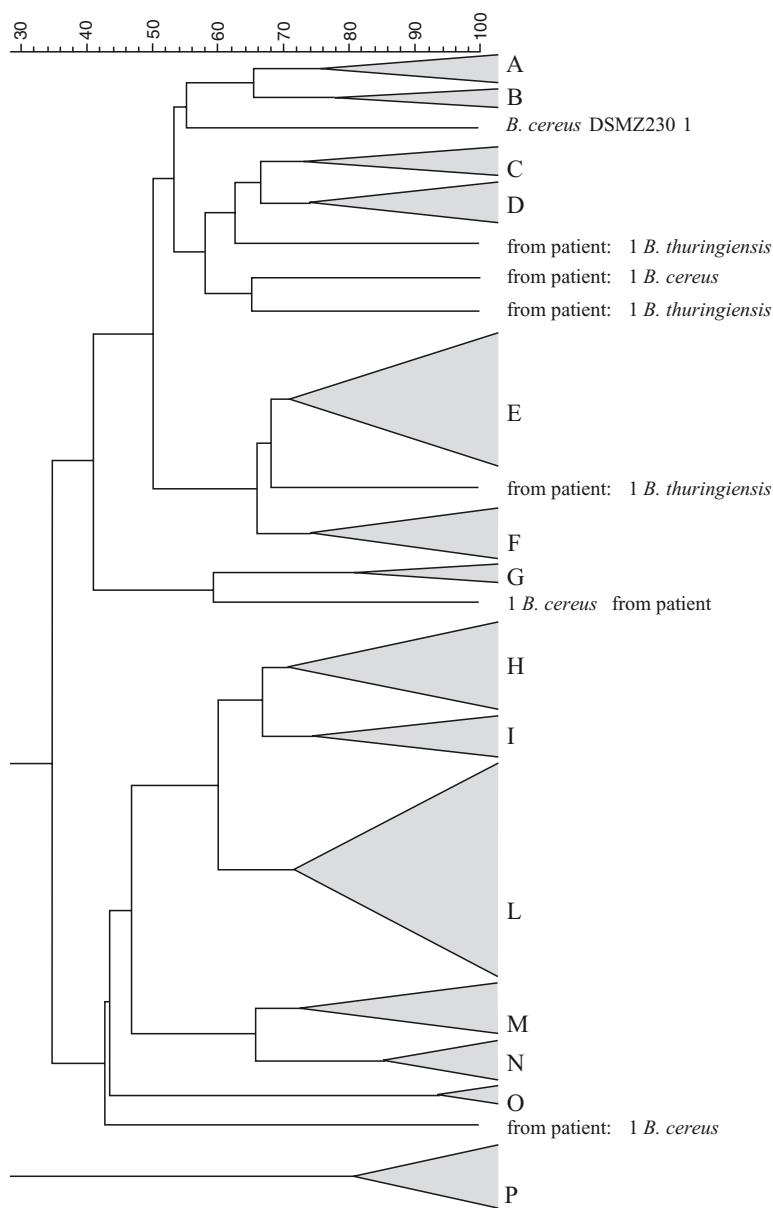


Fig. 3 UPGMA-based dendrogram obtained by RAPD-PCR with primer M13 for *Bacillus cereus* (Bc) and *B. thuringiensis* (Bt) strains using 70% similarity

Although rep-PCR is a suitable method for distinguishing closely related species of bacteria (de Bruijn et al. 1996; Cherif et al. 2002), difficulties are present when *B. cereus* and *B. thuringiensis* have to be identified.

Table 10 Strain distribution in clusters

Cluster	Source
Cluster A	From patient: 1 <i>B. thuringiensis</i> and 1 <i>B. cereus</i> from food: 1 <i>B. cereus</i>
Cluster B	From patient: 1 <i>B. cereus</i> From food: 1 <i>B. cereus</i>
Cluster C	<i>B. thuringiensis</i> DSMZ 350 From pesticide: 1 <i>B. cereus</i> From food: 1 <i>B. thuringiensis</i>
Cluster D	<i>B. thuringiensis</i> DSMZ 6080 From pesticide: 1 <i>B. cereus</i> From food: 1 <i>B. cereus</i> From patient: 1 <i>B. cereus</i>
Cluster E	<i>B. thuringiensis</i> DSMZ 2046 From patient: 1 <i>B. thuringiensis</i> and 7 <i>B. cereus</i> from pesticide: 1 <i>B. cereus</i> From food: 1 <i>B. cereus</i>
Cluster F	From patient 4: <i>B. cereus</i> From food: 1 <i>B. cereus</i>
Cluster G	From patient: 1 <i>B. cereus</i> and 1 <i>B. thuringiensis</i>
Cluster H	<i>B. cereus</i> DSMZ 4282 From patient: 5 <i>B. cereus</i> and 1 <i>B. thuringiensis</i> from food: 1 <i>B. cereus</i>
Cluster I	From patient: 3 <i>B. cereus</i> and 1 <i>B. thuringiensis</i>
Cluster L	<i>B. cereus</i> DSMZ 4222 From patient: 16 <i>B. cereus</i> From pesticide: 2 <i>B. cereus</i>
Cluster M	From pesticide: 1 <i>B. cereus</i> From patient: 2 <i>B. cereus</i> and 2 <i>B. thuringiensis</i>
Cluster N	From food: 4 <i>B. cereus</i>
Cluster O	From patient: 2 <i>B. cereus</i>
Cluster P	From patient: 5 <i>B. cereus</i> From food: 1 <i>B. cereus</i>

Primer REP 1R-Dt of 15 bp and the primer REP 2-Dt of 22 bp were used (Gevers et al. 2001). As reported in Fig. 2, ten clusters (A to L) were obtained by the rep-PCR fingerprints analysis using 80% similarity (Fig. 4). rep-PCR was more effective in clustering *Bacillus* strains. The technique clusters strains from the same origin as reported in cluster C, D, F, I and L. In cluster E are grouped strains from pesticides and in cluster C strains from patients (Table 11) (Manzano et al. 2009). According with Abriouel et al. (2007) “the utilization of conserved repetitive sequences dispersed in the whole genome is useful in contributing to the differentiation of genomic groups and check their association with foods”.

PCR-TTGE Analyses

One molecular method useful for the evaluation of microbial diversity is the application of the Temporal Temperature Gradient gel Electrophoresis (TTGE) or the Denaturing Gradient Gel Electrophoresis (DGGE) to a PCR product (Myers et al. 1987).

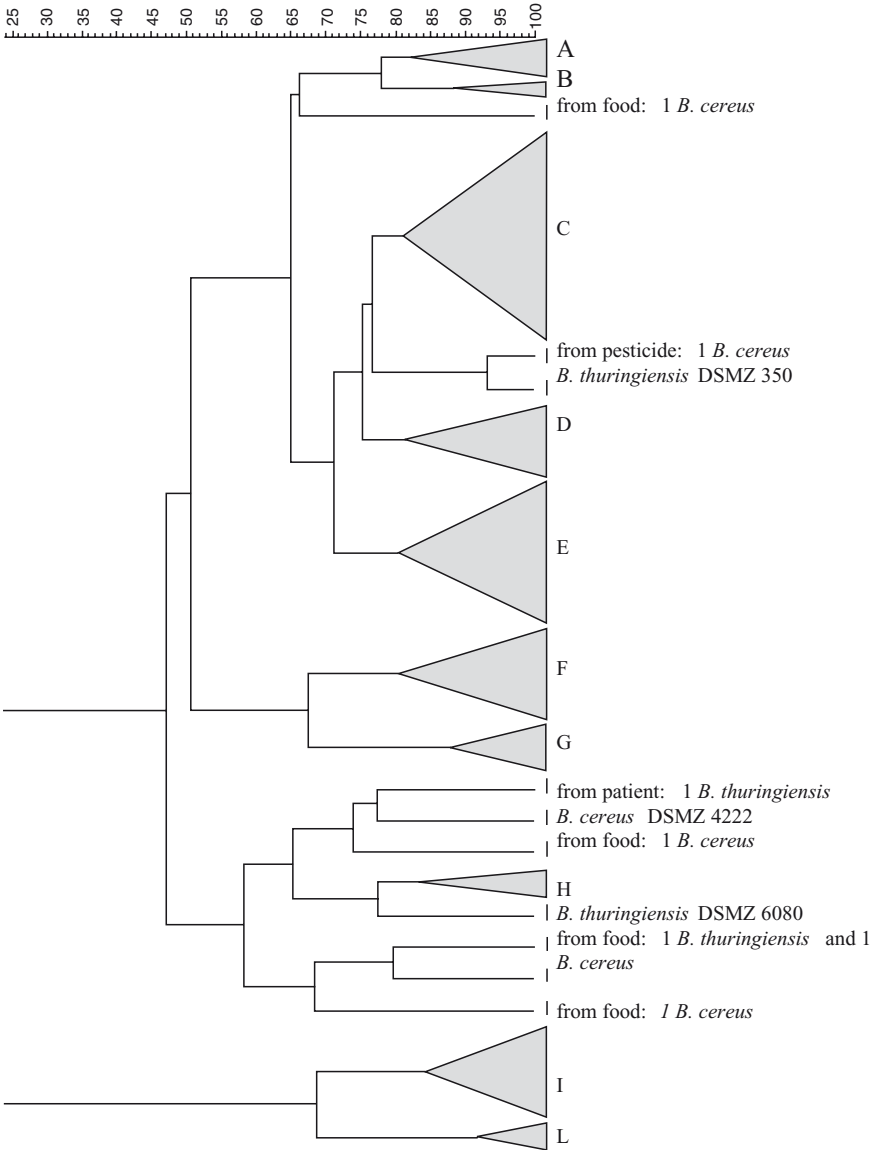


Fig. 4 rep-PCR dendrogram of *Bacillus cereus* and *B. thuringiensis* strains determined by the Unweighted Pair Group Method using Arithmetic Average (UPGMA), correlation coefficient of 80%

The amplicon could be obtained either using primers homologous to conserved regions or primers designed to anneal to a specific region. These techniques have been shown to detect differences in the melting behavior of DNA fragments from 200 to 1,000 bp differing by a single base mutation. One DNA fragment subjected

Table 11 Cluster distribution for strains analysed by rep-PCR using primer REP1RDT and primer REP2DT

Cluster	Source
Cluster A	From patient: 2 <i>B. cereus</i> and 1 <i>B. thuringiensis</i> From food: 1 <i>B. cereus</i>
Cluster B	From patient: 1 <i>B. cereus</i>
Cluster C	<i>B. cereus</i> DSMZ 2301 From patient: 16 <i>B. cereus</i> and 2 <i>B. thuringiensis</i>
Cluster D	From patient: 6 <i>B. cereus</i> and 2 <i>B. thuringiensis</i>
Cluster E	<i>B. cereus</i> DSMZ 4282 From patient: 6 <i>B. cereus</i> From food: 1 <i>B. cereus</i> From pesticide: 5 <i>B. cereus</i>
Cluster F	From patient: 6 <i>B. cereus</i> and 3 <i>B. thuringiensis</i>
Cluster G	From food: 5 <i>B. cereus</i>
Cluster H	<i>B. thuringiensis</i> DSMZ 2046 From patient: 2 <i>B. thuringiensis</i>
Cluster I	From patient: 9 <i>B. cereus</i>
Cluster L	From patient: 3 <i>B. cereus</i>

to an increasingly denaturing environment partially melts till the completely dissociation into single strands at the reaching of extreme denaturing conditions. The position in the gel where a DNA fragment subjected to denaturing gradient, melts and stops as migrating is dependent on the nucleotide sequence in the melted region. Different sequences stops at different positions (levels). DGGE/TTGE is a good method of finding polymorphisms when DNA samples amplified from different individuals are run on denaturing gradient gels. This method has been applied in many protocols for identification or differentiation of strains from food (Vasquez et al. 2001; Ercolini 2004). Primers BCFW1 with a 40 bp GC clamp added, and BCRW1 were used for amplification of 69 amplicons from *B. cereus* strains and 15 amplicons from *B. thuringiensis*. Amplicons were used in a sequence-specific separation obtained by the TTGE technique. *B. cereus* amplicons were splitted in seven levels as reported in Table 12.

B. thuringiensis were splitted in two levels: the first for seven strains from humans and one from food, and the second level for four strains from humans.

PCR products in each gel are widespread, indicating a high variability within each species considered: *B. thuringiensis* and *B. cereus*. No clear differentiation was obtained by TTGE between *B. cereus* and *B. thuringiensis*, in fact when loaded into the same gel more levels are representative of each species. Researchers can fail in the identification of *B. cereus* or *B. thuringiensis* as no clear genetic traits define a species. As reported by Vilas-Bolas et al. (2002) horizontal gene transfer between strains of these different “species” and extrachromosomal DNA transfer (Helgason et al. 2000b) make these species genetically indistinguishable making it impossible to delineate discrete clusters. The presence of subset of genes unique to either species, located at the terminus of replication can explain the genome plasticity

Table 12 Levels obtained by PCR-TTGE analyses applied to *B. cereus* strains

Levels	Number of strains from humans	Number of strains from food	Number of strains from pesticides
1	9	0	0
2	4	1	0
3	24	6	4
4	3	0	0
5	2	1	0
6	7	4	2
7	2	0	0

present in this microbial group (Sayama and Bork 2001; Rasko et al. 2005). These regions can be the result of changes due to the insertion or deletion of mobile elements. Also utilizing molecular methods it is difficult to identify a *Bacillus* strain as the genetic exchange between *Bacillus* strains, in the absence of genetic barriers, rules out this kind of classification at the species and subspecies level.

Conclusions

Bacillus cereus group, responsible for human food-borne illness, exhibits a great variety in physiologic characteristics. Growth and/or survival is possible in various environments from raw and cooked foods to packaging materials. Spore production is of importance in the capacity to survive extreme environmental conditions such as high acidity, low temperature and high temperature treatment used for food stabilization. Contaminate foods could be source of human diseases due to vegetative cell/spore ingestion or to toxin ingestion. Spore germination and toxin production can occur in the intestine. Food monitoring is important to prevent intoxication and toxico-infections. Classical microbiological methods used in *B. cereus* detection and enumeration are time consuming and fail to differentiate *B. cereus* from closely related species. Molecular methods has been developed to obtain fast detection and identification of *B. cereus* and the closely related species in food either by the analyses of isolates from food or by analyses of DNA extracted from food. Principle methods allowing strain detection and identification are PCR-based methods. Specific sets of primers can be used for strain detection and for genes encoding toxin detection. RAPD-PCR and rep-PCR fingerprintings obtained from isolates can be used to obtain differentiation among strains collections. Also PCR-TTGE technique can be used for strains differentiation. Results obtained indicate difficulties in the identification of a clear and simple method able to produce unequivocal results, due to the horizontal plasmid transfer among closely related species belonging to the *B. cereus* group. This variability in strain identification can underestimate food poisoning due to *B. thuringiensis* or other *Bacillus* species able to produce toxins. *B. cereus* can cause two kinds of food-borne disease: an emetic (vomiting)

intoxication due to the ingestion of a toxin (cereulide) preformed in the food, and a diarrhoeal infection due to the ingestion of bacterial cells which produce enterotoxin in the small intestine

The duration of emetic illness is usually 6–24 h whereas the duration of the diarrhoeal illness is usually 12–24 h. To limit food outbreaks due to *B. cereus* it is important the respect of hygiene condition in food industries, the application of treatments to kill vegetative cell or spores and the maintainement of good storage condition. *B. cereus* food poisoning is principally associated with temperature abuse during the storage of cooked foods thus adequate strategies should be undertaken to control this pathogen.

References

- Abriouel H, Omar NB, Rosario L, Cañamero MM, Ortega E, Gálvez A (2007) Differentiation and characterization by molecular techniques of *Bacillus cereus* group isolated from Poto Poto and Degùè, two traditional cereal-based fermented foods of Burkina Faso and Republic of Congo. *J Food Prot* 70:1165–1173
- Agata N, Ohta M, Yokoyama K (2002) Production of *Bacillus cereus* emetic toxin (cereulide) in various foods. *Int J Food Microbiol* 73:23–27
- Andrighetto C, Zampese L, Lombardi A (2001) RAPD-PCR Characterization of *Lactobacilli* isolated from artisanal meat plants and traditional fermented sausages of Veneto region (Italy). *Lett Appl Microbiol* 33:26–30
- Ash C, Farrow JAE, Dorsch M, Stackebrandt E, Collins MD (1991) Comparative analysis of *Bacillus anthracis*, *Bacillus cereus* and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *Int J Syst Bacteriol* 41:343–346
- Cachat E, Baker M, Read TD, Priest FG (2008) A *Bacillus thuringiensis* strain producing a polyglutamate capsule resembling that of *Bacillus anthracis*. *FEMS Microbiol Lett* 285:220–226
- Chen ML, Tsen HY (2002) Discrimination of *Bacillus cereus* and *Bacillus thuringiensis* with 16S rRNA and *gyrB* gene based PCR primers and sequencing of their annealing sites. *J Appl Microbiol* 92:912–919
- Cherif A, Borin S, Rizzi A, Ouzari H, Boudabous A, Daffonchio D (2002) Characterization of a repetitive element polymorphism-polymerase chain reaction chromosomal marker that discriminates *Bacillus anthracis* from related species. *J Appl Microbiol* 93:456–462
- Cherif A, Borin S, Rizzi A, Ouzari H, Boudabous A, Daffonchio D (2003a) *Bacillus anthracis* diverges from related clades of the *Bacillus cereus* group in 16S-23S ribosomal DNA intergenic transcribed spacers containing tRNA genes. *Appl Environ Microbiol* 69:33–40
- Cherif A, Brusetti L, Rizzi A, Boudabous A, Khyami-Horani H, Daffonchio D (2003b) Genetic relationship in the '*Bacillus cereus* group' by rep-PCR fingerprinting and sequencing of a *Bacillus anthracis*-specific rep-PCR fragment. *J Appl Microbiol* 94:1108–1113
- Clavel T, Carlin F, Lairon D, Nguyen-The C, Schmitt P (2004) Survival of *Bacillus cereus* spores and vegetative cells in acid media simulating human stomach. *J Appl Microbiol* 9:214–219
- Comi G, Maifreni M, Manzano M, Lagazio C, Coccolin L (2000) Mitochondrial DNA restriction enzyme analysis of *Saccharomyces cerevisiae* strains isolated from grapes of the wine producing area of Collio (Italy). *Int J Food Microbiol* 58:117–121
- Corich V, Mattiazzi A, Soldati E, Carraio A, Giacomini A (2005) *Sau*-PCR, a novel amplification technique for genetic fingerprinting of microorganisms. *Appl Environ Microbiol* 71:6401–6406
- Daffonchio D, Cherif A, Borin S (2000) Homoduplex and heteroduplex polymorphisms of the amplified ribosomal 16S-23S Internal Transcribed Spacers describe genetic relationships in the "*Bacillus cereus* group". *Appl Environ Microbiol* 66:5460–5468

- Daffonchio D, Raddadi N, Merabishvili M, Cherif A, Carmagnola L, Brusetti L, Rizzi A, Chanishvili N, Visca P, Sharp R, Borin S (2006) Strategy for identification of *Bacillus cereus* and *Bacillus thuringiensis* strains closely related to *Bacillus anthracis*. *Appl Environ Microbiol* 72:1295–1301
- Dagmaard PH, Granum PE, Bresciani J, Torregrossa MV, Eilenberg J, Valentino L (1997) Characterization of *Bacillus thuringiensis* isolated from infections in burn wounds. *FEMS Immunol Mol Microbiol* 18:47–53
- de Been M, Franche C, Moezelaar R, Abee T, Siezen J (2006) Comparative analysis of two-component signal transduction systems of *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis*. *Microbiology* 152:3035–3048
- de Bruijn FJ, Rademaker J, Schneider M, Rossbach U, Louws FJ (1996) rep-PCR Genomic fingerprinting of plant-associated bacteria and computer-assisted phylogenetic analyses. In: Stacey G, Mullin B, Gresshoff P (Eds) *Biology of plant interaction. Proceedings of the 8th international congress of molecular plant–microbe interactions*. APS, Knoxville, pp 497–502
- Dietrich R, Fella C, Strich S, Martlbauer E (1999) Production and characterization of monoclonal antibodies against the hemolysin BL enterotoxin complex produced by *Bacillus cereus*. *Appl Environ Microbiol* 65:4470–4474
- Domig KJ, Mayer HK, Kneifel W (2003) Methods used for the isolation, enumeration, characterization and identification of *Enterococcus* spp. 2. Pheno- and genotypic criteria. *Int J Food Microbiol* 88:165–188
- Ehling-Schulz M, Svensson B, Guinebreteire M-H, Lindback T, Andersson M, Schulz A, Fricker M, Christiansson A, Granum PE, Martlbauer E, Nguyen-The C, Salkinoja-Salonen M, Scherer S (2005) Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. *Microbiology* 151:183–197
- Ercolini D (2004) PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *J Microb Meth* 56:297–314
- Fernandez A, Collaudo J, Cunha LM, Ocio MJ, Martinez A (2002) Empirical model building based on Weibull distribution to describe the joint effect of pH and temperature on the thermal resistance of *Bacillus cereus* in vegetable substrate. *Int J Food Microbiol* 77:147–153
- Forney LJ, Zhou X, Brown CJ (2004) Molecular microbial ecology: land of the one-eyed king. *Curr Opin Microbiol* 7:210–220
- Fricker M, Messelhauser U, Busch U, Scherer S, Ehling-Schulz M (2007) Diagnostic real-time PCR assay for the detection of enteric *Bacillus cereus* strains in foods and recent food-borne outbreaks. *Appl Environ Microbiol* 73:1892–1898
- Gevers D, Huys G, Swings J (2001) Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol Lett* 205:31–36
- Ghelardi E, Salvetti S, Celandroni F, Senesi S (2000) Le tossinfezioni alimentari da *Bacillus cereus*. *Riv Ital Ig* 60:171–181
- Gilbert RJ, Kramer JM (1984) *Bacillus cereus* enterotoxins: present status. *Biochem Soc Trans* 12:198–200
- Giraffa G, Neviani E (2000) Molecular identification and characterization of food-associated *Lactobacilli*. *Int J Food Sci* 12:403–423
- Giraffa G, Neviani E (2001) DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystem. *Int J Food Microbiol* 67:19–34
- Giusto C, Iacumin L, Comi G, Buiatti S, Manzano M (2006) PCR-TTGE and RAPD-PCR Techniques to analyze *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* isolate from craft beers. *J Inst Brewing* 112:340–345
- Gonzales MJ, Brown BS, Carlton BC (1982) Transfer of *Bacillus thuringiensis* plasmids coding for δ -endotoxin among strains of *Bacillus thuringiensis* and *Bacillus cereus*. *Proc Nat Acad Sci U S A* 79:6951–6955
- Granum PE (2001) *Bacillus cereus*. In: Doyle MP (Ed) *Microbiology: Fundamentals and frontiers*, 2nd edn. ASM Press, Washington, DC, pp 373–381
- Gurtler V, Stanisich VA (1996) New approaches to typing and identification of bacteria using the 16S-23S rDNA space region. *Microbiology* 142:3–16

- Gurtler V (1999) The role of recombination and mutation in 16S-23S rDNA spacer rearrangement. *Gene* 238:241–252
- Hansen BM, Hendriksen NB (2001) Detection of enterotoxigenic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. *Appl Environ Microbiol* 67:185–189
- Hauge S (1955) Food poisoning caused by aerobic spore-forming bacilli. *J Appl Bacteriol* 18:591–595
- Health Protection Agency (2005) Conteggio di *Bacillus cereus* e di altre specie *Bacillus*. Standards Unit, Unit, Evaluations and Standards Laboratory, CDSC, F 15i1.4
- Helgason E, Caugant DA, Olsen I, Kolsto AB (2000a) Genetic structure of population of *Bacillus cereus* and *B. thuringiensis* isolates associated with peridontitis and other human infections. *J Clin Microbiol* 38:1615–1622
- Helgason E, Okstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, Hegna I, Kolsto AB (2000b) *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*- One species on the basis of genetic evidence. *Appl Environ Microbiol* 66:2627–2630
- Henderson I, Dongzheng Y, Turnbull PCB (1995) Differentiation of *Bacillus anthracis* and other *Bacillus cereus* group bacteria using IS231-derived sequences. *FEMS Microbiol Lett* 128:113–118
- Hill KK, Ticknor LO, Okinaka RT, Asay M, Blair H, Bliss KA, Laker M, Pardington PE, Richardson AP, Tonks M, Beecher DJ, Kemp JD, Kolsto AB, Lee Wong AC, Keim PJ (2004) Fluorescent amplified fragment length polymorphism analysis of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* isolates. *Appl Environ Microbiol* 70:1068–1080
- Jakson SG, Goodbrand RB, Ahmed R, Kasatiya S (1995) *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. *Lett Appl Microbiol* 21:103–105
- Jensen MA, Hubner RJ (1996) Use of homoduplex ribosomal DNA spacer amplification products and heteroduplex cross-hybridization products in the identification of *Salmonella* serovars. *Appl Environ Microbiol* 62:2741–2746
- Jensen GB, Hansen BM, Eilenberg J, Mahillon J (2003) The hidden lifestyles of *Bacillus cereus* and relatives. *Environ Microbiol* 5:631–640
- Kaneko T, Nozaki R, Aizawa K (1978) Deoxyribonucleic acid relatedness between *Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis*. *Microbiol Immunol* 22:639–641
- Kim Y-R, Czajka J, Batt A (2000) Development of a fluorogenic probe-based PCR assay for detection of *Bacillus cereus* in nonfat dry milk. *Appl Environ Microbiol* 66:1453–1459
- Kramer JM, Gilbert RJ (1989) *Bacillus cereus* and other *Bacillus* species. In: Doyle MP (ed) Foodborne bacterial pathogens. Marcel Dekker Inc, New York, pp 21–64
- La Duc MT, Satomi M, Agata N, Venkateswaran (2004) *gyrB* as a phylogenetic discriminator for members of the *Bacillus anthracis-cereus-thuringiensis* group. *J Microbiol Met* 56:383–394
- Mackay JM (2004) Real-Time PCR in the microbiology laboratory. *Clin Microbiol Infect* 19:190–212
- Mantynen V, Lindstrom K (1998) A rapid PCR-based DNA test for enterotoxigenic *Bacillus cereus*. *Appl Environ Microbiol* 64:1634–1639
- Manzano M, Cocolin L, Cantoni C, Comi G (1998) A rapid method for the identification and partial serotyping of *Listeria monocytogenes* in food by PCR and restriction enzyme analysis. *Int J Food Microbiol* 42:207–212
- Manzano M, Cocolin L, Cantoni C, Comi G (2003a) *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus mycoides* differentiation using a PCR-RE technique. *Int J Food Microbiol* 81:249–254
- Manzano M, Giusto C, Iacumin L, Cantoni C, Comi G (2003b) A molecular method to detect *Bacillus cereus* from a coffee concentrate sample used in industrial preparations. *J Appl Microbiol* 95:1361–1366
- Manzano M, Giusto C, Medrala D, Cantoni C, Comi G (2003c) Optimization of DNA extraction to detect *Bacillus cereus* from food using a PCR technique- Short Communication. *Polish J Food Nutr Sci* 12:69–71
- Manzano M, Giusto C, Iacumin L, Cantoni C, Comi G (2008) Molecular methods for *Bacillus cereus* and *Bacillus thuringiensis* from humans, pesticides and foods, differentiation. *Acta Alim* 10:1–9

- Manzano M, Giusto C, Iacumin L, Cantoni C, Comi G (2009) Molecular methods to evaluate biodiversity in *Bacillus cereus* and *Bacillus thuringiensis* strains from different origins. *Food Microbiol* 26:259–264
- Maukonen J, Matto J, Wirtanen G, Raaska L, Mattila-Sandholm T, Saarela M (2003) Methodologies for the characterization of microbes in industrial environments: a review. *J Ind Microbiol Biotechnol* 30:327–356
- Myers RM, Maniatis T, Lerman LS (1987) Detection and localization of single base pair changes by denaturing gradient gel electrophoresis. *Meth Enzymol* 155:501–527
- Pannucci J, Okinaka RT, Williams E, Sabin R, Ticknor LO, Kuske CR (2002) DNA sequence conservation between the *Bacillus anthracis* pXO2 plasmid and genomic sequence from closely related bacteria. *BMC Genomics* 3:34 (electronic version : www.biomedcentral.com/1471-2164/3/34)
- Peerbooms PG, Kuijt P, Maclaren DM (1987) Application of chromosomal restriction endonuclease digest analysis for use as typing method for *Clostridium difficile*. *J Clin Pathol* 40:771–776
- Pirttijarvi TS, Andersson MA, Salkinoja-Salonen MS (2000) Properties of *Bacillus cereus* and other bacilli contaminating biomaterial-based industrial processes. *Int J Food Microbiol* 60:231–239
- Priest FG, Kaji DA, Rosato YB, Canhos VP (1994) Characterization of *Bacillus thuringiensis* and related bacteria by ribosomal RNA gene restriction fragment length polymorphisms. *Microbiology* 140:1015–1022
- Radhika B, Padmapriya BP, Chandrashekar A, Keshava N, Varadaraj MC (2002) Detection of *Bacillus cereus* in foods by colony hybridization using PCR-generated probe and characterization of isolates for toxins by PCR. *Int J Food Microbiol* 74:131–138
- Radnedge L, Agron PG, Hill KK, Jackson PJ, Ticknor LO, Keim P, Andersen GL (2003) Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis*. *Appl Environ Microbiol* 69:2755–2764
- Rasko DA, Altherr MR, Han CS, Ravel J (2005) Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiol Rev* 29:303–329
- Rossetti L, Giraffa G (2005) Rapid identification of dairy lactic acid bacteria by M13-generated, RAPD-PCR fingerprint databases. *J Microbiol Meth* 63:135–144
- Ryan PA, Maacmillan JD, Zilinskas BA (1997) Molecular cloning and characterization of the genes encoding the L₁ and L₂ components of hemolysin BL from *Bacillus cereus*. *J Bacteriol* 179:2551–2556
- Samarzija D, Sikova S, Redzepovic S, Antunac N, Havranek J (2002) Application of RAPD analysis for identification of *Lactococcus lactis* subsp. *cremoris* strains isolated from artisanal cultures. *Microbiol Res* 157:13–17
- Sayama M, Bork P (2001) Evolution of prokaryotic gene order: genome rearrangements in closely related species. *Trends Gen* 17:10–13
- Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* 62:774–806
- Sergeev N, Distler M, Varga M, Chizhikov V, Herold KE, Rasooly A (2006) Microarray analysis of *Bacillus cereus* group virulence factors. *J Microbiol Meth* 65:488–502
- Setlow B, Setlow P (1998) Heat killing of *Bacillus subtilis* spores in water is not due to oxidative damage. *Appl Environ Microbiol* 64:4109–4112
- Sorokin A, Candelon B, Guilloux K, Galleron N, Wackerow-Kouzova N, Dusko Ehrlich S, Bourguet D, Sanchis V (2006) Multiple-locus sequence typing analysis of *Bacillus cereus* and *Bacillus thuringiensis* reveals separate clustering and a distinct population structure of psychrotrophic strains. *Appl Environ Microbiol* 72:1569–1578
- Ticknor LO, Kolsto AB, Hill KK, Keim P, Laker MT, Tonks M, Jackson PJ (2001) Fluorescent amplified fragment length polymorphism analyses of Norwegian *Bacillus cereus* and *Bacillus thuringiensis* soil isolates. *Appl Environ Microbiol* 67:4863–4873
- Tiecco G (2000) Microbiologia degli Alimenti di Origine Animale. In: Edagricole C (ed) Edizioni Agricole della Calderini. Bologna, Italia, pp 345–359

- Valero M, Hernandez-Herrero LA, Fernandez PS, Salmeron MC (2002) Characterization of *B. cereus* isolates from fresh vegetables and refrigerated minimally processed foods by biochemical and physiological tests. *Food Microbiol* 19:491–499
- van Netten P, van de Moosdijk A, van Hoensel P, Mossel DAA, Perales I (1990) Psychrotrophic strains of *Bacillus cereus* producing enterotoxins. *J Appl Bacteriol* 69:73–79
- Vasquez A, Ahnè S, Pettersson B, Molin G (2001) Temporal temperature gradient gel electrophoresis (TTGE) as a tool for identification of *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus zeae* and *Lactobacillus rhamnosus*. *Lett Appl Microbiol* 32:215–219
- Versalovic J, Schneider M, de Bruijn FJ, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). *Meth Mol Cell Biol* 5:25–40
- Vilas-Bolas G, Sanchis V, Lereclus D, Lemos MVF, Bourguet D (2002) Genetic differentiation between sympatric populations of *Bacillus cereus* and *Bacillus thuringiensis*. *Appl Environ Microbiol* 68:1414–1424
- Ward ES, Ellard DJ (1983) Assignment of the delta-endotoxin gene of *Bacillus thuringiensis* var. *israeliensis* to a specific plasmid by curing analysis. *FEBS Lett* 180:45–49
- Wayne L, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, WEC, Murray RGE, Stackebrandt E, Starr MP, Truper HG (1987) International committee on systematic bacteriology: report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* 37:463–464
- Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucl Acid Res* 18:7213–7218
- Wilcks A, Jayaswal N, Lereclus D, Andrup I (1998) Characterization of plasmid pAW63, a second self-transmissible plasmid in *Bacillus thuringiensis* subsp. *kurstaki* HD73. *Microbiol* 144:1263–1270
- Yamada S, Ohashi E, Agata N, Venkateswaran K (1999) Cloning and nucleotide sequence analysis of *gyrB* of *Bacillus cereus*, *B. thuringiensis*, *B. mycoides*, and *B. anthracis* and their application to the detection of *B. cereus* in rice. *Appl Environ Microbiol* 65:1483–149
- Yamamoto S, Harayama S (1995) PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl Environ Microbiol* 61:1104–1109

Control of *Listeria monocytogenes* in San Daniele Dry Cured Ham by Different Technologies: Reduction of *L. Monocytogenes* in Dry Cured Ham

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Abstract Dry cured ham can be contaminated by *L. monocytogenes*. It originates from raw meat, from processing plants and room. For this reason, it is sometimes impossible to eliminate it from dry cured ham. However, it is possible to prevent or minimize contamination, and to inhibit or stop its growth. The aim of this paper was to produce and validate different methods for post-processing application in San Daniele dry-ham in order to reduce the concentration of *L. monocytogenes* by 2–3 log CFU/cm², or to try and completely eliminate it (0 tolerance). An additional goal was to validate the degree of lethality. The methods used included chemical solutions (1.5% sodium lactate, 1% sodium diacetate, a mix solution of 1.5/1.0% sodium lactate/diacetate), ionized air, water and ozonized air, hydrogen peroxide solution, essential oils, and microbial protective starter (*Leuconostoc carnosum*). The samples, represented by surfaces of San Daniele dry cured ham (both meat and pig-skin parts), were inoculated with different concentrations of a mix of five *L. monocytogenes* biotypes. After the inoculum, the samples were treated with the above-mentioned technologies. To assess the effects of each treatment, the survived cells of *L. monocytogenes* were counted by EN/ISO 11290-2 method. Chemical solutions reduced the concentration of *L. monocytogenes* by 2–3 log CFU/cm², ionized air by 3 log CFU/cm², water and ozonized air respectively by 1–2 log CFU/cm² and 1–3 log CFU/cm², hydrogen peroxide solution by 2–3 log CFU/cm², essential oils by 1–3 log CFU/cm², and microbial protective starter by 3 log CFU/cm². The resulting data demonstrated that the investigated technologies allowed to eliminate from 1 to 3 log of *L. monocytogenes* from dry cured ham, both meat and pig skin. The results showed high reproducibility and repeatability, so we recommend using these methods as a post-processing application in San Daniele dry cured ham production.

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Introduction

Listeria monocytogenes has been recognized as one of the most important food-borne pathogens, because it can cause severe diseases in humans. Although the incidence of listeriosis and the mortality rate are considered low (Gianfranceschi et al. 2002; Mead et al. 1999; Salamina et al. 1996), *L. monocytogenes* has produced several major outbreaks, which have been associated with consumption of contaminated food (Farber and Peterkin 1991; McLauchlin 1997). Food-borne listeriosis is commonly regarded as an invasive disease sometimes associated with bacteremia and rarely associated with gastrointestinal symptoms (Slutsker and Schuchat 1999) in immune-depressed individuals (Cocolin et al. 2005). However, recent reports of a new, non-invasive form of listeriosis causing febrile gastroenteritis clearly indicate that people with no predisposing conditions may be affected (Franciosa et al. 2001; Aureli et al. 1998). These findings, coupled with the large diffusion of *L. monocytogenes* in food, have increased the significance of this microorganism for public health (Hong et al. 2007; Bell and Kyriakides 2003; Aureli et al. 1998; Miettinen et al. 1999; Salamina et al. 1996; Parodi et al. 1990; Bell and Kyriakides 2003). In Italy, listeriosis became a patent disease in 1991, while in 1993, a program was established to monitor the pathogen in food. Furthermore, the lack of minimum infectious dose in food led many countries to take a “zero-tolerance” approach in ready-to-eat food promoting its growth, as opposed to a tolerance threshold of a 100 units per gram in those products where it cannot grow (GUCE 2002).

L. monocytogenes can easily contaminate and colonize raw material and food processing facilities (Hong et al. 2007; Comi et al. 2005a, b). As a matter of fact, it has been found in different parts along the meat processing chain, from slaughterhouses to industrial facilities. The bacterium is difficult to eliminate in the facilities, it survives during the meat production phase, and is often detected in the final products (Shabala et al. 2008; Blackman and Frank 1996). This may have a big economic impact on the food industry because of monitoring costs and recalls if the bacterium is found in food for sale (Shabala et al. 2008).

The problem may escalate because *L. monocytogenes* can grow in meat products in the presence of high salt concentration, at refrigerated temperatures (Shabala et al. 2008; Grisenti et al. 2004; Farber and Peterkin 1991; Duggan and Philips 1998; Farber and Harwing 1996). In addition, it is ubiquitous in nature and a large number of foods could get contaminated and pose a risk. A particularly severe problem stems from food eaten without further cooking and with a long shelf-life, for example delicatessen meats, fresh sausages (Lado and Yousef 2007; Swaminathan 2001). In recent years, thanks to the extensive international debate on *L. monocytogenes* in food, this bacterium has been isolated less frequently, but the percentage of positives in some kinds of food is still high (Grisenti et al. 2004; Tompkin 2002). Fortunately, despite the massive presence of this microorganism

in food and particularly in meat products, the total number of recorded listeriosis cases is relatively small.

L. monocytogenes can be detected in dry cured ham (Wooi Fang et al. 1997; Grisenti et al. 2004). It originates from raw meat, from environmental sources, processing plants, drying and ripening room and machinery (Robbins et al. 2005; Nørrung 2000; Kathariou 2002; Comi et al. 2005a, b). During the production cycle of dry cured ham, *L. monocytogenes* cannot grow because of decreasing *Aw*, which reaches values below 0.89, because of long ripening times, up to 12 months, and because of the refrigeration temperatures during the first processing phase. So, even though it may be present in raw meat, the concentration of *L. monocytogenes* tends to decrease during all ripening phases of dry cured ham and never grows back (Grisenti et al. 2004). For this reason, dry cured ham is considered a safe product. Dry cured ham is a ready-to-eat food and can be exposed to cross recontamination (Tompkin 2002) at the end of ripening, during deboning, slicing, and packaging in Modified Atmosphere or under vacuum. To prevent contamination or recontamination, dry cured ham facilities have implemented different plans that is, HACCP (Hazard analysis critical control point), GMP (Good Manufacturing Practice), SOP (Standard Operation Procedure), and SSOP (Standard Sanification Organization Program). These plans have decreased the presence of *L. monocytogenes* both in the production rooms and in dry cured ham (Comi et al. 2005a, b), thus reducing the percentage of contaminated dry cured ham to less than 0.3%. So the risk of listeriosis is very low and so far, cured ham has never been involved in listeriosis. However, the dry ham contamination with *L. monocytogenes* causes sales problems in Italy/Europe, and in the US. As a matter of fact, European regulations (GUCE 2002) require absence of *L. monocytogenes* in 25 g of food with a tolerance of less than 100 CFU/g at the expiry date in products such as dry cured ham, whereas US regulations require absence in 25 g (0 tolerance). This is because in the USA *L. monocytogenes* is considered a big and real problem and every year many cases of listeriosis occur due to contaminated food. More specifically, *L. monocytogenes* in cooked or ready-to-eat products is responsible for 40% of listeriosis cases in the USA (Bell and Kyriakides 2003; Tappero et al. 1995). The absence of *L. monocytogenes* in food was introduced as a requirement in the new FSIS rules passed on 6 June 2003 and made applicable as at 6 October 2003 (FDA/FSIS 2003a). The rule does not apply to sampling limits and systems, while it deals with preventive strategies implemented in the facilities. In other words, producers are obliged to introduce both measures aimed at inhibiting growth and inactivation treatments – known as “post-lethality treatments” – which must be applied to the finished product possibly after packaging (Gola et al. 2003). In addition, the USA/FSIS document on “Listeria risk assessment” (FDA/FSIS 2003b) recommends and requires that different combination methods be used in order to control *L. monocytogenes* in ready-to-eat food products, which are exposed to recontamination after the inactivation treatment. Pursuant to the FSIS rules, producers of ready-to-eat food must validate their production process and specifically the anti-listeria treatments to periodically monitor their effectiveness and productivity. Also dry cured ham facilities must implement anti-listeria treatments. The most widely used plan to achieve this purpose is the

“challenge test”, which must be applied according to USDA/FSIS (1995, <http://www.foodsafety.gov/~dms/lmr2-1.html>). Data of the USDA/FSIS document on “*Listeria Risk Assessment*” show a synergic effect achieved by applying a combination of different methods to control microorganisms in food. For dry cured ham, a combination of $a_w < 0.92$ and a storage temperature below 2–4°C is sufficient to prevent growth of *L. monocytogenes* (FDA/FSIS 2003b), even though these methods do not allow to reach the required zero-tolerance level. So, until now it seems impossible to completely eliminate microorganisms from dry cured ham, but it is possible to obtain a very low contamination level without any risk for consumers.

Different methods are applied as post-lethality treatments to prevent, eliminate, or reduce the presence of *L. monocytogenes* or other pathogens in ready-to-eat food. They are used as stand-alone methods or in combination and include pasteurization or heat treatments (Porto-Fett et al. 2008), mix of organic or inorganic salts, organic acids (Seman et al. 2008; Shabala et al. 2008; Stopforth et al. 2008; Geornaras et al. 2006; Comi et al. 2005a), mix of essential oils (O’Byrne et al. 2008; Turgis et al. 2008), a combination peroxyacetic acid/nisine and protective starter (Minei et al. 2008), hydrostatic pressure (Carlson et al. 2008; Gola et al. 2003); ozone (Emer et al. 2008; Comi et al. 2005a, b, 2006; Kim et al. 1999; Khadre and Yousef 2001; Sheldon and Brown 1986), active antimicrobial food packaging (Quintavalla and Vicini 2002), a combination of MAP or vacuum-packaged and storage temperature (Grisenti et al. 2004; Wooi Fang et al. 1997). However, these methods can prevent growth or reduce the presence of *L. monocytogenes* and other pathogens in food but, with the exception of heat treatments, only rarely do they manage to achieve a zero-tolerance level.

Considering that the zero tolerance requirement brought about many problems in terms of export of San Daniele dry cured ham to the USA because of a remote chance that it may be affected by a very low contamination with *L. monocytogenes* (<10 CFU/g) as it is impossible to completely eliminate it during production or in the finished product, the aim of the work was to produce and validate different methods to eliminate or reduce *L. monocytogenes* from San Daniele Dry cured ham. The methods used included chemical solutions (1.5% sodium lactate, 1% sodium diacetate, a 1.5%/1.0% sodium lactate/diacetate mixed solution), ionized air, water and ozonised air, hydrogen peroxide solution, essential oils and microbial protective starter (*Leuconostoc carnosum*).

Material and Methods

Samples

Different slices of San Daniele dry cured ham (DCH) (A_w 0.91) and slices of pig skin (PS) were obtained from a facility in San Daniele, Italy (Fig. 1). Each sample was inoculated with a suspension of *L. monocytogenes* and exposed to different treatments.

A_w was evaluated by ISO-FDIA 21807 (2004)

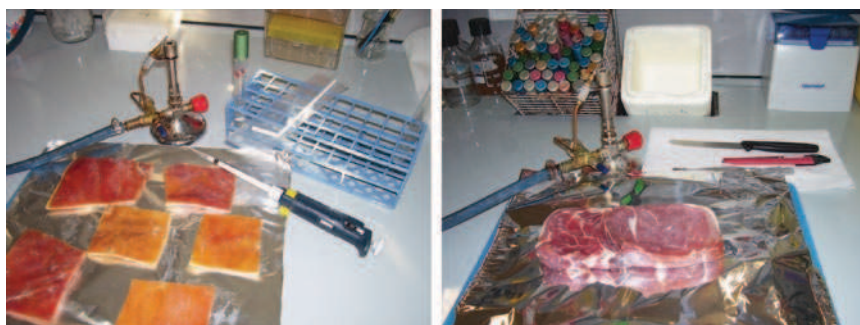


Fig. 1 Slices of dry cured ham and pig skin

Preparation of Inocula

Five strains of *L. monocytogenes*, including *Listeria monocytogenes* Scott A, *Listeria monocytogenes* NCTC 7979 (serotype 1/2a), *Listeria monocytogenes* NCTC 10887 (serotype 1/2b), *Listeria monocytogenes* NCTC 10527 (serotype 4b), *Listeria monocytogenes* DSA from meat products, were used. Each strain was grown in Brain Heart Infusion Broth (Oxoid, Italy). After a growing period of 18 h at 37°C, the broth cultures were mixed and the mix was centrifuged at 4,000 rpm at 4°C for 15 min. The sediment was re-suspended in 100 ml of peptoned water (NaCl 7 g, peptone 1 g and distilled water 1 l). The concentration of the suspension was about 10^8 – 10^9 CFU/ml. After carrying out proper dilutions, 100 μ l of the diluted mixture was inoculated by plating in each sample (40 cmq each).

Analysis Method

In order to valuate the effects of the treatments, each inoculated sample was diluted in peptoned water and 0.1 of the dilution was plated in Palcam agar according to the ISO 11290-2 method. The enrichment method (ISO 11290-1) was used in order to assess the presence of *L. monocytogenes* in a 25 g sample.

Statistical Analysis

Statistical calculations were carried out using Stata 8 (Stata Corp LP 2005) as a program to analyze data. To verify the homogeneity of variance, the Barlett test was applied, then One-way analysis of Variance (ANOVA) was performed, and significant differences ($P < 0.05$) among samples were determined with Tukey's honestly significant difference range.

Treatments

Chemical Solution

The samples were inoculated and treated as follows:

- a) Slices of dry cured ham (DCH) were inoculated, treated as described below, and packaged in Modified atmosphere (MAP, 15% CO₂/85% N₂, O₂ 0.05%).
 1. Fifteen samples were packaged without any treatments.
 2. Fifteen samples were treated with 5 ml of a sodium lactate solution (1.5% p/v in water).
 3. Fifteen samples were treated with 5 ml of a sodium diacetate solution (1% p/v in water).
 4. Fifteen samples were treated with a mixed solution of sodium lactate/sodium diacetate (1.5%/1% in water).
- b) Slices of dry cured ham (DCH) were inoculated, treated as described below and packaged under vacuum
 1. Fifteen samples were packaged without any treatments (control).
 2. Fifteen samples were treated with 5 ml of a sodium lactate solution (1.5% in water).
 3. Fifteen samples were treated with 5 ml of a sodium diacetate solution (1% in water).
 4. Fifteen samples were treated with a mixed solution of sodium lactate/sodium diacetate (1.5%/1% in water).

No pig skins (PS) were used in order to assess the activity of the chemical solutions.

The chemical solutions were plated and distributed on the inoculated slices, the packaging was PET12/PE/EVOH/PE a.f. for the upper film and PET R 200/PE/EVOH/PE/PEEL 50 for lower film, the packaging device was Foodpack Basic V/G (I.L.P.R.A.).

All treated and control samples were stored at 4°C and analyzed at 0, 15, 30, 45, 60 days to assess the behavior of *L. monocytogenes*.

Ionized Air

Six slices of dry cured ham (DCH) and six slices of pig skin (PS) were inoculated. Three samples of each (DHC and PS) were put in a box, without any treatment (control samples) and three samples were put in a second box and treated for 24 h with an ionizer (Bioxigen “TRIS” model, Sital Klima, TV, Italy, Fig. 2). Both treated and non-treated samples were analyzed in order to assess the behavior of *L. monocytogenes*. The level of initial inoculum in the samples was assessed by analyzing the slices of DCH and PS (three slices each) after adding the suspension of *L. monocytogenes*.

Ozonated Water

27 DCH and 27 PS were inoculated and used for the test. 9 DCH and 9 PS were inoculated and not treated (control samples), 9 DCH and 9 PS, after inoculum, were washed with sterile water, while 9 DCH and 9 PS were washed with ozonated water obtained with an ozone generator (Del Ozone, San Luis Obispo, Ca, USA). The concentration of aqueous ozone (0.2 ppm) was measured using the indigo trisulfonate method by Bader and Hoigne (1981). The control and treated samples were analyzed in order to assess the behavior of inoculated *L. monocytogenes*.

Ozonated Air

18 DCH and 18 PS were inoculated and used for the test. 9 DCH and 9 PS were inoculated (control samples), kept in a box for 24 h without any treatment, 9 DCH and 9 PS, after inoculum, were kept for 24 h in a box (Fig. 2) and treated with ozonated air obtained with an ozone generator (Del Ozone, San Luis Obispo, Ca, USA).

Hydrogen Peroxide

40 DCH and 40 PS samples were inoculated. 10 ml of water (control samples) or 10 ml of three different solutions of hydrogen peroxide (20%, 10%, and 3%) were added to the samples (ten each). The samples were split in two groups. One group was kept in air, the second group was packaged under vacuum in PET. After 10 min



Fig. 2 Box and ionizer system

both the control samples and the treated samples were analyzed. The color of both treated samples of DCH and PS was assessed by scanner because the treatment with hydrogen peroxide produced a color change (whitening). The photos of the treated samples were compared with the control by C.I.E. (Commission International de l'Eclairage) "Lab" system (Image-Pro-Plus; Media Cybernetics, 4.1), based on the theory of opposite colors by García-Esteban et al. (2002). The peroxide activity on fats of the hydrogen peroxide treatments was assessed by measuring the number of peroxides according to the method described in Attachment III to the Official Journal of European Communities (5 September, 1991).

Essential Oils

A mix of essential oils and natural vegetable extract (Pat. Pend.) was tested in inoculated DCH and PS. Essential oils included solution A (Pat. Pend.), solution B (Pat. Pend.), and solution C (A: 25% v/v + B: 25% v/v + sterile water: 50% v/v). Solutions A and B were used "in toto" as prepared by the facility. All solutions (A, B, C) were plated on the inoculated samples. After the treatments, the samples were analyzed after 0, 3, and 6 h (3 replicates of each). Control samples without any treatments were also prepared.

Protective Starter

15 DCH and 15 PS samples were inoculated with a mixed suspension of *L. monocytogenes* at a final concentration of 1,000 CFU/cm². After 1 h, a suspension of *Leuconostoc carnosum* (Christian Hansen, Italy) was plated on the same inoculated samples. The final concentration was 100,000 CFU/g. All samples were packaged under vacuum in PET, split into three groups, and stored at 4°C. The first group of each was analyzed at 0 days, the second at 15 days, and the third at 30 days in order to assess the behavior of *L. monocytogenes*.

Results and Discussion

Chemical Solution

The results concerning the behavior of *L. monocytogenes* in sliced cured ham packaged in MAP and under vacuum are reported in Figs. 3 and 4. Samples were inoculated with *L. monocytogenes* concentrations of 4.38 log CFU/g, which are much higher than the concentrations that could usually be found in reality after manipulation-induced re-contamination. As a matter of fact, the level of re-contamination due to manipulation usually amounts to approximately 1.5 log CFU/g (Grisenti et al. 2004). Experimental data show that the concentration of inoculated *L. monocytogenes* gradually and remarkably decreases over time. An observation of the relevant

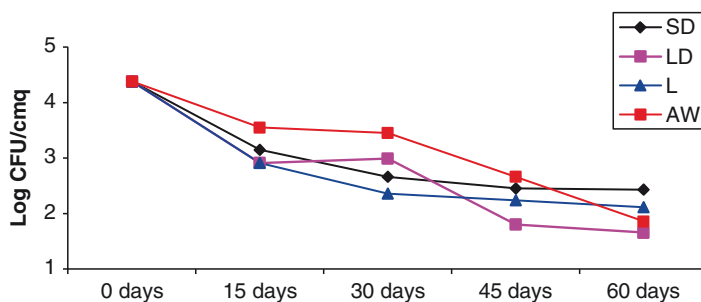


Fig. 3 Behaviour of *Listeria monocytogenes* in dry cured ham packaged in MAP at 4°C. Legend: SD – sodium diacetate; LD – sodium diacetate/sodium lactate; L: sodium lactate; Aw: control sample

diagrams shows that in all samples, regardless of the applied treatment, of the sample and of the packaging type, the load of *L. monocytogenes* decreases by an average of 1 or 2 log units. In MPA samples at the end of the storage period (60 days), the concentration of *L. monocytogenes* decreased by over 2 log units in the control samples (Aw) and upon treatment with LD, whereas it dropped by approximately 2 log units upon SD and L treatments (Fig. 3). In samples that were packaged under vacuum, the concentration of *L. monocytogenes* at the end of the storage period (60 days) dropped by over 2 log units in the controls (Aw) and after SD treatment, while it decreased by approximately 2 log units after LD and L treatments (Fig. 4). However, all anti-microbial agents used and the control showed an average decrease of over 1 or 1.5 log units already after 45 days. Therefore, the data show that also the control samples, which were never exposed to any treatment based on anti-microbial solutions, not only inhibit the growth of *L. monocytogenes* but they even promote a reduction. This proves that an Aw of 0.88–0.92 in dried cured ham, by itself, can not only inhibit the growth but also inactivate the cells of *L. monocytogenes*. As a matter of fact, the literature shows that, based on experimental data, *L. monocytogenes* can only develop in food when Aw in the food itself is higher than 0.92 (A.A.V.V. 1996). Therefore, *L. monocytogenes* will never develop in deboned or sliced dried cured ham since the Aw value of such products falls within the 0.88–0.92 range. Fang et al. (1997) demonstrated that *L. monocytogenes* in cold meat cuts drops from 4.6 log to 1.5 log CFU/g and therefore does not proliferate as one would sensibly expect considering that the Aw value of slices amounts to 0.90–0.91. Grisenti et al. (2004) largely demonstrated that no increases in *L. monocytogenes* can be observed in MAP sliced or cut under-vacuum samples after 60 days of storage at 3°C, 8°C, and 20°C. They also showed that the concentration of this micro-organism gradually decreased over time, especially in those samples where Aw was equal to 0.90. The statistical analysis (data not shown) carried out on under-vacuum samples showed no significant difference between the treatments (SD, L, LD) and the control (Aw), regardless of storage time. Conversely, significant differences were found between the concentration of *L. monocytogenes* in treated ham samples and MAP packaged control samples.

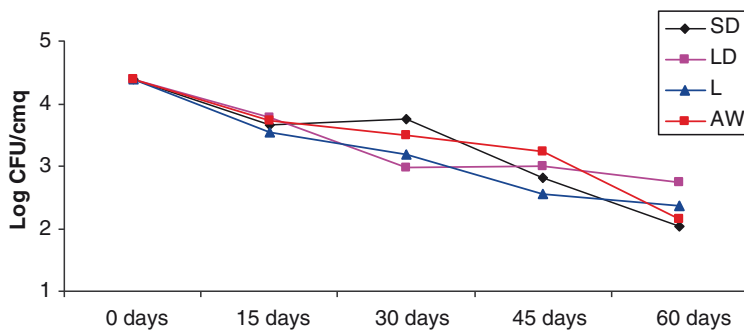


Fig. 4 Behaviour of *Listeria monocytogenes* in dry cured ham packaged under vacuum at 4°C. Legend: SD – sodium diacetate; LD – sodium diacetate/sodium lactate; L: sodium lactate; Aw: control sample

After 30 and 45 days, the decreases observed in the concentration of *L. monocytogenes* differed significantly depending on the treatment. After 30 days, the concentration of surviving *L. monocytogenes* was lower in samples that were treated with sodium lactate. Such concentration showed a significant difference ($p < 0.05$) compared to the concentration observed in untreated samples or samples treated with a sodium lactate/diacetate mixture, whereas no statistically significant difference was observed with samples treated with sodium diacetate only.

After 45 days, the lowest concentration of *L. monocytogenes* was observed in samples treated with a sodium lactate/diacetate mixture. However, it did not differ significantly from the concentration observed in samples treated with sodium diacetate or sodium lactate, whereas it was significantly different from the concentration observed in untreated samples (Aw only). A higher number of surviving *L. monocytogenes* was found in these untreated samples. Finally, after 60 days, no significant difference could be observed in terms of *L. monocytogenes* behavior between the different treatments and the control. This proves the effectiveness over time of the treatment combination, Aw, and modified atmosphere. Indeed, in this case, the maximum drop in concentration of *L. monocytogenes* was observed in samples treated with a sodium lactate/diacetate mix, while the smallest decrease was observed in samples that were only treated with sodium diacetate. However, such average decreases were not significantly different from one another because of a high standard deviation.

The acid or salt preservatives, including sodium lactate and sodium diacetate, have been considered formula-based methods for controlling *L. monocytogenes* (Seman et al. 2008). More specifically, they are used as anti-microbial agents in ready-to-eat meats, to prolong their shelf-life, or to inhibit pathogen growth, even though some molecules have not been approved. Glass et al. (2007) screened a large number of organic acids to prevent and inhibit *L. monocytogenes* in turkey and pork-beef bologna. In addition, Geornaras et al. (2006) investigated the activity of chemical solutions, including nisin, acetic acid, lactic acid, potassium lactate, and

sodium diacetate, as anti-listeria post-processing treatments in smoked sausages. Their data agree with ours. As a matter of fact, those authors believe that apparently the anti-listeria activity of chemical solutions was greatly enhanced when applied to products formulated with anti-microbial agents, and specifically the growth of *L. monocytogenes* was completely inhibited on sausages treated with acetic or lactic acid alone, and in sequence with nisin.

However, the variability in growth or inhibition levels of *L. monocytogenes* in food depends on many parameters, including the type of chemicals and the sequence of the lethal process. The inactivation rates, from fastest to slowest, result from lethal pH then by lethal water activity, or lethal water activity and then lethal pH, lethal pH and lethal water activity applied simultaneously, lethal pH alone, or water activity alone (Shabala et al. 2008). For this reason, in order to be sure of preventing *L. monocytogenes* growth, it is crucial to validate the process and apply a severe control on the operation procedure and on the anti-microbial concentration. Reynolds et al. (2001) reduced the concentration of *L. monocytogenes*, which was intentionally added, by a severe validation and control of the dry cured ham process. The aim of these controls is to limit the adaptative capacity of *L. monocytogenes* to lethal stress and to survive in hostile environments. As shown by our data, a control of dehydration and ripening during dry cured ham production lead to inhibition of *L. monocytogenes* as does the use of preservatives. Indeed, to increase the anti-listeria effect a modified atmosphere and packaging process should also be carefully selected (Grisenti et al. 2004).

Ionized Air

The results relating to the behavior *L. monocytogenes* on sliced dry cured ham and on pig skin treated with ionized air are shown in Figs. 5 and 6. The treatments produced a decrease of up to 2 log CFU/g both in sliced dry cured ham and in pig skin. A minor decrease was observed in untreated (control) samples. The number of *L. monocytogenes* found in treated DCH and in PS was two logs lower than in the control samples. Significant differences ($P < 0.05$) were observed between the treated and untreated samples, both in DCH and PS.

Odour and color of both treated samples were not changed by the ionized treatment. Only a minor dehydration on the surface of both treated samples was noticed. Indeed, no oxidative products were released by the ionized treatments. The number of peroxides in the treated DCH slices must be considered low, being 7.4–7.9 meq O_2 /Kg. This value was less than or similar to the untreated samples, that is 7.7–10.3 meq O_2 /Kg. The differences might stem from the applied method and from the difficulties in extracting fat from dry cured ham slices. The peroxide products were not assessed in treated pig skin samples because their oxidation does not lead to a loss in the product quality.

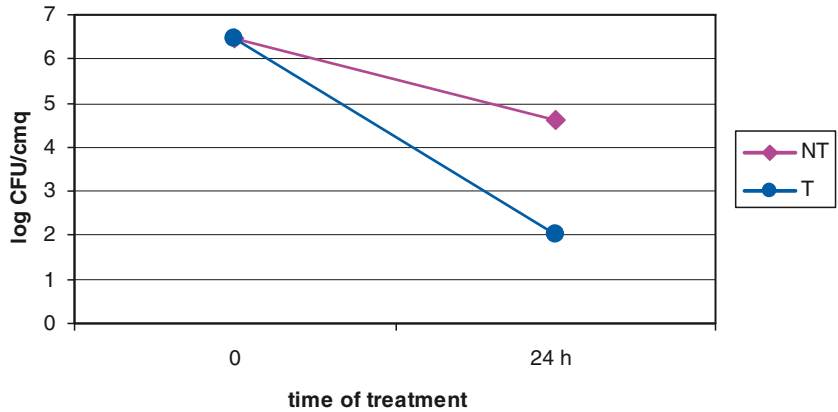


Fig. 5 Behaviour of *L. monocytogenes* in pig skin treated with ionized air. Legend: NT: not treated; T: treated

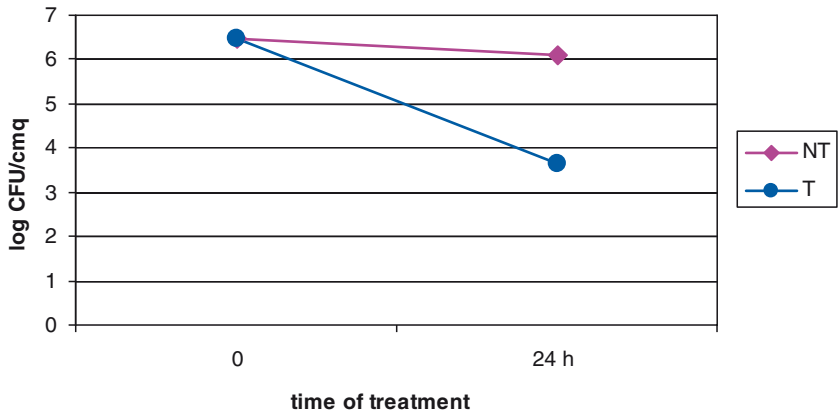


Fig. 6 Behaviour of *L. monocytogenes* in dry cured ham treated with ionized air. Legend: NT: not treated; T: treated

The pig skin was cut off before slicing, selling, or eating the dry cured ham. According to the data, ionized air could be used as a viable method because it is safe. Ionised air does not leave behind any residual compound in the sample. It is effective as an anti-microbial method as demonstrated by Comi et al. (2005b, c, 2006) and Gabbay (1990).

Ozonated Water and Air

Data about treatments with ozonized water are shown in Tables 1 and 2. Ozonated water produced a decrease in the number of inoculated *Listeria monocytogenes*,

Table 1 Behaviour of *L. monocytogenes* in dry cured ham treated with ozonated water

Samples	Average	Standard deviation
Not treated	6.5 ^a	0.6
Treated with water	5.4 ^a	0.5
Treated with ozonated water	4.5 ^a	0.3

^a Mean values with different letters are statistically different ($P \leq 0.001$)

Table 2 Behaviour of *L. monocytogenes* in pig skin treated with ozonated water

Samples	Average	Standard deviation
Not treated	5.4	0.3
Treated with water	4.2 ^a	0.4
Treated with ozonated water	4.3 ^a	0.3

^a Mean values with different letters are statistically different ($P \leq 0.001$)

but the same decrease was observed in the control, where the samples were treated with water without added ozone. The samples obtained with water without added ozone showed a lower concentration of *L. monocytogenes* than the untreated samples. Indeed, viable *L. monocytogenes* was found in ozonized water obtained from the treated samples. Significant differences ($P < 0.001$) were observed when comparing the treated (with or without added ozone) and the untreated samples. Conversely, there are no significant differences between the samples treated with and without added ozone. Therefore, the decrease might be due to the mechanical action (washing) of water on the samples. The lack activity of the ozonized water might be due to the water temperature (15°C) and to a low ozone concentration in water (0.2 ppm). The ozone generator that we used did not allow to cool water down to 4°C or to increase the ozone concentration. The bactericidal activity of ozonized water could be improved if water at 4°C was added (Kaothien et al. 2001) and if its concentration were equal to or higher than 20 ppm (Wade et al. 2003).

Indeed, the matrix (DCH or PS) played a role in the ineffectiveness of the treatment. The presence of organic compounds in the matrix may degrade the ozone and consequently its oxidative activity (Moore et al. 2000). For this reason, Beltràn et al. (2005) suggested to maintain a constant level of ozone during a treatment, because it could be inactivated by organic compounds.

The use of ozonated air did not produce any decrease in *L. monocytogenes* concentration in DCH or the PS samples (data not shown). No significant differences were observed when comparing the untreated to the treated samples. In addition, the treatment produced high fat oxidation. Fat showed a yellow colour after 5 h from the start of the treatment.

Ozone is a strong anti-microbial agent with several potential applications in the food industry. High reactivity and spontaneous decomposition to a non-toxic product (i.e. Oxygen) make it a viable disinfectant, ensuring microbial safety of food products (Fisher et al. 2000; Kim et al. 1999; Restaino et al. 1995; Horvath et al. 1985). It is recognized as safe (GRAS). It can be used as a gas or mixed in water. It was successfully used to inactivate mycotoxins, pesticide residues,

and contaminating microflora on meat, poultry, eggs, fruits, vegetables, and dry foods (Kim et al. 1999; Sheldon and Brown 1986). The results of its use were often different and depended on the treatment, on the ozone concentration, and on the matrix. Despite the response variability, it is recommended as an anti-microbial agent. Our data demonstrated that ozone mixed in water produced a low anti-listeria effect, while no activity was identified when it was used as a gas. It would probably be necessary to conduct further investigations.

Hydrogen Peroxide

The results relating to the behaviour of *L. monocytogenes* on sliced dry cured ham and on pig skin treated with different concentrations of hydrogen peroxide are reported in Tables 3 through 6. As shown, the treatment with different hydrogen peroxides succeeded in reducing the concentration of *L. monocytogenes*. Table 3 shows data relating to the treatment of DCH samples kept in air. A decrease of about 4 log CFU/g was observed using a 20% hydrogen peroxide. A minor reduction was observed using a 10% and a 3% solution of hydrogen peroxide. Conversely, washing the PS with a 20% hydrogen peroxide solution achieved a decrease of 2.4 log CFU/g, while a 10% solution produced a decrease of 2 log CFU/g (Table 4). The decrease observed when using a 3% hydrogen peroxide solution was higher than the levels achieved with a 20% or 10% solution. Although no explanation could be found for such data, the matrix might have had a role in such anomaly.

Tables 5 and 6 show data about the behaviour of *L. monocytogenes* in PS treated with different concentrations of hydrogen peroxide and packaged after treatment under vacuum. Vacuum was thought to influence the activity of hydrogen peroxide because, by eliminating air, vacuum might improve the activity of hydrogen peroxide. This ipothesis was wrong. As shown, the data obtained by packaging the treated DCH samples under vacuum were similar to those obtained in samples kept in air. No significant differences were observed ($p > 0.05$) between the data of Tables 3 and 4 and those of Tables 5 and 6. Furthermore, it seems that the best results were obtained using a 20% hydrogen peroxide solution, even though the best data were obtained when using a 10% (Table 4) and a

Table 3 Behaviour of *L. monocytogenes* in dry cured ham treated with different concentrations of H₂O₂ and kept in air

% H ₂ O ₂ solution (v/v)	Average log CFU/cm ^q	Standard Deviation
20%	2.0	0.0
10%	4.5	0.4
3%	5.3	0.2
0%	6.3	0.2

Table 4 Behaviour of *Listeria monocytogenes* in pig skin treated with different concentrations of H_2O_2 and kept in air

% H_2O_2 solution (v/v)	Average log CFU/cm ²	Standard Deviation
20%	3.8	0.0
10%	2.6	0.6
3%	3.6	0.2
0%	5.4	0.5

Table 5 Behaviour of *L. monocytogenes* in dry cured ham treated with different concentrations of H_2O_2 and kept under vacuum

% H_2O_2 solution (v/v)	Average log CFU/cm ²	Standard Deviation
20%	2.0	0.0
10%	4.4	0.2
3%	4.4	0.6
0%	6.2	0.2

Table 6 Behaviour of *L. monocytogenes* in pig skin treated with different concentrations of H_2O_2 and kept under vacuum

% H_2O_2 solution (v/v)	Average log CFU/cm ²	Standard Deviation
20%	3.0	0.0
10%	3.5	0.4
3%	2.6	0.5
0%	5.4	0.3

3% (Table 5) hydrogen peroxide solution respectively in PS kept in air and packaged under vacuum.

All hydrogen peroxide solutions produced a color change (whitening) both in DCH and in PS (Figs. 7a through c and 8a through c). Comparing the colour of the treated and not treated samples (7a through b and 8a through b) it can be noted whitening produced by the hydrogen peroxide treatment. Such change was due to the oxidative activity of the compound. However, after 7 days of storage at 4°C in PET packaging and under vacuum, the colour of the treated samples was similar to that of the untreated samples. The CIE system (L^* , a^* , b^*) confirmed the results of the sensorial analysis. So, DCH and PS reverted to the typical colour. The colour of dry cured ham is important for its quality, and any change might decrease its value and sensorial quality (Garcia-Esteban et al. 2004). Indeed, color is a fundamental quality parameter for fresh and ripened meat, especially for buyers (Vorst et al. 2004). Therefore, even the slightest color change perceived by human eye is unacceptable.

Since the treatment with hydrogen peroxide may produce fat oxidation, the peroxide number was measured. Data showed that the number of peroxides in treated samples was low, namely 8.4 meq O_2 /Kg, with no difference compared to untreated samples, which showed a level of 8.9 meq O_2 /Kg). Therefore, the hydrogen peroxide treatment did not produce any fat oxidation. In conclusion, we

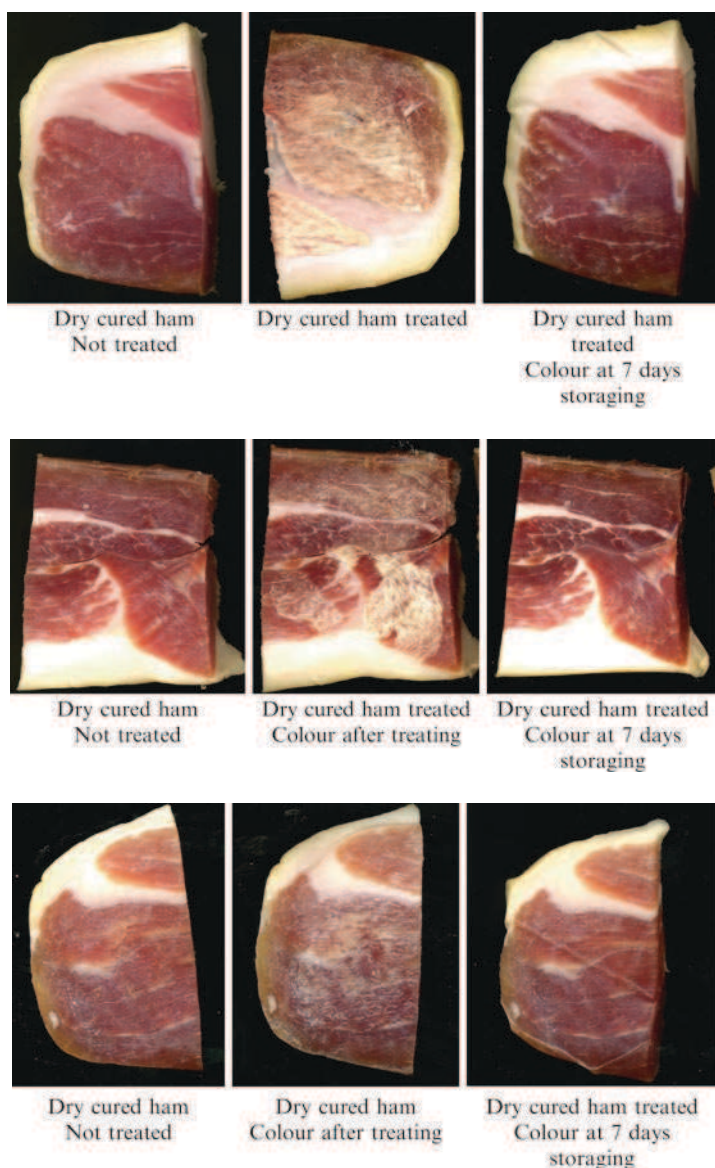


Fig. 7 (a) Dry cured ham treated with H_2O_2 (20% v/v) (b) Dry cured ham treated with H_2O_2 (10% v/v) (c) Dry cured ham treated with H_2O_2 (3% v/v)

suggest using hydrogen peroxide to eliminate *L. monocytogenes* from dry cured ham because it is safe and does not produce any sensorial changes. Indeed, the use of hydrogen peroxide to eliminate microorganisms from the environment is well known (Robbins et al. 2005) and JECFA in 2004 (<http://www.jecfa.ilsa.org>)

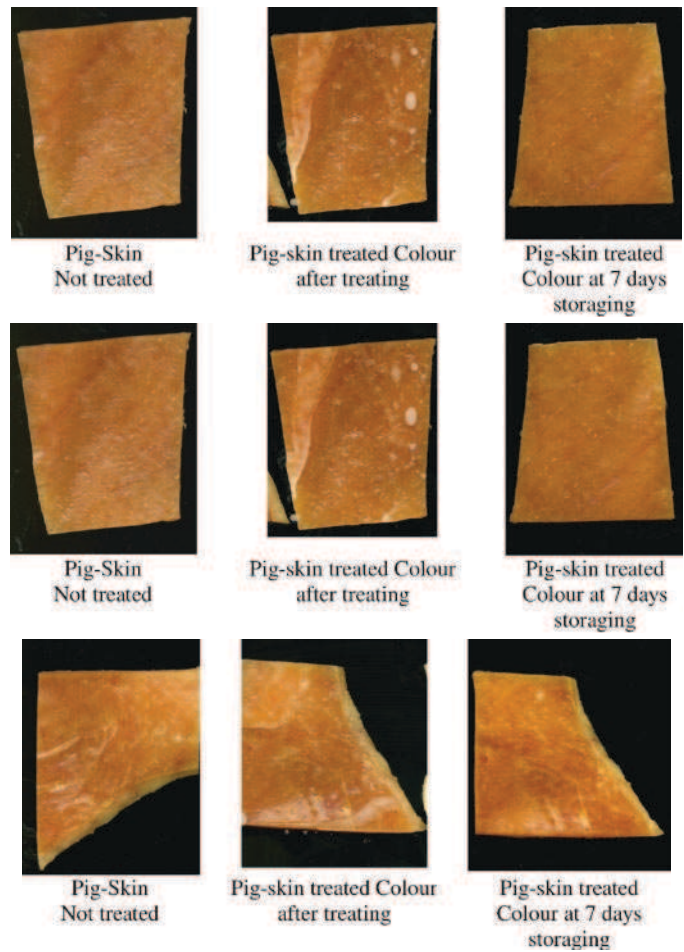


Fig. 8 (a) Pig-skin treated with H₂O₂ (20% v/v). (b) Pig-skin treated with H₂O₂ (10% v/v). (c) Pig-skin treated with H₂O₂ (3% v/v)

stated that hydrogen peroxide is safe for human beings. According to our data, a 10% hydrogen peroxide solution could be considered sufficient to eliminate *L. monocytogenes* from dry cured ham. A lower concentration is not effective and may produce resistance, as demonstrated by Lou and Yousef (1996). However, in order to eliminate contaminating microorganism spores or cells on foods, hydrogen peroxide must be used, which is a commonly used sanitizer and it is an alternative to preservatives and ozone. According to our studies, the use of hydrogen peroxide has produced better results than ozonized water and air. So, it is a real and effective alternative to anti-microbial agents and ozone, as it leaves behind no residues in food.

Essential Oils

Essential oils were used as such or diluted at 50% in sterile water. Additional solutions of both essential oils (A, B) were also prepared (20%, 10%, 3%), but they were not included in this paper because they were ineffective at decreasing the level *L. monocytogenes* both in DCH and in PS. On the other hand, it was necessary to use a mix of whole or 50% diluted oil in order to achieve a tiny reduction.

The results are shown in Table 7. The level of *L. monocytogenes* in the inoculated samples decreased during storage. A greater decrease was observed after 6 h from the beginning of the treatments. After 3 h, the treatment with solution A produced a reduction of 2.6 log CFU/g in PS and 2.8 log CFU/g in DCH compared to the untreated samples. With solution B, after 3 h the reduction was 1.8 log CFU/g in PS samples and 0.7 log CFU/g in DCH compared to the untreated samples. Using both the solutions diluted at 50% in water did not produce any decrease in *L. monocitogenes* inoculated in both samples. After 6 h, the treatment with solution A produced a reduction of 3.0 log CFU/g in PS and 2.7 log CFU/g in DCH compared to the untreated samples. With solution B, after 6 h the reduction was 2.9 log CFU/g in PS samples and 1.6 log CFU/g in DCH compared to the untreated samples. In contrast with the data at 3 h, at 6 h both mixes diluted at 50% in water produced a decrease of 3.0 log CFU/g in PS samples and of 1.2 log CFU/g in DCH samples (Table 4).

Such decrease depends on the mix of the essential oil concentration and on the duration of contact between the mix solution and the samples, even though there is also a matrix effect. Indeed, the drop was higher on the pig skin compared to dry cured ham. The presence of a higher concentration of protein compounds in dry cured ham compared to pig skin might have weakened the activity of essential oils. Once again, our data confirm the potential activity of essential oils against microorganisms and *L. monocytogenes* in food (Graumann and Holley 2008;

Table 7 Behaviour of *Listeria monocytogenes* in DCH and PS treated with different mixes of essential oil

Samples	0		3 h		6 h	
	X	SD	X	SD	X	SD
Control DCH	6.0	0.2	5.8	0.4	5.8	0.4
DCH – A	6.1	0.2	3.1	0.6	3.1	0.4
DCH – B	5.9	0.2	5.1	0.6	4.3	0.5
DCH – C	6.0	0.2	5.8	0.2	4.7	0.3
Control PS	5.9	0.1	5.3	0.6	5.5	0.4
PS – A	5.9	0.1	2.5	0.4	2.4	0.2
PS B	5.9	0.1	3.5	0.6	2.5	0.3
PS C	6.1	0.1	5.9	0.4	2.4	0.1

Legend: DCH: dry cured ham; PS: Pig skin; A: solution A
B: Solution B; C: solution (mix A + B + water)
X: average; SD: standard deviation.

O'Bryan et al. 2008; Turgis et al. 2008; Giatrakou et al. 2008). However, its effectiveness must be validated by challenge tests, being the activity largely dependent on different parameters, as reported above. Despite the results, we suggest using essential oils as anti-listeria agents because they are natural and considered safe.

Protective Cultures

The effect of *Leuconostoc carnosum* 4010 (CHR Hansen, Italy) is reported in Table 8. As shown, the concentration of *L. monocytogenes* decreases to <1 CFU/g. Indeed, enrichment cultures managed to cut the concentration of the inoculated microorganism to less than 0 CFU/25 g. In other words, it seems that, after 30 days, protective cultures manage to achieve the 0 tolerance level recommended by FDA/FSIS (2003b).

Food safety now largely depends on the efficacy of food preservation systems based on combined technologies, on the intrinsic properties of the food products, and on the target microorganism. The protective starter, the bacteriocins alone or in combination with other systems (i.e. dehydration, ripening, chemicals), represent one of the best technologies to prevent or inhibit pathogens. In recent years, many technologies based on the use of a starter culture or bacteriocins were used (Minei et al. 2008; Albano et al. 2007; Drosinos et al. 2006; Urso et al. 2006; Leroy and De Vuyst 2005; Brashears 2000; Coffey et al. 1998). Specifically, protective cultures were used as a starters to improve ripening and aroma, and to prevent or inhibit the growth of *L. monocytogenes*. Interesting results emerged when using a starter as an anti-listeria agent. Brashears (2000) demonstrated that the use of lactic acid bacteria in ready-to-eat food (hot dogs and sliced ham) could have a bacteriostatic effect on intentionally added *L. monocytogenes*. Their data agree with ours because there was a difference of more than 4 log in the amount of *L. monocytogenes* in ready-to-eat products containing this microorganism compared to the controls after 28 days of storage. In this case, the anti-listeria effect has been assessed vis-a-vis the competition level. The growth of lactic acid bacteria inhibited the growth of *L. monocytogenes*. Conversely, several authors demonstrated that in order to improve the anti-listeria effect starter cultures – bacteriocin producers – must be used. Bacteriocins are anti-microbial peptides produced by lactic acid bacteria, coagulase negative cocci, that have antimicrobial and specifically anti-listeria

Table 8 Behaviour of *Leuconostoc carnosum* CH versus *Listeria monocytogenes* in Data: UFC/cm²

Time of storage (days)	<i>L. carnosum</i>	<i>L. monocytogenes</i>
0	100.000	1.000
15	80.00	<1
30	75.00	<1/25 cm ²

effects (Jofrè et al. 2008; Comi et al. 2008). Jofrè et al. (2008) demonstrated that the application of enterocins A and B, sakacin K and nisin could extend the shelf life of pressurized ready-to-eat meat products.

Hence, our data confirmed that many lactic acid bacteria are food grade and can safely be added to food products, because they continue to produce inhibitory compounds during the product ripening and storage and therefore guarantee a continuous inhibition of the pathogens (Comi et al. 2008; Albano et al. 2007; Drosinos et al. 2006; Urso et al. 2006; Leroy and De Vuyst 2005; Brashears 2000; Villegas and Gilliland 1998; Winkowski and Montville 1992; Schillinger et al. 1990). In our case, a *Leuconostoc carnosum* 4010 was used (CHR Hansen, Italy). It produces a powerful bacteriocin with a great effect on *L. monocytogenes*. The bacteriocins could completely inhibit *L. monocytogenes* both in vitro and in vivo. As the data demonstrate, its effect is not only bacteriostatic but also bacteriocidal. Indeed, using *Leuconostoc carnosum* as protective culture has completely eliminated the total concentration of the *L. monocytogenes*, which was intentionally added both on DCH and on PS. The inhibition does not imply a growth of the protective culture on both inoculated samples. So, it did not produce the typical white slime of lactic acid bacteria observed on the surface of meat products treated with starter bacteria. In addition, data are highly reproducible and repeatable, so we recommend using this protective culture as anti-listeria method in ready-to-eat food.

Conclusions

Listeria monocytogenes is a ubiquitous micro-organism and therefore may contaminate dry cured ham during the production phase. The technology used in production poses a few hurdles such as salting and dehydration, which prevent the germ from growing.

Consequently, the product must be considered safe. However, the presence of even low concentrations of *L. monocytogenes* in whole, de-boned, or sliced dry cured ham, amounting to 1 CFU/g or cmq, may pose problems in terms of sales in some markets such as the US, where a 0 tolerance standard is required for such micro-organism. The purpose of our study was to produce and optimize methods that can minimize or completely remove *L. monocytogenes* in dry cured ham. The methods we used included anti-microbial agents, as compared with dehydration only, ionized air, hydrogen peroxide, essential oils, and protective starter cultures. The data obtained with this study show that the A_w level in the product at the end of the curing period (0.90) cannot support the growth of intentionally added *L. monocytogenes*. Quite conversely, an A_w of 0.90 in the tested samples reduced the load of such germ by 2 log units of more over time. Such reduction was observed both in under-vacuum packaged slices and in MAP packaged slices. Also anti-microbial additives reduced the number of intentionally added *L. monocytogenes*. In this case, the reduction largely depended on the type of additive and on the type of packaging, but in any case amounted to 2 log units or more. Adding

consumer-safe anti-microbial solutions such as those used in our tests further enhances the safety level of finished products. As a matter of fact, the effect of a low A_w on *L. monocytogenes* might be fostered by adding appropriate solutions of such additives on the surface of dry cured ham. For this reason, we can undoubtedly conclude that the data obtained demonstrate that no *L. monocytogenes* can grow on sliced dry cured ham or on dry cured ham with an A_w value equal to or lower than 0.90 and stored at refrigeration temperatures.

Moreover, using other methods including solutions of hydrogen peroxide (20% v/v), ionized air, essential oils, allowed to eliminate from 1 to 3 log of *Listeria monocytogenes* from dry ham meat and pig skin. In contrast, *Leuconostoc carnosum* added to inoculated samples as protective starter brought about a reduction of up to 4 log in the level of intentionally added *L. monocytogenes*. So, an optimization of the concentration of the *Lc. carnosum* could lead to the 0 tolerance requirement posed by FDA/FSIS (2003a, b). Poor results were obtained when using ozonated water and air.

For the production of San Daniele dry cured ham, these treatments could be defined as post-lethality processes to be applied to the finished product after deboning and before packaging under vacuum. The treatments showed a high level of repeatability and reproducibility.

Considering their anti-listeria effects and the level of reduction, the investigated treatments allowed to comply with the concept “absence of risk” and with the limit for *L. monocytogenes* (less than 100 CFU/g) recommended by the Official Journal of European Communities (GUCE 2002). All the investigated methods are safe. No toxic residues, no changes in odor and flavor, and no oxidative compounds were found after their application. For these reasons, they could be safely used for several Italian DOP or IGP products. In any case, since the maximum level of initial contamination of raw meat cannot exceed 100 CFU of *Listeria monocytogenes* per gram of meat, in order to completely eliminate the risk of listeriosis it would be enough to implement a severe control of the operating standard procedures such as salting, drying, and ripening during the production of San Daniele dry cured ham. Our data have largely demonstrated this, and consequently no other treatments are required.

References

- A.A.V.V. (1996) ICMSF, Microorganisms in foods 5, Microbial specifications of food pathogens. Blackie & Academic & Professional, Chapman and Hall, London, pp 141–182
- Albano H, Todorov S, van Reenen CA, Hogg T, Dicks LMT, Teixeira P (2007) Characterization of two bacteriocins produced by *Pediococcus acidilactici* isolated from “Alheira”, a fermented sausage traditionally produced in Portugal. *Int J Food Microbiol* 116(2):239–247
- Aureli P, Franciosa G, Pourshaban M, Gattuso A, Gianfranceschi M (1998) Laboratory findings on *Listeria monocytogenes* strains involved in a large outbreak of febrile gastroenteritis occurred in schools served by a mass catering system. XIII International symposium on problems of Listeriosis, Halifax, Canada, 28/6, 2–7

- Bader H, Hoigne J (1981) Determination of ozone in water by the indigo method. *Water Res* 15:449–452
- Bell C, Kyriakides A (1998) *Listeria*: a practical approach to the organism and its control in foods. Blackwell Science, Oxford
- Bell C, Kyriakides A (2003) *Listeria monocytogenes*. In: C de W Blackburn, McClure PJ (eds) Foodborne pathogens, chapter 12. CRC Press, Boca Raton, FL, pp 337–358
- Beltràn D, Selma MV, Marin A, Gil MI (2005) Ozonated water extends the shelf life of fresh-cut lettuce. *J Agric Food Chem* 53(14):5654–5663
- Blackman IC, Frank JF (1996) Growth of *Listeria monocytogenes* as a biofilm on various food-processing surfaces. *J Food Prot* 59:556–565
- Brashears M (2000) Competitive inhibition of *Listeria monocytogenes* in ready-to-eat pork products. Research Rep. 99-216, 1–10. [http://www.pozk.org/porkScience Documents/CompetitiveInhibition.pdf](http://www.pozk.org/porkScience/Documents/CompetitiveInhibition.pdf)
- Carlson BA, Geornaras I, Yoon Y, Scanga JA, Sofos JN, Smith GC, Belk KE (2008) Studies to evaluate chemicals and conditions with low-pressure applications for reducing microbial counts on cattle hides. *J Food Prot* 71(7):1343–1348
- Cocolin L, Stella S, Nappi R, Bozzetta E, Cantoni C, Comi G (2005) Analysis of PCR-based methods for characterization of *Listeria monocytogenes* strains isolated from different sources. *Int J Food Microbiol* 103:167–178
- Coffey A, Ryan M, Ross RP, Hill C, Arendt E, Schwartz G (1998) Use of a broad-host-range bacteriocin-producing *Lactococcus lactis* transconjugant as a alternative starter for salami manufacture. *Int J Food Microbiol* 43:231–235
- Comi G, Urso R, Paiani M, Ottaviani S (2005a) Dry cured ham cured and packaged in modified atmosphere or under vacuum: effects of different additives and of Aw on the behavior of *L. monocytogenes*. *Ind Alim XLIV*, Marzo, 272–278
- Comi G, Lovo A, Bortolussi N, Paiani M, Berton A, Bustreo G (2005b) Use of an ionizing device for air decontamination in cells used for the production of San Daniele dry cured ham. *Ind Alim XLIV*, Ottobre, 1–9
- Comi G, Osualdini M, Manzano M, Lovo A, Bortolussi N, Berton A, Bustreo G (2006) Decontamination of facilities and equipment used in food processing companies by means of ionizers. *Ind Alim XLV*, giugno, 661–669
- Comi G, Manzano M, Giusto C, Iacumin L (2008) Feasible methods for controlling hazards in the food chain. *Ital J Agron-Riv Agron* 3(suppl. 1):167–172
- Drosinos EH, Mataragas M, Metaxopoulos J (2006) Modeling of growth and bacteriocin production by *Leuconostoc mesenteroides* E131. *Meat Sci* 74(4):690–696
- Duggan J, Philips CA (1998) *Listeria* in the domestic environment. *Nutr Food Sci* 213(6):73–79
- Emer Z, Akbas MY, Ozdemir M (2008) Bactericidal activity of ozone against *Escherichia coli* in whole and ground black peppers. *J Food Prot* 71(5):914–917
- Fang WNG, Langlois BE, Moody WG (1997) Fate of pathogens in vacuum-packaged dry-cured (Country style) ham slices stored at 2 and 25°C. *J Food Prot* 60(12):1541–1547
- Farber JM, Harwing J (1996) The Canadian position on *Listeria monocytogenes* in ready-to-eat foods. *Food Control* 7(4/5):253–258
- Farber JM, Peterkin PI (1991) *Listeria monocytogenes*, a food-borne pathogen. *Microbiol Rev* 55(3):476–511
- FDA/FSIS (2003a) Control of *Listeria monocytogenes* in ready-to-eat meat and poultry products. 9 CFR Part 430, June 2003
- FDA/FSIS (2003b) Food and Drug Administration Food Safety and Inspection Service February, 10th 2003– Draft FSIS Risk Assessment for *Listeria* in Ready-to-eat-Meat and Poultry product
- Fisher CW, Lee D, Dodge BA, Hamman KM, Robbins JB, Martin SE (2000) Influence of catalase and superoxide dismutase on ozone inactivation of *Listeria monocytogenes*. *Appl Environ Microbiol* 66(4):1405–1409
- Franciosa G, Tartaro S, Wedell-Neergaard C, Aureli P (2001) Characterization of *L. monocytogenes* strains involved in invasive and non-invasive listeriosis outbreaks by PCR-based fingerprinting techniques. *Appl Environ Microbiol* 67(4):1793–1799

- G.U.C.E. (2002) Gazzetta Ufficiale delle Comunità Europee (Official Journal of the European Union) (2002/C115E/061)
- Gabbay J (1990) Effect of ionization on microbial air pollution in the dental clinic. *Environ Res* 52:1–59
- Garcia-Esteban M, Ansorena D, Gimeno O, Astiasarán I (2002) Optimization of instrumental color analysis in dry-cured ham. *Meat Sci* 63:287–292
- Garcia-Esteban M, Ansorena D, Astiasarán I (2004) Comparison of modified atmosphere packaging and vacuum packaging for long period storage of dry-cured ham: effects on colour, texture and microbiological quality. *Meat Sci* 67:57–63
- Geornaras I, Skandamis PN, Belk KE, Scanga JA, Kendall PA, Smith GC, Sofos JN (2006) Post-processing application of chemical solutions for control of *Listeria monocytogenes*, cultured under different conditions, on commercial smoked sausage formulated with and without potassium lactate-sodium diacetate. *Food Microbiol* 23:762–771
- Gianfranceschi M, Pourshaban M, Gattuso A, Wendell-Neergaard C, Aureli P (2002) Characterization of *Listeria monocytogenes* strains isolated from food and humans in Italy by pulsed-field electrophoresis. *Food Microbiol* 19:47–55
- Giatrakou V, Kikkidou S, Papavergou A, Kontominas MG, Savvaidis IN (2008) Potential of oregano essential oil and MAP to extend the shelf-life of fresh swordfish: a comparative study. With ice storage. *J Food Microbiol Safety* 71:167–173
- Glass K, Preston D, Veessenmeyer J (2007) Inhibition of *Listeria monocytogenes* in turkey and pork-beef bologna by combinations of sorbate, benzoate and propionate. *J Food Prot* 70(1):214–217
- Gola S, Frustoli M, Rovere P, Miglioli L (2003) Inattivazione di *Listeria monocytogenes* in prosciutto crudo trattato con la pressione idrostatica. – Inactivation of *Listeria monocytogenes* in dry cured ham by hydrostatic pressure treatment. *Ind Conserve* 78:441–449
- Graumann GH, Holley RA (2008) Inhibition of *Escherichia coli* O157H7 in ripening dry fermented sausage by ground yellow mustard. *J Food Prot* 71(3):486–493
- Grisenti MS, Lori D, Vicini L, Bovis N, Pedrelli B, Barbuti S (2004) Comportamento di *Listeria monocytogenes* in prosciutto crudo stagionato in rapporto all'atmosfera di confezionamento e alla temperatura di conservazione – behavior of *Listeria monocytogenes* in dry cured ham depending on the packaging atmosphere and storage temperature. *Ind Conserve* 79:3–12
- Hong E, Doumith M, Duperrier S, Giovanacci I, Morvan A, Glaser P, Buchrieser C, Jacquet C, Martin P (2007) Genetic diversity of *Listeria monocytogenes* recovered from infected persons and pork, seafood and dairy products on retail sale in France during 2000 and 2001. *Int J Food Microbiol* 114:187–194
- Horvath M, Bilizky L, Huttner J (1985) Fields of utilization of ozone. In: Clark RJH (ed) *Ozone*. Elsevier Science Publishing Co., Inc., New York, pp 257–316
- ISO-FDIA 21807 (2004) Microbiology of food and animal feedings stuffs. Determination of water activity. International Organization for Standardization, Geneva, Switzerland
- Jofrè A, Aymerich T, Monfort JM, Garriga M (2008) Applications of enterocins A and B, sakacin K and nisin to extend the safe shelf-life of pressurized ready-to-eat meat products. *Eur Food Res Technol* 228(1):159–162
- Kaothien P, Jhala R, Henning D, Julson JL, Muthukumarappan K, Dave RI (2001) Effectiveness of ozone for controlling *Listeria monocytogenes* in ready to eat cured ham. IFT Annual meeting-New Orleans, Louisiana
- Kathariou S (2002) *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *J Food Prot* 65(11):1811–1829
- Khadre MA, Yousef AE (2001) Sporicidal action of ozone and hydrogen peroxide: a comparative study. *Int J Food Microbiol* 71:131–138
- Kim JG, Yousef AE, Dave S (1999) Application of ozone for enhancing the microbiological safety and quality of foods: a review. *J Food Prot* 62(9):1071–1087
- Lado BH, Yousef AE (2007) Characteristic of *Listeria monocytogenes* important to food processors. In: Ryser ET, Marth H (eds) *Listeria, Listeriosis, food safety*, 3rd edn. CRC Press, Boca Raton, FL, pp 157–213

- Leroy F, De Vuyst L (2005) Simulation of the effect of sausage ingredients and technology on the functionality of the bacteriocin-producing *Lactobacillus sakei* CTC 494 strain. *Int J Food Microbiol* 100(1):141–152
- Lou Y, Yousef AE (1996) Resistance of *Listeria monocytogenes* to heat after adaptation to environmental stresses. *J Food Prot* 59:465
- McLauchlin J (1997) The pathogenicity of *Listeria monocytogenes*. A public health perspective. *Rev Med Microbiol* 8:1–14
- Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV (1999) Food-related illness and death in the United States. *Emerg Infect Dis* 5:607–625
- Miettinen MK, Björkroth KJ, Korkeala H (1999) Characterization of *Listeria monocytogenes* from an ice cream plant by serotyping and pulsed-field gel electrophoresis. *Int J Food Microbiol* 46:187–192
- Minei CC, Gomes BC, Ratti RP, D'Angelis CEM, De Martinis ECP (2008) Influence of peroxyacetic acid and nisin and coculture with *Enterococcus faecium* on *Listeria monocytogenes* biofilm formation. *J Food Prot* 71(3):634–638
- Moore G, Griffith C, Peters A (2000) Bactericidal properties of ozone and its potential application as a terminal disinfectant. *J Food Prot* 63(8):1100–1106
- Woo F Ng, Langlois BE, Moody WG (1997) Fate of selected pathogens in vacuum-packaged dry-cured (country-style) ham slices stored at 2 and 25°C. *J Food Prot* 60(12):1541–1547
- Nørung B (2000) Microbiological criteria for *Listeria monocytogenes* in foods under special consideration of risk assessment approaches. *Int J Food Microbiol* 62:217–221
- O'Bryan CA, Crandall PG, Chalova VI, Ricke SC (2008) Orange essential oils antimicrobial activities against *Salmonella* spp. *J Food Sci* 73(6): 264–267
- Parodi M, Marino P, Balzaretti C, Mauri A, Cainarca M, Cantoni C (1990) A case report of sporadic listeriosis related to pork meat in healthy man, *Giorn. Malattie Inf E Parassitarie* 42:115–116
- Porto-Fett ACS, Call JE, Luchansky JB (2008) Validation of commercial process for inactivation of *Escherichia coli* O157H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on the surface of whole muscle beef jerky. *J Food Prot* 71(5):918–926
- Quintavalla S, Vicini L (2002) Antimicrobial food packaging in meat industry. *Meat Sci* 62:373–380
- Restaino L, Frampton EW, Hemphill JB, Palnikar P (1995) Efficacy of ozonated water against various food-related microorganisms. *Appl Environ Microbiol* 61(9):3471–3475
- Reynolds AE, Harrison MA, Rose-Morrow R, Lyon CE (2001) Validation of dry cured ham process for control of pathogens. *J Food Sci* 66(9):1373–1379
- Robbins JB, Fisher CW, Moltz AG, Martin SE (2005) Elimination of *Listeria monocytogenes* biofilms by ozone, chlorine, and hydrogen peroxide. *J Food Prot* 68(3):494–498
- Salamina G, Delle Donne E, Piccolini A, Poda G, Cesaroni D, Bucci M, Fini R, Maldini M, Schuchat A, Swaminathan B, Bibb W, Rocurt J, Binkin N, Salmaso S (1996) A food borne outbreak of gastroenteritis involving *Listeria monocytogenes*. *Epidemiol Infect* 117:429–436
- Schillinger U, Kaya M, Lucke FK (1990) Behaviour of *Listeria monocytogenes* in meat and its control by a bacteriocin-producing strain of *Lactobacillus sakei*. *J Appl Bacteriol* 70(6):473–478
- Seman DL, Quickert SC, Borger AC, Meyer JD (2008) Inhibition of *Listeria monocytogenes* growth in cured ready-to-eat meat products by use of sodium benzoate and sodium diacetate. *J Food Prot* 71(7):1386–1392
- Shabala L, Lee SH, Cannesson P, Ross T (2008) Acid and NaCl limits to growth of *Listeria monocytogenes* and influence of sequence of inimical acid and NaCl levels on inactivation kinetics. *J Food Prot* 71(6):1169–1177
- Sheldon BW, Brown AL (1986) Efficacy of ozone as a disinfectant for poultry carcasses and chill water. *J Food Sci* 51(2):305–309
- Slutsker L, Schuchat A (1999) Listeriosis in humans. In: Ryser ET, Marth EH (eds) *Listeria*, listeriosis and food safety. Marcel Dekker, New York, pp 75–95

- Stata (2005) Statistical software for professionals. Stata Corp LP, Texas, USA. www.stata.com/support/updates/stata8.html
- Stopforth JD, Mai T, Kottapalli B, Samadpour M (2008) Effect of acidified sodium chlorite, chlorine, and acidic electrolyzed water on *Escherichia coli* O157H7, *Salmonella* and *Listeria monocytogenes* inoculated onto leafy greens. *J Food Prot* 71(3):625–628
- Swaminathan B (2001) *Listeria monocytogenes*. In: Doyle MP, Beuchat LR, Montville TJ (eds) *Food microbiology: fundamentals and frontiers*, 2nd edn. ASM Press, Washington, DC, pp 383–410
- Tappero JN, Schuchat A, Deaver KA et al (1995) Reduction in the incidence of human listeriosis in the United States. Effectiveness of prevention efforts. *J Am Med Assoc* 273:1118–1122
- Tompkin RB (2002) Control of *Listeria monocytogenes* in the food processing environment. *J Food Prot* 65(4):709–725
- Turgis M, Han J, Borsa J, Lacroix M (2008) Combined effect of natural essential oils, modified atmosphere packaging and gamma radiation on the microbial growth on ground beef. *J Food Prot* 71(6):1237–1243
- Urso R, Rantsiou K, Cantoni C, Comi G, Coccolin L (2006) Technological characterization of a bacteriocin-producing *Lactobacillus sakei* and its use in fermented sausages production. *Int J Food Microbiol* 110(3):232–239
- Villegas E, Gilliland SE (1998) Hydrogen peroxide production by *Lactobacillus delbrueckii* subsp. *Lactis* a 1–5°C. *J Food Sci* 63(6):1070–1073
- Vorst KL, Clarke RH, Allison CP, Booren AM (2004) A research note on radio frequency transponder effects on bloom of beef muscle. *Meat Sci* 67:179–182
- Wade WN, Scouten AJ, McWatters KH, Wick RL, Demirci A, Fett WF, Beuchat LR (2003) Efficacy of ozone in killing *Listeria monocytogenes* on Alfalfa seed and sprouts and effects on sensory quality of sprouts. *J Food Prot* 66:44–51
- Winkowski K, Montville TJ (1992) Use of meat isolate, *Lactobacillus bavaricus* MN, to inhibit *Listeria monocytogenes* growth in a model meat gravy system. *J Food Safety* 12:19–22

Part III

Viral Infections

Epidemiology and Diagnosis of Hepatitis B in the Mediterranean Region and Elsewhere

Nurdan Tözün, Özdal Ersoy, Fatih Eren, and Veysel Tahan

Abstract After cancer, viral infections constitute a great threat to human health throughout the world and among these viral hepatitis constitutes one of the most important public threats in the world. Despite considerable advances in medical technology and attempts to eradicate the diseases, viral hepatitis still remain as major public health problem with their high morbidity and mortality in the Mediterranean, Eastern Europe, Middle East and elsewhere. Uncontrolled migratory flow from developing countries to regions of low endemicity gave rise to changes not only in the prevalence of viral infection but also the genotype, clinical course and modes of transmission. Among several viruses which may affect the liver those which constitute important public health problems are hepatitis A, B, C, D, and E. All of these viruses can cause an acute disease with symptoms lasting several weeks however HBV can cause asymptomatic infection which can silently progress to chronic infection in which the patient never gets rid of the virus and many years later develops cirrhosis of the liver or liver cancer. Of the two billion people who have been infected with HBV, more than 350 million have chronic (lifelong) infections. In this chapter, we reviewed the present situation, epidemiology, clinical presentation, prevention, diagnostic techniques and the universal importance of Hepatitis B.

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Keywords Hepatitis B infection • hepatocellular carcinoma, cirrhosis • epidemiology • HBV

Abbreviations

ALT	Alanine aminotransferase
anti-HBc	Antibody to hepatitis B virus core antigen
anti-Hbe	Antibody to hepatitis B virus e antigen
bps	Bits per second
DNA	Deoxyribonucleic acid
EU	European Union
HCW	Health care workers
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
anti-HBs	Hepatitis B surface antibody
HbeAg	Hepatitis B virus e antigen
HbsAg	Hepatitis B virus surface antigen
HIV	Human immune deficiency virus
RNA	Ribonucleic acid
USSR	Union of Soviet Socialist Republics
WHO	World Health Organization

After cancer, viral infections constitute a great threat to human health throughout the world. Over the last decades many European countries have been a seat of a high rate of immigration triggered by economic crisis, wars, political instabilities or geographical events. With the falling down of the “so-called” impassable walls between the countries, the segregation of the USSR, ease of mobilisation from one country to another and the creation of new job opportunities with the development of the EU there has been a great flow of immigrants especially to the countries like Spain, Italy, and Greece and a substantial proportion of the estimated 20 millions of immigrants have moved to Europe over the last 15 years. This movement has been inevitably accompanied by the change in the prevalence of infectious diseases in the host country. Some recent studies have demonstrated that the prevalent infectious diseases in immigrants are HIV, tuberculosis and chronic viral hepatitis, more frequently caused by HBV, particularly in African people coming from sub-Saharan areas.

Viral hepatitis constitutes one of the most important public threats in the world. As stated above and shown by Esteban et al. (2008), Palumbo et al. (2008), Magdzik (2000) and Ni et al. (2001) uncontrolled migratory flow from developing countries to regions of low endemicity gave rise to changes not only in the prevalence of viral infection but also the genotype, clinical course and modes of transmission.

Among several viruses which may affect the liver those which constitute important public health problems are hepatitis A, B, C, D, and E. Whilst hepatitis A and E transmitted by oral-fecal route can cause serious epidemics especially

among some special risk and age groups, the morbidity and mortality associated with parenterally transmitted hepatitis B, C and Delta virus infections make their priorities at the level of health organisations when long term complications of these viruses are taken into consideration. All of these viruses can cause an acute disease with symptoms lasting several weeks including yellowing of the skin and eyes (jaundice); dark urine, extreme fatigue, nausea, vomiting and abdominal pain and it can take several months to a year to feel fit again. But especially HBV can cause asymptomatic infection which can silently progress to chronic infection in which the patient never gets rid of the virus and many years later develops cirrhosis of the liver or liver cancer. Viral hepatitis costing life to millions of individuals especially in endemic areas like East Asia, Africa and the Mediterranean region show differences in terms of prevalence according to the socioeconomical and geographical status of the countries.

Of the two billion people who have been infected with HBV, more than 350 million have chronic (lifelong) infections. Likewise, 170 million people are suffering from hepatitis C infection throughout the world. The chronicity rate in hepatitis C infection is much higher than hepatitis B. Chronically infected persons are at high risk of death from cirrhosis of the liver and liver cancer, and the estimated worldwide mortality of hepatitis B is 0.5–1.2 million deaths a year, recently shown by Lavanchy (2004), Perz et al. (2006a, b) and Parkin et al. (2001)

Efforts aimed at rising the public awareness about the magnitude of the problem and the spread of information by the authorities to the population under threat are the essential elements of the battle against viral hepatitis. However most of the time, viral hepatitis does not seem to take place among the priorities of National Health associations and health policy makers. First of all, accurate numbers reflecting the true prevalence of hepatitis B and C in the EU are lacking. This is largely due the lack of population wide screening studies and there is a worldwide need of appropriate national registries. The most prevalent infections which constitute a public challenge are often discovered by the laboratory in asymptomatic patients either by chance, as a more or less unexpected finding in a medical investigation, or as part of a screening programme. Thus, efforts directed to “case finding” prevails to screening studies in most of the countries. This is particularly important in childhood infections since over 90% of childhood HBV infections are asymptomatic, thus the true incidence of childhood disease is underrepresented by most surveillance data, which reflect reported cases of clinically apparent disease (Alter 2003).

In fact, a report elaborated from the European Centre for Disease Prevention and Control and (ECDC) edited by Amato-Gauci and Ammon (2007) emphasizes that the national incidence figures often reflect activity to find asymptomatic patients rather than reflecting the ‘true’ incidence of infection.

As of 2007, 171 of the 193 member countries of the WHO had implemented the recommendations of the Expanded Programme on Immunization to offer universal hepatitis B vaccination to infants. (Expanded Programme on Immunization of the Department of Immunizations, Vaccines and Biologicals. WHO vaccine-preventable diseases: monitoring system, 2007 global summary. Geneva (Switzerland): World Health Organization; 2007. Available: http://whqlibdoc.who.int/hq/2007/WHO_

[IVB_2007_eng.pdf](#); accessed: 2008 Dec 3). With the establishment of an effective screening program by more accurate techniques in blood banks and the implementation of universal vaccination for HBV to all newborns; the incidence and prevalence of HBV were dramatically reduced but there is still a need for much effort notably in countries of high endemicity.

The approach and the management of hepatitis has been subject to revolutionary changes over the past 10 years. Recent advances in the molecular biological techniques, definition of genotypes and their different consequences, better recognition of viral genome, host virus interactions, diseases behavior and the introduction of new antivirals in the therapeutic armamentarium have created optimism in the control of these debilitating diseases. However, this optimism generated is tempered by the limitations of sustained response to antivirals, frequent occurrence of breakthroughs and relapses over time and viral resistance problems under medication. Now we better know whom to treat, how long and what are the end points of the treatment; but we still do not know to which degree histological improvement, 1 or 2 log decrease in viral load, ALT normalization or in better saying viral clearance upon which our therapies are based are satisfactory in decreasing liver disease related mortality and making us feel secure about the prevention hepatocellular carcinoma.

Despite considerable advances in medical technology and attempts to eradicate the diseases, viral hepatitis still remain as major public health problem with their high morbidity and mortality in the Mediterranean, Eastern Europe, Middle East and elsewhere.

In this chapter, we reviewed the present situation, epidemiology, clinical presentation and importance of Hepatitis B.

Characteristics of the Virus

Hepatitis B virus infection is an important and major disease of mankind and as stated before about 1/3 of world population, are seropositive (having seromarkers for ongoing or past infection) for HBV, mentioned by WHO 2000; Mahoney 1999; Lee 1997; Lok and McMahon 2007.

Hepatitis B virus is a double-stranded DNA virus of the hepadnaviridae family. The virus has an envelope, and a viral DNA genome of about 3,200 bps within its core. After the virus enters a hepatocyte, the viral genome is delivered to the nucleus, and the relaxed circular DNA is converted to covalently-closed-circular DNA (cccDNA). That cccDNA plays a major role in the persistence of the virus since it serves as a template for the transcription of the viral RNA. The replication cycle of HBV demonstrated by Summers and Mason (1982) includes reverse transcription of RNA intermediates to prime DNA synthesis and translation of the hepatitis B proteins, including hepatitis B surface antigen (HBsAg) and e antigen (HBeAg).

The virus has at least eight major genotypes (A to H), based on an intergroup divergence of more than 8% in the complete nucleotide sequence. Except for genotypes E and G, the genotypes have sub-genotypes with a sequence difference of at

least 4% but their clinical significance remains to be determined. The geographical distribution of genotypes across the world varies significantly. In studies concerning genotypes of HBV (Chu et al. 2003; Ture et al. 2005; Ni et al. 2004; Yuen et al. 2004) it was shown that; Genotype A is prevalent in northwestern Europe and the USA; genotypes B and C in Asia; genotype D in the Mediterranean basin (Italy, Greece, Turkey), Middle East and India; genotype E in west Africa; genotype F in South and Central America; genotype G in the USA and France; and genotype H in Mexico and South America.

Genotypes may define the characteristic of HBV progression and response to treatment. In general, genotype B is associated with less progressive liver disease than genotype C, and genotype D has a less favorable prognosis than genotype A as mentioned by Kao (2007). In the studies of Kao et al. (2000) and Chan et al. (2004), it has been shown that Genotype C is associated with an increased risk of hepatocellular carcinoma (HCC) and Genotype D with a more severe liver disease.

HCC is preventable by HBV vaccine. Although the vaccine will not cure chronic hepatitis, it is 95% effective in preventing chronic infections and is the first vaccine against a major human cancer. In 1991, the World Health Organization (WHO) called for all children to receive the hepatitis B vaccine, and almost 170 countries have added this vaccine to their routine immunization programmes. However, the children in the poorest countries, who need the vaccine the most, have not been receiving it because their governments could not afford it. Fortunately, hepatitis B vaccine will soon be available in these countries with the assistance of the Global Alliance for Vaccines and Immunization (GAVI) and the Global Fund for Children's Vaccines.

Epidemiology

The prevalence of HBV infection varies markedly in three different geographic areas of the world, as well as in different population subgroups. Overall, approximately 45% of the global population live in areas of high HBV prevalence, mentioned by Mahoney (1999a, b). In sub-Saharan Africa, the Pacific, and particularly Asia, HBV infection is highly endemic (prevalence >8%), with the majority of individuals becoming infected during childhood shown again by Lavanchy (2004). Outside of the endemic areas, regions with intermediate rates (prevalence 2–8%) of chronic HBV infection include the southern parts of Eastern and Central Europe, the Middle East and Turkey, the Amazon basin, and the Indian subcontinent. In western and northern European countries and North America, HBV infection is relatively rare (low endemic region, prevalence <2%) and acquired primarily in adulthood summarised by Lok et al. (2001). However in low endemic regions, demography and prevalence of HBV infection may sometimes vary (increase) due to immigrations as mentioned before. In some regions of Europe and Mediterranean where there is high rate of immigration, concealed HBV infection may be present particularly among certain ethnic and migrant communities. Furthermore, the influx of HBV carriers from southeast Asia and China (predominantly genotypes B and C) into the

European and Mediterranean region (where genotypes A and D are more common) may result in a shift towards the more progressive liver disease than the characteristics of genotypes B and C.

Data about the epidemiology of hepatitis B in the Mediterranean region are abundant. However most of these studies show differences in methodology, data compilation and analysis which make a statistical comparison impossible. In seroepidemiological studies, markers of hepatitis B infection (anti-HBs, anti-HBc or HBsAg) were observed in 11.7% (58/497) in healthy Spanish population demonstrated by Dal-Re et al. (1991). HBsAg and anti-HBc prevalences were 3.7% and 34.2% in Italy respectively shown in the study of Da Villa et al. (1998). In various studies from Greece (Lionis et al. 1997; Mazokopakis et al. 2000; Kyriakis et al. 2000), the prevalence of HBsAg and anti HBs were reported between 1% and 1.2% and 17% and 24.5% respectively.

Epidemiological data of 2002 (<http://www.cdc.gov/ncidod/diseases/hepatitis/slides/hepb>) indicate that in northern, western and central Europe, 0.2–0.5% of the population is HBsAg-positive and 4–6% of the population have anti-HBs antibodies. In eastern Europe, Russia, the Mediterranean region and southwest Asia, 2–7% of the population is HBsAg-positive and 20–55% of the population have anti-HBs antibodies. Areas of highest endemicity include Bulgaria, Moldova, the Caucasus region, Jordan and Saudi Arabia.

Turkey represents a bridge between the two continents and as a country with special demographic and socioeconomic features, not only geographically but also in terms of epidemiology, disease behaviour and ethnical differences it deserves special attention. Considerable improvements in hygienic measures and positive developments in community care in Turkey had profound influence over the prevalence of infectious diseases notably hepatitis B over the last few years. National vaccination programme endorsed by the Ministry of Health mandates all the newborns to be vaccinated at birth and a second catch up vaccination is offered at the secondary school level for those who missed the vaccine in the previous years. Nevertheless the burden of HBV is still high in the region especially in the background of chronic progressive liver diseases and hepatitis B remains to be a major reason for liver transplantation in Turkey. A recent meta- analysis reported by Toy et al. (2009) on the pooled data of 109 studies carried out between the years 1989–2009 showed that HBV prevalence in adults is 4.9 in Turkish population the highest prevalence being in the 35–44 age group (5.79%) and among the pregnant women. However, the authors said it would be a mistake to interpolate the results into the entire population. A new epidemiologic survey on hepatitis prevalence is under way with the leadership of the Turkish Association for The Study of the Liver. This population wide study based on based on 2-stage systematic cluster sampling method conform to “Eurostat” population sampling programme, will show the variations of hepatitis prevalence within Turkey.

Seropositivity and chronicity for HBV infection is closely related to the age of getting infected. The more early the infection is acquired, the higher rate of chronicity is to be expected; thus, if perinatally acquired the risk of chronicity is over 90%, if infected between ages 1–5 the risk is between 20% and 50% but if infected late in adulthood the risk is decreased down to 5% as shown in the study of Chu et al. (2003).

Antenatal hepatitis B screening for all pregnant women followed by active and passive immunization of all infants born to HBsAg positive mothers is recommended. This procedure has a success rate of 90–97% and also provides long-lasting immunity, as mentioned by Lok 2002 and Marion et al. 1994.

In Europe and the Mediterranean region a significant proportion of chronic hepatitis B patients are infected with mutant viral strain, a mutation in the core promoter or pre-core region of the viral genome. The pre-core variant is the most common variant in the Mediterranean area, whereas the core promoter variant is prevalent in Asia. HBeAg negative hepatitis with high HBV DNA levels and mutations in the core promoter or precore regions seems to be less common in children than in adults. In areas where mutant viruses are prevalent Genotypes A and D are more prominent. The clinical implications of these variants and genotypes are still under investigation. Therefore, the burden of hepatitis B disease in country-specific populations is one of the most important factors, in approach to viral hepatitis B as shown by Manesis (2006).

Modes of Transmission

HBV is transmitted by percutaneous and mucous membrane exposures to infectious blood and body fluids that contain the virus. A highly resilient virus, HBV is resistant to breakdown, can survive outside the body, and is easily transmitted through contact with infected body fluids. Hepatitis B virus is present in the blood, saliva, semen, vaginal secretions, menstrual blood, and to a lesser extent, perspiration, breast milk, tears, and urine of infected individuals which is shown by many studies (Alter et al. 1976, 1977; Bancroft et al. 1977). The presence of hepatitis B e antigen (HBeAg) is associated with higher levels of HBV DNA in serum and denotes a higher potential of infectivity. Perinatal transmission is much higher in HBe Ag positive pregnant women with high viral load.

Hepatitis B transmission can only be prevented by a thorough understanding of the routes of transmission. The virus is transmitted by contact with blood or body fluids of an infected person in the same way as human immunodeficiency virus (HIV). However, HBV is 50–100 times more infectious than HIV. In brief, the main ways of getting infected with HBV are perinatal (from mother to baby at the birth), child-to-child transmission, unsafe injections (Hutin et al. 1999), transfusions and sexual contact. The virus is not spread by contaminated food or water, and cannot be spread casually in the workplace.

The transmission route vary according to the endemicity rates. In areas of high endemicity, the most common route of transmission is perinatal or the infection is acquired horizontally during the preschool years. Lin et al. (2003) demonstrated that the prevalence of HBeAg is higher in Asian than in African HBsAg carrier mothers (40% versus 15%), so perinatal transmission is greater in Asians, but mainly horizontal in Africans. In areas of intermediate endemicity, transmission is either perinatal or horizontal as also mentioned by Mele et al. (2001).

Table 1 HBV endemicity regions

Factor	High endemicity(>8%)	Intermediate endemicity (2–8%)	Low endemicity (<2%)
Chronic infection rate (%)	5–20	2–5	0.1–2
Regions	Southeastern Asia, China, Alaska, Sub-Saharan Africa	Eastern Europe, Mediterranean region, Central Asia, Latin and Southern America, Middle East and Turkey	USA, Canada, Western Europe, Australia, New Zealand
The age of being infected	Perinatally, early childhood	Childhood	Adulthood
Route of transmission	Maternally versus perinatally	Percutaneous	Sexual activity*, percutaneous

* *Alter and Margolis (1990)*

Other sources of infection include contaminated surgical instruments and donor organs. Health care workers, dentists, and others who have frequent contact with infected blood or blood products are at highest risk. HBV transmission from infected healthcare personnel to patients is relatively uncommon, but has occurred during invasive surgical, obstetrical, or dental procedures. Renal dialysis mentioned by Alter et al. (1983), and long-term household or intimate non-sexual contact with an HBsAg-positive individual also exposes people to the risk of acquiring HBV unless necessary precautions have been taken beforehand (HBV vaccination, using separate dialysis equipment etc.).

Finally some “trendy” applications such as tattooing, ear/body piercing or cosmetic injections, manicure, pedicure, shaving or traditional procedures such as circumcision performed by unsafe or shared instruments and the use of non disposable equipment in acupuncture or diagnostic procedures such as angiography or electro-myography may spread the virus as mentioned in the studies of Limentani and Elliott (1979) and Kent et al. (1988).

With respect to transmission among health care workers (HCW), from HCW to the patients or in hospital setting in general, the major motif for the spread of the virus is the non-compliance with recommended infection control practices, shown again in the studies of Simonsen et al. (1999) and Kane et al. (1999).

The endemicity, transmission routes and the age being infected is summarised in Table 1.

Clinical Presentation

Hepatitis B may cause acute and chronic infection. In acute infection, clinical hepatitis B becomes apparent after an incubation period of 45–180 days. The elimination of hepatitis B virus by non-cytopathic mechanisms starts several weeks

before the disease onset. Hepatitis B virus DNA clearance is largely mediated by antiviral cytokines that are produced by cells of the innate and adaptive immune responses – including tumour necrosis factor α , interferon alfa, or interferon beta mentioned in many articles (Murray et al. 2005; Guidotti et al. 1999; Liaw and Chu 2009). A strong CD4 and CD8 response is necessary for viral clearance in patients with acute hepatitis B. Patients who exhibit insufficient CD4 and CD8 response, develop a persistent inflammatory response and cannot eliminate the virus as shown by Maini et al. (2000).

Chronic hepatitis B infection (CHB) is defined by the persistence of serum hepatitis B surface antigen (HBsAg) for 6 months or longer. The classification of different phases of chronic HBV infection has facilitated our understanding of the disease activity and target groups for the management. As mentioned in many articles (Lok and McMahon 2007; Fattovich et al. 2008; Hui et al. 2007) and revised recently in EASL guidelines (2009); HBV infection has five phases based on the virus–host interaction: **immune tolerance, immune clearance, low or non-replication, e- antigen negative and s-antigen negative phase.**

The first phase (immune tolerant phase) is defined by the presence of HBeAg and high levels of serum HBV DNA. Patients in the immune-tolerant phase are usually young, hepatitis B e antigen (HBeAg) seropositive with high viral loads (2–20 million IU/ml or 10^7 – 10^8 copies/ml) but normal serum alanine aminotransferase (ALT) and no or minimal changes in liver histology. This phase represents active viral replication and immune system tolerance and is most commonly seen in children. High levels of viremia makes these patients highly contagious. The immunotolerant phase is generally short-lived or absent in childhood or adult-acquired HBV infection but may persist for 10–30 years in perinatally infected subjects.

The second phase (immunoactive phase or immune clearance phase) is described by the two variants: the e-antigen positive chronic hepatitis B patients due to wild type virus and e antigen negative patients due to mutant type virus. The second group may be antibody positive to HBeAg. Seroconversion from HBeAg to anti-HBe occurs spontaneously in 50–70% of patients with elevated ALT levels after 5–10 years and point out the end of the immunoactive phase and the beginning of the inactive HBsAg carrier state. In e-antigen positive cases serum HBV-DNA levels generally exceed 20,000 IU/ml (10^5 copies/ml). Patients at the immunoactive phase of the disease have usually high DNA levels but lower than in the immune tolerant phase ($>10^5$ copies/ml or $>2,000$ IU). These individuals have transitioned to a precore or a core promoter mutant of variant virus; their ALT levels may be persistently or intermittently elevated and need to be checked repeatedly. They often are asymptomatic, but they may have mild symptoms of fatigue or malaise. In general, e-antigen negative chronic hepatitis B tends to occur in older individuals, in males, and in those who more often have severe disease requiring long term management. In brief at this phase, immune tolerance to the virus is lost and the immune system mounts sufficient immunologic response to cause inflammation, hepatic injury and commonly symptomatic hepatitis. *The third phase (inactive HBsAg carrier state)* is characterized by the presence of

anti-HBe, low or undetectable serum HBV DNA levels (HBV DNA $< 2,000$ IU/ml or $< 10^4$ copies/ml), normal ALT and inactive liver histology. In this phase HBeAg is cleared and a small percentage of these patients may eventually lose surface antigen in the long run. In *the fourth phase*; (HBeAg negative chronic hepatitis B) some inactive HBsAg carriers may eventually develop HBV reactivation with recrudescence of liver disease. Reactivation may occur with the wild type virus with seroreversion of HBeAg but most often these patients are HBeAg-negative, and harbor HBV variants with nucleotide substitutions in the precore and/or the basal core promoter regions unable to express or expressing low levels of HBeAg. This chronic phase typically runs a fluctuating course, mentioned by Fung and Lok (2004). Usually the treatment is offered at the fourth phase when active disease brings the patient to clinical attention. These patients have elevated ALT, high HBV DNA levels (2,000–20 million IU/ml (10^4 – 10^8 copies/ml) with moderate to severe necroinflammation and fibrosis on their liver biopsy. Patients who have fluctuating levels of transaminases and viremia require a minimum follow-up of 1 year with serum ALT and HBV DNA levels every 3 months in order to differentiate a reactivation of HBeAg-negative chronic hepatitis B from inactive HBV carriers who have a good prognosis as mentioned in the article by Martinot-Peignoux et al. (2002).

The fifth phase (HBsAg [-] phase), some of the inactive HBsAg carriers may eventually lose HBsAg and develop neutralizing HBs antibodies (anti-HBs), but low levels of HBV-DNA may be persisting as detected by PCR in serum and liver biopsy specimens. Loss or immunity to HBsAg is associated with good prognosis (reduced risk of cirrhosis, decompensation and HCC).

Prognosis of Chronic Hepatitis B Infection

Chronic hepatitis B is a progressive disease. Several studies have shown that after diagnosis, the 5-year cumulative incidence of developing cirrhosis ranges from 8% to 20% with the 5-year cumulative incidence of hepatic decompensation being approximately 20%. The 5 year survival of patients with compensated cirrhosis is 80–86%. Once hepatic decompensation occurs, mortality rate increases remarkably ranging from 70% to 85% at 5-year follow-up in different studies (Fattovich et al. 2002; Fattovich 2003; Hui et al. 2002). Fattovich et al. also mentioned that, in the cirrhotic patient the 5-year cumulative risk of developing hepatocellular carcinoma is 17% in East Asia and 10% in the Western Europe and the United States and the 5-year liver related death rate is 15% in Europe and 14% in East Asia. The risk is higher in patients with a family history of HCC.

Factors predicting disease progression may be host or virus related and can be classified as accordingly: older age, male gender, family history and disease expression (e antigen + versus – cases) (host factors), viral load, ALT levels and genotype (virus related factors). Other contributing factors are habitual alcohol consumption, cigarette smoking, and aflatoxin exposure.

Prevention of HBV/Vaccination

As Benjamin Franklin truly pointed out “An Ounce of Prevention is Worth A Pound of Cure”; Hepatitis B is a preventable disease in two ways: by public awareness about how to protect from the transmission of the virus and by vaccination.

The implementation of screening for HBV in blood banks since 1970s has greatly reduced the transfusion related infection. Screening of plasma, organ, tissue, and semen donors, virus inactivation of plasma-derived products and maintenance of strict control by the authorities over the precautions that need to be followed in healthcare, dental and cosmetic application centres are mandatory as shown by Schmunis et al. (2001).

Public awareness can be raised by the national health authorities, non governmental organizations; notably professional medical societies, patients’ associations and foundations. Special “alert days” are very efficient. The use of audiovisual and printed media via TV programmes and press are indispensable for diffusing the information to the public and particularly individuals under risk. Finally education at any level at schools is the master goal of the strategic planning in this field.

Syringe exchange programmes for IV drug users are successfully applied in many countries and have greatly contributed to the decrease in the number of transmission of blood borne pathogens, as described by McKnight et al. (2007).

Hepatitis B vaccine has an outstanding record of safety and effectiveness and the vaccine prophylaxis is the most effective and the cheapest approach to prevent HBV infection globally. Since 1982, over one billion doses of hepatitis B vaccine have been used worldwide. In the early 1990s WHO recommended the addition of the hepatitis B vaccination to all national immunisation programmes. As the year 2008, the vaccine is given as a series of three intramuscular doses. Studies (Mele et al. 2001; Xu et al. 1995) have shown that the vaccine is 95% effective in preventing children and adults from developing chronic infection if they have not yet been infected. HBV positive mothers often infect their infants during the prenatal or postnatal period

The vast majority of infants born to HBeAg positive mothers become infected, and most become chronic carriers if not vaccinated, as mentioned by Hsieh et al. (1992). The risk of horizontal transmission of HBV is especially important during elementary school years between the ages of 7 and 11 years according to a survey carried out on 1,500 school children by Degertekin et al. (2000).

In many countries where 8–15% of children used to become chronically infected with HBV, the rate of chronic infection has been reduced to less than 1% in immunized groups of children. Further more in a study done in Taiwan vaccination has reduced the occurrence of HCC in the long run as shown by Chang (2009). Vaccination programmes will contribute to a decrease in hepatitis B seroprevalence over the next decade, as shown by Perz et al. (2006a, b). Immediate postnatal vaccination of infants at high risk effectively prevents prenatal infection, particularly when added protection is provided by hepatitis B immune globulin given shortly after birth as described by Wong et al. 1984 and Wheeley et al. 1990.

Nevertheless, a small proportion of those receiving complete passive-active prophylaxis still become infected. Supplementary serological control of successful immunization of the infant is recommended at the age of 9–15 months as an aid in the long-term medical management of the few vaccine failures in patients who become infected.

More than 170 countries have adopted universal vaccination strategy at the present time. In western Europe, most countries started with a universal infant/neonate or adolescent immunisation programme. Belgium (1999), Germany (1995), Italy (1991), Portugal (adolescents: 1994; neonates: 2000) and Spain (adolescents: 1993; neonates: 1998) have both universal programmes in place.

Hepatitis B vaccine for infants has been introduced in 163 WHO member states by the end of 2006. Global HBV vaccine coverage is estimated at 60% and is as high as 89% in the region of USA in contrast to 28% in the South East Asian Region and 49% in the African Region, given by WHO/Department of Immunisation, Vaccines and Biologicals Database. One hundred and ninety three member States including GAVI 74 Alliance support eligible countries. (www.whqlibdoc.who.int/publications/2008)

Some developed countries such as the United Kingdom, Ireland, the Netherlands, and the Nordic countries with a very low prevalence of HBV do not apply universal vaccination programme to infants but choose to provide hepatitis B vaccines only to well-defined risk groups.

Briefly, the application of universal vaccine and a further catch up vaccination at school age has dramatically reduced HBV rate in some countries. Several lines of evidence suggest that the incidence and prevalence of HBV related chronic liver diseases and HCC has also declined in countries adhering to the immunisation programme. Much effort is needed to eradicate the virus and hence the only preventable cancer (HCC) in the world especially in high and moderately endemic areas.

Diagnosis

During the past decade, there have been substantial progresses in the diagnosis of viral hepatitis. The development of molecular biological methods has made tremendous contribution to this evolution. The standardization and automation of enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) methods have changed the way of the physicians to diagnose and monitor patients with viral hepatitis. DNA sequencing has revealed the mutations that cause the drug resistance. Moreover this method has allowed the genotyping of hepatitis viruses based on their nucleotide sequence of specific gene region. More sensitive molecular biological methods such as reverse dot blot hybridization and PCR-restriction fragment length polymorphism (RFLP) have been used for the identification of these drug resistance mutations and genotypes. All the methods mentioned above used to detect virological parameters have allowed clinicians

to accurately define the targets of the treatment and the patients at the target. Currently, viral hepatitis is diagnosed and managed by the combination of biochemical and virological parameters.

Virological assays that detect virological parameters are classified into two categories: serological and molecular tests.

Serological Markers

Viral antigens of hepatitis viruses or antibodies against viral epitopes of the host in plasma or serum are detected by ELISA tests described in many studies (Chevaliez and Pawlotsky 2007, 2009; Worman 1997). These assays constitute the mainstay of screening and are pivotal for the initial diagnosis. The principle of the assays is based on sandwich ELISA. Antigens or antibodies (target) in serum of patients infected with hepatitis viruses are captured by target specific recombinant antibodies or antigens that coat the wall of microtiter plates. Secondary enzyme linked antibodies (in the case of antigen) or antigens (in the case of antibodies) bind to the immobilized target. Substrates are added and converted by this conjugated enzyme into a color compound. The rate of color formation (sample optical density [OD]/internal control OD) is proportional with the amount of the target in the medium.

The serological markers of HBV are HBs antigen (HBsAg), anti-HBs antibodies (AntiHBs), HBe antigen (HBeAg), anti-HBe antibodies, and anti-HBc antibodies (including total anti-HBc antibodies and anti-HBc IgM). Newer generations of ELISA's detect these markers with high sensitivity but have limitations. In diagnosing patients with chronic hepatitis B, ELISA may not detect HBsAg because of mutations within and/or outside of the "a" determinant epitope that affect conformational epitope recognition or HBsAg secretion or expression. The most common and known escape mutation of HBsAg variant is the glycine to arginine mutation at amino acid position 145 of "a" determinant. However, previous commercial assays did not recognize mutant HBsAg epitopes, the ability of newer HBsAg assays to detect HBV variants has been improved, shown by Gibb et al. (2007), Muhlbacher et al. (2008) and Weber et al. (2006).

Other conditions where HBsAg may not be detected by ELISA are occult HBV infection and HDV-HBV co-infection. ELISA test may also exceptionally exhibit false positivity in individuals with autoimmune hepatitis, pregnant women and chronic liver disease other than hepatitis B infection.

Serum HBsAg levels were shown to correlate well with the HBV-DNA, cccDNA and intrahepatic HBV DNA. Low pretreatment HBsAg is better than HBV DNA to predict good response to peginterferon and lamivudine treatment shown by Chan et al. (2007), Brunetto et al. (2009) and Ozaras et al. 2008. It was proposed that HBsAg quantification might be used as a surrogate marker for monitoring the efficacy of HBV treatment in the future.

Molecular Tests

Molecular tests consist of numerous molecular methods. They are collected under two groups of tests: the first category of tests have been designed to detect viral replication (quantitative or qualitative) in peripheral blood or the liver and the second have been designed to identify genotype specific sequence or sequence variation of clinical or pathophysiological importance in the hepatitis virus genome.

Viral replication is detected by two types of tests: target nucleic acid amplification based tests and hybridization (signal amplification) based tests.

Signal Amplification Techniques

The technique is described by Attila (2006) based on an increase or amplification of signal generated from probe molecule hybridized target nucleic acid sequence. It has avoided target amplification and hence minimized the possibility of contamination by target amplification products. The commonly used signal amplification technologies include branched DNA (bDNA) and hybrid capture assays. The bDNA method was initially developed by Chiron (Emeryville, CA, USA) and marketed by Bayer Diagnostics (Emeryville, CA, USA), whilst the hybrid capture method was developed and marketed by Digene Corporation (Gaithersburg, MD, USA). Currently, all available signal amplification based commercial kits for detection of HBV are quantitative. The most important characteristics of these hybridization assays are precise and accurate quantification.

Digene Hybrid capture method is only used for the quantitative detection of HBV DNA. Target DNA hybridizes with an RNA probe mix specific to HBV ad and ay strains. RNA:DNA hybrids are captured onto surface microtiter plates or tube coated with antibodies for RNA:DNA hybrids. Multiple conjugated antibodies are added and bind to each captured hybrid resulting in substantial signal amplification. These antibodies are conjugated with reporter molecule (alkaline phosphatase) that cleaves chemiluminescent substrate. The emitted light is measured as relative light units (RLUs) on a luminometer. Quantitative data is generated with the use of calibration curve of calibrators.

There are three commercial version of hybrid capture method for quantitative detection of HBV-DNA mentioned in many studies (Yao et al. 2004; Ho et al. 1999; Barlet et al. 1994; Hendricks et al. 1995; Marin et al. 2001; Lopez et al. 2002; Sakugawa et al. 2003; Brechtbuehl et al. 2001; Loeb et al. 2000; Ronsin et al. 2006): Digene Hybrid Capture Assay 1, 2 standart and 2 ultrasensitive (Digene Corporation, Gaithersburg, MD, USA). The most sensitive of them is the Ultra sensitive version which detects HBV-DNA concentrations down to 0.02 pg/ml.

Branched DNA (bDNA) assays are commercially available for detection of HBV-DNA as detailed by Attila (2006) and Benner et al. (2005). This assay consists of a variety of bDNA associated with signal amplification reporter molecule. Solid support is coated with capture probe that hybridizes with capture

extenders (CEs). A family of oligonucleotides called CE's is used to immobilize the target (HBV-DNA) to the solid support. Immobilized target then is captured by target probes which in turn hybridize to pre-amplifier molecule, thereby 'sandwiching' the target between the target probes and pre-amplifier. The pre-amplifier captures amplifier probes, forming bDNA complex so that each of its branch is conjugated with alkaline phosphatase. The enzyme cleaves chemiluminescent substrate that can generate light (detectable in a luminometer). As a consequence of the branching, a single target assembles a large number of signalling molecules in the microwell. Therefore, the result is stronger signal amplification and lower detection limits.

The design of the branched DNA and the way it is hybridized to the nucleic acid to be investigated differs between different generations of the bDNA assay. The branching of the DNA allows for very dense decorating of the DNA with the enzyme which is important for the high sensitivity of the assay. In all versions of the assay, the linearly amplified signal is directly related to the number of targets present in the original sample. Therefore quantitative results can be generated when this assay is performed with quantitative standards. Versant™ HBV DNA 3.0 Assay (HBV bDNA 3.0 assay) (Bayer Corporation, Tarrytown, New York, USA) is the most sensitive assay among all signal amplification-based assays with a lower detection limit of 350 IU/ml. It has a broad dynamic range of 350–17,850,000 IU/ml for quantitative detection of HBV-DNA.

Target Nucleic Acid Amplification Techniques

Nucleic acid amplification techniques use nucleic acid polymerase target specific oligonucleotides (primers) and (deoxy) ribonucleotidetriphosphate (dNTP) mix to amplify a specific nucleic acid sequence by means of cyclic enzymatic reaction leading to the generation of large number of detectable copies (Lorinez 2006). Target amplification techniques used for qualitative and/or quantitative detection of HBV-DNA are polymerase chain reaction [PCR, mainly by Roche (Basel, Switzerland)], "real time" PCR and "transcription mediated- amplification" (TMA). These target amplification-based techniques are different in terms of reaction components and of other reaction conditions such as temperature.

PCR-based assays that have been developed by Roche Molecular Systems (Pleasanton, CA, USA) are used for quantitative detection of HBV-DNA. Amplicor HBV monitor and its semiautomated version Cobas Amplicor HBV Monitor has lower detection limit of 1,000 copies/ml and 200 copies/ml respectively.

Real-Time PCR

It is the newest of the assays that uses target amplification-based technique and had a significant impact on diagnosis and management of HBV infection as reported by Wong and Medrano 2005; Bernard and Wittwer 2002; Mackay 2004;

Stelzl et al. 2004) Non-real-time-based assays have lower detection limit of 50–200 IU/ml (250–1,000 copies/ml) and limited dynamic range up to 4–5 $\log_{10}$ IU/ml. Real-time PCR has increased sensitivity (3.5–10 IU/ml) and provided wider dynamic range (up to 8–9 $\log_{10}$ IU/ml). In the monitoring of the HBV-DNA level, viral load has been expressed at a logarithmic level because the assay can detect very high viral load levels (up to 10^8 – 10^9 copies/ml). Moreover, logarithmic reduction or increase in HBV-DNA level is used to identify the response to antiviral therapy and virological breakthrough Lok and McMahon (2007).

The method of the assay also called quantitative real time pcr (qPCR) relies on detection and quantification of the fluorescent signal emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real time) as opposed to the endpoint detection. Fluorescent dyes that intercalate with double-stranded DNA, and modified DNA oligonucleotide probes (Taqman, Beacons, hybridization probes, e.g.) which fluoresce when hybridized with a complementary DNA are used for the development of the fluorescent signal. At a certain point during cycling, the product accumulates enough to increase fluorescence above the background. This point is referred to as cycle threshold (C_t) or crossing point (C_p) and correlates with the initial amount of target template. Consequently, the greater the quantity of target DNA in the initial material, the faster will be the increase in fluorescent signal yielding a lower C_t . As Real-time PCR assay does not require post amplification manipulations, for example gel electrophoresis, ELISA or blotting, analysis can be completed rapidly allowing greater sample throughput. The analysis of cycle-fluorescence time course is performed with computer software. Amounts of HBV- DNA are then determined by comparing the results to a standard curve produced by universal standards

Artus Biotech and Roche Molecular Systems have real-time-based assays for detection and quantification of HBV-DNA. Currently Cobas Tagman and Abbott platforms which use Cobas Taqman and $m2000_{RT}$ amplification platforms together with automated sample preparations instruments with the AmpliPrep® system (CAP-CTM, Roche Molecular System, Pleasanton, California) and $m2000_{SP}$ (Abbott Diagnostic, Chicago Illinois), respectively are used for detection of both HBV-DNA. The most promising approach for the future is fully automated real-time PCR assays.

There are numerous assays based on different techniques for detection and quantification of viral genomes. As a result of this, the levels given in different units (pg/ml, Meq/ml, genome equivalent/ml, copies/ml e.g.) can not be compared with each other. However, conversion factor has allowed that non standardized units can be converted to copies/ml. But this application is not useful and does not exactly resolve the standardization problem because assays based on different techniques have been calibrated with different standards. Therefore, HBV International Units (IU) established by World Health Organization has been used for universal standardization of all commercial diagnostic kits. Thereby viral levels can be expressed in IU/ml and standardized. However, all the commercial kits have standard deviation of 0.5 $\log_{10}$ IU/ml. Moreover the reduction and increase of viral genome of at least 1 $\log_{10}$ IU/ml is clinically significant.

Sequence Analysis

Direct sequence analysis is the “gold standard” for the determination of the viral genotype and sequence variation (precore/core, resistance mutations e.g.) within viral genome. S regions are sequenced directly after the PCR amplification to determine HBV genotypes, respectively. However, this analysis is labor intensive and prohibitively expensive, especially for routine clinical use. Moreover it only identifies viral variants representing at least 20–25% of the circulating viral populations, as mentioned by [Gauthier et al. \(1999\)](#).

There are three alternative methods to rapidly and less expensively determine genotype and sequence variation: PCR-RFLP and reverse dot blot hybridization. The first method is based on digesting amplified PCR products with specific restriction enzymes and determining the differential migration of the resulting nucleic acid fragments using agarose gel electrophoresis. PCR-RFLP can reliably detect viral variants representing at least 5% of circulating viral populations discussed by [Pawlotsky \(2002\)](#). In second method, the biotinylated amplicons produced in PCR are hybridized with genotype or sequence variation-specific probes which are immobilized on a nitrocellulose strip. After hybridization, streptavidin labeled with alkaline phosphatase is added and bound any biotinylated hybrid previously formed. Incubation with chromogen results in purple a brown band that represents the presence of genotype or mutation. This assay is referred to as “line probe assay”. There are currently commercial available line probe-based assays: INNO-LiPA HBV Genotyping, INNO-LiPA HBV PreCore and The Trugene® HBV Genotyping Kit (Siemens Medical Solutions Diagnostics, Tarrytown, New York). They are not able to identify genotype subtypes.

Treatment Objectives

The topic of treatment will not be dealt at large in this manuscript but today it is well accepted that the crucial principles in the management of chronic HBV are: selection of patient, medication and physician all that with good collaboration and for sufficient duration.

The goals of treatment in chronic HBV are to convert patients from high replicative status to low replicative status as confirmed by HBeAg to anti-HBe seroconversion in addition to loss of HBV DNA, histological improvement and normalization of ALT. The ultimate goals are prevention of cirrhosis and hepatocellular carcinoma, provide a good quality of life and increase survival. A number of the pivotal treatment trials have shown an excellent correlation between all of these end points; individuals who have improved histology also have improvement in these other end points.

The therapeutic armamentarium in this field is enlarging with promising results but at the expense of viral resistance and cost. The drugs used in HBV treatment are mainly interferons (mostly pegylated), and nucleos(t)ide analogs (Lamivudine, Adefovir and the more recent ones: Telbivudine, Entecavir and Tenofovir). Different

treatment guidelines published so far are basically very similar (The more they change the more they look the same). The common message of these guidelines is that the treatment should be curtailed according to different parameters such as the age of the patient, HBe Ag/Ab status, HBV DNA levels, genotype histology, the severity of liver disease and concomitant illnesses. Patients compliance and motivation as well as his/her physical and psychological state should be taken into consideration.

After treatment, even in patients who had sustained response (HBV DNA negative 6 months after treatment) or were luckier enough to achieve HBs seroconversion, there is still a danger of developing HCC in the long term.

There will be a time in the future we will no more talk on HBV, a time when all the newborns would have been vaccinated against hepatitis B, and as stated some centuries ago, measures for prevention would prevail over efforts to treat!

“Act, before disease becomes persistent through long delays...”

Publius Ovidius Naso, Roman poet, (43 BC–17 AD)

References

- Alter MJ (2003) Epidemiology of hepatitis B in Europe and worldwide. *J Hepatol* 39:64–69
- Alter MJ, Margolis HS (1990) The emergence of hepatitis B as a sexually transmitted disease. *Med Clin N Am* 74:1529–1541
- Alter HJ, Seeff LB, Kaplan PM, McAuliffe VJ, Wright EC, Gerin JL et al (1976) Type B hepatitis: the infectivity of blood positive for e antigen and DNA polymerase after accidental needlestick exposure. *N Engl J Med* 295:909–913
- Alter HJ, Purcell RH, Gerin JL, London WT, Kaplan PM, McAuliffe VJ et al (1977) Transmission of hepatitis B to chimpanzees by hepatitis B surface antigen-positive saliva and semen. *Infect Immun* 16:928–933
- Alter MJ, Ahtone J, Maynard JE (1983) Multiple-dose vial associated with transmission of hepatitis B virus in a hemodialysis unit. *Ann Intern Med* 99:330–333
- Amato-Gauci A, Ammon A (2007) ECDC to launch first report on communicable diseases epidemiology in the European Union. *Euro Surveill.* 12(6):E070607.2
- Attila L (2006) Nucleic acid testing for human disease by Taylor & Francis group, chapter 2, pp 19–65
- Bancroft WH, Snitbhan R, Scott RM, Tingpalapong M, Watson WT, Tanticharoenyos P, et al (1977) Transmission of hepatitis B virus to gibbons by exposure to human saliva containing hepatitis B surface antigen. *J Infect Dis* 135:79–85
- Barlet V, Cohard M, Thelu MA, Chaix MJ, Baccard C, Zarski JP, Seigneurin JM (Sep 1994) Quantitative detection of hepatitis B virus DNA in serum using chemiluminescence: comparison with radioactive solution hybridization assay. *J Virol Methods* 49(2):141–151
- Benner SA, Steven A, Sismour M (July 2005) Synthetic biology. *Nat Rev Gen* 6:533–543
- Bernard PS, Wittwer CT (Aug 2002) Real-time PCR technology for cancer diagnostics. *Clin Chem* 48(8):1178–1185 (Review)
- Brechtbuehl K, Whalley SA, Dusheiko GM, Saunders NA (Apr 2001) A rapid real-time quantitative polymerase chain reaction for hepatitis B virus. *J Virol Methods* 93(1–2):105–113
- Brunetto MR, Moriconi F, Bonino F, Lau GK, Farci P, Yurdaydin C, Piratvisuth T, Luo K, Wang Y, Hadziyannis S, Wolf E, McCloud P, Batrla R, Marcellin P (Apr 2009) Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. *Hepatology* 49(4):1141–1150
- Chan HL, Hui AY, Wong ML, Tse AM, Hung LC, Wong VW, Sung JJ (2004) Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. *Gut* 53:1494–1498

- Chan HL, Wong VW, Tse AM, Tse CH, Chim AM, Chan HY, Wong GL, Sung JJ (Dec 2007) Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response. *Clin Gastroenterol Hepatol* 5(12):1462–1468
- Chang MH (2009) Cancer prevention by vaccination against hepatitis B. *Recent Results Cancer Res* 181:85–94 (Review)
- Chevaliez S, Pawlotsky JM (7 May 2007) Hepatitis C virus: virology, diagnosis and management of antiviral therapy. *World J Gastroenterol* 13(17):2461–2466 (Review)
- Chevaliez S, Pawlotsky JM (Jan–Mar 2009) Virological techniques for the diagnosis and monitoring of hepatitis B and C. *Ann Hepatol* 8(1):7–12 (Review)
- Chu CJ, Keeffe EB, Han SH, Perrillo RP, Min AD, Soldevila-Pico C, Carey W, Brown RS Jr, Luketic VA, Terrault N, Lok AS (2003) Hepatitis B virus genotypes in the United States: results of a nationwide survey. *Gastroenterology* 125:444
- Da Villa G, Piccinino F, Scolastico C, Fusco M, Piccinino R, Sepe A (1998) Long-term epidemiological survey of hepatitis B virus infection in a hyperendemic area (Afragola, Southern Italy): results of a pilot vaccination project. *Res Virol* 149:263–270
- Dal-Re R, Aguilar L, Coronel P (1991) Current prevalence of hepatitis B, A and C in a healthy Spanish population. A seroepidemiological study. *Infection* 19:409–413
- Degertekin H, Tuzcu A, Yalcin K (2000) Horizontal transmission of HBV infection among students in Turkey. *Public Health* 114:411–412
- EASL (2009) EASL clinical practice guidelines: management of chronic hepatitis B. *J Hepatol* 50:227–242
- Esteban JI, Sauleda S, Quer J (2008) The changing epidemiology of hepatitis C infection in Europe. *J Hepatol* 48:148–162
- Fattovich G (2003) Natural history and prognosis of hepatitis B. *Semin Liver Dis* 23:47–68
- Fattovich G, Pantalena M, Zagni I, Realdi G, Schalm SW, Christensen E (2002) Effect of hepatitis B and C virus infections on the natural history of compensated cirrhosis: a cohort study of 297 patients. *Am J Gastroenterol* 97:2886–2895
- Fattovich G, Bortolotti F, Donato F (2008) Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. *J Hepatol* 48:335–352
- Fung SK, Lok ASF (2004) Treatment of chronic hepatitis B: who to treat, what to use, and for how long? *Clin Gastroenterol Hepatol* 2:839–848
- Gauthier J, Bourne EJ, Lutz MW, Crowther LM, Dienstag JL, Brown NA, Condreay LD (Dec 1999) Quantitation of hepatitis B viremia and emergence of YMDD variants in patients with chronic hepatitis B treated with lamivudine. *J Infect Dis* 180(6):1757–1762
- Gibb R, Nimmo GR, O'Loughlin P, Lowe P, Drummond D (2007) Detection of HBsAg mutants in a population with a low prevalence of hepatitis B virus infection. *J Med Virol* 79:351–355
- Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV (1999) Viral clearance without destruction of infected cells during acute HBV infection. *Science* 284:825–829
- Hendricks DA, Stowe BJ, Hoo BS, Kolberg J, Irvine BD, Neuwald PD, Urdea MS (Nov 1995) Perrillo RP Quantitation of HBV DNA in human serum using a branched DNA (bDNA) signal amplification assay. *Am J Clin Pathol* 104(5):537–546
- Ho SK, Chan TM, Cheng IK, Lai KN (Aug 1999) Comparison of the second-generation digene hybrid capture assay with the branched-DNA assay for measurement of hepatitis B virus DNA in serum. *J Clin Microbiol* 37(8):2461–2465
- Hsieh CC, Tzonou A, Zavitsanos X, Kaklamani E, Lan SJ, Trichopoulos D (1992) Age at first establishment of chronic hepatitis B virus infection and hepatocellular carcinoma risk. A birth order study. *Am J Epidemiol* 136:1115–1121
- Hui AY, Chan HL, Leung NW, Hung LC, Chan FK, Sung JJ (2002) Survival and prognostic indicators in patients with hepatitis B virus-related cirrhosis after onset of hepatic decompensation. *J Clin Gastroenterol* 34:569–572
- Hui CK, Leung N, Yuen ST, Zhang HY, Leung KW, Lu L et al (2007) Natural history and disease progression in Chinese chronic hepatitis B patients in immune-tolerant phase. *Hepatology* 46:395–401
- Hutin YJ, Harpaz R, Drobeniuc J, Melnic A, Ray C, Favorov M et al (1999) Injections given in health-care settings as a major source of acute hepatitis B in Moldova. *Int J Epidemiol* 28:782–786

- Kane A, Lloyd J, Zaffran M et al (1999) Transmission of hepatitis B, hepatitis C and human immunodeficiency viruses through unsafe injections in the developing world: model- based regional estimates. *Bull World Health Organ* 77(10):801–807
- Kao JH (2007) Role of viral factors in the natural course and therapy of chronic hepatitis B. *Hepatol Int* 1:415–430
- Kao JH, Chen PJ, Lai MY, Chen DS (2000) Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 118:554–559
- Kent GB, Brondum J, Keenlyside RA, LaFazia LM, Scott HD (1988) A large outbreak of acupuncture- associated hepatitis B. *Am J Epidemiol* 127:591–598
- Kyriakis KP, Foudoulaki LE, Papoulia EI, Sofroniadou KE (2000) Seroprevalence of hepatitis B surface antigen (HBsAg) among first-time and sporadic blood donors in Greece: 1991–1996. *Transfusion Med* 10:175–180
- Lavanchy D (2004) Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepatol* 11:97–107
- Lee WM (1997) Hepatitis B virus infection. *N Engl J Med* 337(24):1733–1745
- Liaw JF, Chu CM (2009) Hepatitis B virus infection. *Lancet* 373:582–592
- Limentani AE, Elliott LM (1979) An outbreak of hepatitis B from tattooing. *Lancet* 2:86–88
- Lin HH, Kao JH, Chang TC, Hsu HY, Chen DS (2003) Secular trend of age-specific prevalence of hepatitis B surface and e antigenemia in pregnant women in Taiwan. *J Med Virol* 69:466–470
- Lionis C, Koulentaki M, Biziagos E, Kouroumalis E (1997) Current prevalence of hepatitis A, B and C in a well-defined area in rural Crete, Greece. *J Viral Hepatitis* 4:55–61
- Loeb KR, Jerome KR, Goddard J, Huang M, Cent A, Corey L (Sep 2000) High-throughput quantitative analysis of hepatitis B virus DNA in serum using the TaqMan fluorogenic detection system. *Hepatology* 32(3):626–629
- Lok AS (2002) Chronic hepatitis B. *N Engl J Med* 346(22):1682–1683
- Lok AS, McMahon BJ (2007) Chronic hepatitis B. *Hepatology* 45:507–539
- Lok AS, Heathcote EJ, Hoofnagle JH (2001) Management of hepatitis B: 2000 – summary of a workshop. *Gastroenterology* 120(7):1828–1853
- Lopez VA, Bourne EJ, Lutz MW, Condreay LD (Jun 2002) Assessment of the COBAS Amplicor HBV Monitor Test for quantitation of serum hepatitis B virus DNA levels. *J Clin Microbiol* 40(6):1972–1976
- Lorinez A (2006) Nucleic acid testing for human disease in nucleic acid technology. CRC Press/ Taylor & Francis, Boca Raton, FL co. 616.075 LOR. (ISSN 1-57444-543-X)
- Mackay IM (2004) Real-Time PCR in the microbiology laboratory. *Clin Microbiol Infect* 10:190–212
- Magdzik W (2000) Hepatitis B epidemiology in Poland, Central and Eastern Europe and the newly independent states. *Vaccine* 18:S13–S16
- Mahoney FJ (1999) Update on diagnosis, management, and prevention of hepatitis B virus infection. *Clin Microbiol Rev* 12(2):351–366
- Maini MK, Boni C, Lee CK et al (2000) The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. *J Exp Med* 191:1269–1280
- Manesis EK (2006) HBeAg-negative chronic hepatitis B: from obscurity to prominence. *J Hepatol* 45:343–346
- Marin IJ, Poljak M, Seme K, Meglic-Volkar J, Maticic M, Lesnicar G, Brinovec V (Feb 2001) Comparative evaluation of semiautomated COBAS AMPLICOR hepatitis B virus (HBV) monitor test and manual microwell plate-based AMPLICOR HBV MONITOR test. *J Clin Microbiol* 39(2):758–761
- Marion SA, Pastore MT, Pi DW, Mathias RG (1994) Longterm follow-up of hepatitis B vaccine in infants of carrier mothers. *Am J Epidemiol* 140:734–746
- Martinot-Peignoux M, Boyer N, Colombat M, Akreimi R, Pham BN, Ollivier S et al (2002) Serum hepatitis B virus DNA levels and liver histology in inactive HBsAg carriers. *J Hepatol* 36:543–546

- Mazokopakis E, Vlachonikolis J, Philalithis A, Lionis C (2000) Seroprevalence of hepatitis A, B and C markers in Greek warship personnel. *Eur J Epidemiol* 16:1069–1072
- McKnight CA, Des Jarlais DC, Perlis T, Eigo K (2007) Syringe exchange programs—United States, 2005. *MMWR Morb Mortal Wkly Rep* 56:1164–67
- Mele A, Tancredi F, Romano L, Giuseppone A, Colucci M, Sangiuolo A, Lecce R, Adamo B, Tosti ME, Taliani G, Zanetti AR (2001) Effectiveness of hepatitis B vaccination in babies born to hepatitis B surface antigen-positive mothers in Italy. *J Infect Dis* 184:905–908
- Muhlbacher A, Weber B, Burgisser P, Eiras A, Cabrera J, Louisirothanakul S, Tiller FW et al (2008) Multicenter study of a new fully automated HBsAg screening assay with enhanced sensitivity for the detection of HBV mutants. *Med Microbiol Immunol* 197:55–64
- Murray JM, Wieland SF, Purcell RH, Chisari FV (2005) Dynamics of hepatitis B virus clearance in chimpanzees. *Proc Natl Acad Sci USA* 102:17780–17785
- Ni Y-H, Chang M-H, Huang L-M, Chen HL, Hsu HY, Chiu TY et al (2001) Hepatitis B virus infection in children and adolescents in a hyperendemic area: 15 years after mass hepatitis B vaccination. *Ann Intern Med* 135:796–800
- Ni YH, Chang MH, Wang KJ et al (2004) Clinical relevance of hepatitis B virus genotype in children with chronic infection and hepatocellular carcinoma. *Gastroenterology* 127:1733–1738
- Ozars R, Tabak F, Tahan V, Ozturk R, Akin H, Mert A, Senturk H (2008) Correlation of quantitative assay of HBsAg and HBV DNA levels during chronic HBV treatment. *Dig Dis Sci* 53:2995–2998
- Palumbo E, Scotto G, Cibelli D, Faleo G, Saracin A, Angarano G (2008) Immigration and hepatitis B virus: epidemiological, clinical and therapeutic aspects. *La Revue de Santé de la Méditerranée orientale* 14(4):784–790
- Parkin DM, Bray F, Ferlay J, Pisani P (2001) Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 94(2):153–156
- Pawlotsky JM. Molecular diagnosis of viral hepatitis. *Gastroenterology* 2002;122:1554–1568
- Perz JF, Elm JL Jr, Fiore AE, Huggler JI, Kuhnert WL, Effler PV (2006a) Near elimination of hepatitis B virus infections among Hawaii elementary school children after universal hepatitis B vaccination. *Pediatrics* 118:1403
- Perz JF, Armstrong GL, Farrington LA et al (2006b) The contribution of hepatitis B and hepatitis C virus infection to cirrhosis and primary liver cancer worldwide. *J Hepatol* 45:529–538
- Ronsin C, Pillet A, Bali C, Denoyel GA (Apr 2006) Evaluation of the COBAS Ampli Prep-total nucleic acid isolation-COBAS TaqMan hepatitis B virus (HBV) quantitative test and comparison to the VERSANT HBV DNA 3.0 assay. *J Clin Microbiol* 44(4):1390–1399
- Sakugawa H, Kobashigawa K, Nakayoshi T, Yamashiro T, Maeshiro T, Tomimori K, Kinjo F, Saito A, Mukaide M (Aug 2003) Monitoring low level hepatitis B virus by a newly developed sensitive test. *Hepatol Res* 26(4):281–286
- Schmunis GA, Zicker F, Cruz JR, Cuchi P (2001) Safety of blood supply for infectious diseases in Latin American countries, 1994–1997. *Am J Trop Med Hyg* 65(6):924–930
- Simonsen L, Kane A, Lloyd J, Zaffran M, Kane M (1999) Unsafe injections in the developing world and transmission of bloodborne pathogens: a review. *Bull World Health Organ* 77(10):789–800
- Stelzl E, Muller Z, Marth E, Kessler HH (Jun 2004) Rapid quantification of hepatitis B virus DNA by automated sample preparation and real-time PCR. *J Clin Microbiol* 42(6):2445–2449
- Summers J, Mason WS (1982) Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell* 29:403–415
- Toy M, Önder FM, Wormann T, Richardus JH, Schalm S, dilman H, De ertekin H, Yurdaydın C (2009) Pevalance of hepatitis B in turkey: meta-analysis -abstract. 7th National Meeting of Hepatology, Izmir, Turkey, 10–14 June 2009 (abstract book)
- Ture Ozdemir F, Duman D, Ertem D, Avsar E, Ozdogan O, Eren F, Kalaycı C, Aslan N, Bozdayı AM, Tozun N (Dec 2005) Determination of hepatitis B genotypes in patients with chronic hepatitis B virus infection in Turkey. *Turk J Gastroenterol* 16(4):183–187

- Weber B, Van der Taelem-Brule N, Berger A, Simon F, Geudin M, Ritter J (2006) Evaluation of a new automated assay for hepatitis B surface antigen (HBsAg) detection Vidas HBsAg ultra. *J Virol Methods* 135:109–117
- Wheelely SM, Boxall EH, Tarlow MJ, Gatrad AR, Anderson J, Bissenden J, Chin KC, Mayne A (1990) Hepatitis B vaccine in the prevention of perinatally transmitted hepatitis B virus infection: final report on a west midlands pilot study. *J Med Virol* 30:113–116
- Wong ML, Medrano JF (July 2005) Real-time PCR for mRNA quantitation. *Biotechniques* 39(1):75–85 (Review)
- Wong VCW, Ip HMH, Reesink HW, Kuhns MC (1984) Prevention of the HBsAg-carrier state in newborn infants of mothers who are chronic carriers of HBsAg and HBeAg by administration of hepatitis vaccine and hepatitis-B immunoglobulin. *Lancet* 8383:921–926
- World Health Organization (2000) Hepatitis B. World Health Organization Fact Sheet 204 (Revised October 2000). WHO Web site. 2000. <http://who.int/inf-fs/en/fact204.html>
- Worman HJ (Aug 1997) Molecular biological methods in diagnosis and treatment of liver diseases. *Clin Chem* 43(8 Pt 2):1476–1486
- Xu ZY, Duan SC, Margolis HS (1995) Long-term efficacy of active postexposure immunization of infants for prevention of hepatitis B virus infection. United States-people's republic of China study group on hepatitis B. *J Infect Dis* 171:54–60
- Yao JD, Beld MG, Oon LL, Sherlock CH, Germer J, Menting S, Se Thoe SY, Merrick L, Ziermann R, Surtihadi J, Hnatyszyn HJ (Feb 2004) Multicenter evaluation of the VERSANT hepatitis B virus DNA 3.0 assay. *J Clin Microbiol* 42(2):800–806
- Yuen MF, Sablon E, Tanaka Y, Kato T, Mizokami M, Doutreloigne J, Yuan HJ, Wong DK, Sum SM, Lai CL (2004) Epidemiological study of hepatitis B virus genotypes, core promoter and precore mutations of chronic hepatitis B infection in Hong Kong. *J Hepatol* 41:119–125

Hepatitis E Virus (HEV) – An Emerging Viral Pathogen

Avrelija Cencič and Walter Chingwaru

Abstract Hepatitis E virus (HEV) is the second major etiologic agent of enterically transmitted non-A, non-B hepatitis worldwide. HEV is an unenveloped, single-stranded RNA virus having genome of approximately 8 kb in size. It is the sole member of the genus *Hepevirus* in the family of *Hepeviridae*. Although it is most commonly recognised to occur in large outbreaks, HEV infection accounts for >50% of acute sporadic hepatitis in both, children and adults in some high endemic areas as Northern Africa and most of Asia. Whereas it was long believed that it is prevalent in the areas where hygiene standards are poor, HEV is expected in non-endemic countries as well. Although HEV is mainly transmitted via the faecal-oral route, a high potential exist for water and foodborne transmission, parenteral and perinatal routes. Person to person contact have been implicated. Cases of acute hepatitis of novel HEV variants have been reported in humans in Europe, Japan and USA, showing that HEV is not limited or geographically distributed. Recently an avian HEV was found to be transmitted via the egg yolk. HEV infection can lead to deadly hepatic failure in 1–4% in the common population, but the mortality incidence reaches 0.5–3% among young adults and 15–20% among pregnant women. A chronic infection has developed in patients with liver transplants and in immune compromised people. Due to the lack of practical models for HEV, the mechanisms of HEV pathogenesis and replication are still not well understood. The diagnosis of HEV infection can be made using serological tests but still, RT-PCR is considered as the gold standard. Up to date, no treatment for HEV is available, but recently, a new vaccine, ending the phase 2 trials has showed promising outcomes. Here, we discuss the current knowledge on the molecular biology, pathology, clinical features, transmission, diagnosis, epidemiology, and prevention of HEV.

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Introduction

Foodborne and waterborne viral infections are increasingly recognised as causes of illness in humans. This increase is partly explained by changes in food processing and consumption patterns, e.g. increased consumption of ecologically produced food. The European directive establishes no specific microbiological criteria concerning the presence of viruses, mainly because quantitative methods for their detection are not yet available, although it is shown that the presence of viruses has been detected in foods that met bacteriological standards. It is not clear whether routine monitoring of food specimens for viral contamination will be feasible. A major problem concerning viruses in organic and conventional production is the use of irrigation water that is fecally contaminated and in most cases contains enteric viruses that persist inside watered vegetables that are eaten raw (Cencic and Borec 2007). One of the threatening and emerging foodborne viruses is without doubt Hepatitis E virus (HEV). Hepatitis is a general term meaning inflammation of the liver. Hepatitis is a disease that can be caused by a variety of different viruses such as hepatitis A, B, C, D and E. Hepatitis E virus (HEV), is the major cause for enterically transmitted non-A, non-B hepatitis worldwide, spherical, non-enveloped, and has a single stranded RNA virus that is approximately 32–34 nm in diameter. HEV has been provisionally classified in the *Caliciviridae* family based on similar physicochemical and biologic properties; recently however, it has been found that the organization of the HEV genome is substantially different from that of other caliciviruses, so it is now classified in a separate family. Taniguchi et al. 2009 have emphasized that HEV is endemic in, and tends to accumulate in developing countries. Not futuristic, it can be used as a potential bio-threat.

Hepatitis E Virus (HEV) Epidemiology

Although hepatitis E is most commonly recognized to occur in large outbreaks, HEV infection accounts for >50% of acute sporadic hepatitis in both children and adults in some high endemic areas (Krawczynski et al. 2001). It is believed that about two billion people live in areas endemic for HEV (reviewed by Chandra et al. 2008). Risk factors for infection among persons with sporadic cases of hepatitis E have not been defined. Unlike hepatitis A virus, which is also transmitted by the faecal-oral route, person-to-person transmission of HEV appears to be uncommon. However, nosocomial transmission, presumably by person-to-person contact, has been reported to occur. Virtually all cases of acute hepatitis E in the United States have been reported among travellers returning from high HEV-endemic areas. Although HEV is often a self-limiting disease with a relatively low overall death rate (0.5–3.0%), the

death rate during pregnancy approaches 15–25% with possibilities of foetal death, abortion, premature delivery, or death of a live-born baby soon after birth (Smith 2001; Chibber et al. 2004). HEV is transmitted primarily by the faecal-oral route and waterborne epidemics are characteristics of hepatitis E. HEV, known for being the cause of major outbreaks of waterborne hepatitis in Asia and Africa, is an emerging pathogen in industrialized countries (Bihl and Negro 2008). Clinical cases of HEV infection in developed countries have generally been associated with travel to tropical or subtropical countries. There has also been an increasing number of reports from Europe and the United States of sporadic hepatitis attributable to HEV but not associated with travel, leading to suggestions that HEV may be endemic at low levels in developed countries. HEV has been reported in Netherlands (Widdowson et al. 2003), Spain (Peralta et al. 2009), France (Legrand-Abravanel et al. 2009), Greece (Stefanidis et al. 2004), Sweden (Olsen et al. 2006), UK (Ijaz et al. 2009), Italy (Di Bartolo et al. 2008), United States (Atiq et al. 2009), Japan (Tanaka et al. 2001) more cases are likely to be reported (Fig. 1).

It has been recognised that HEV and HEV related viruses circulate in domestic animals native to industrial countries and should be considered as a potential public health hazard (Yazaki et al. 2003; Widdowson et al. 2003). This is well documented in swine, but also in chicken (Clemente-Casares et al. 2003). Like swine HEV, avian HEV is also related genetically and antigenically to human HEV.

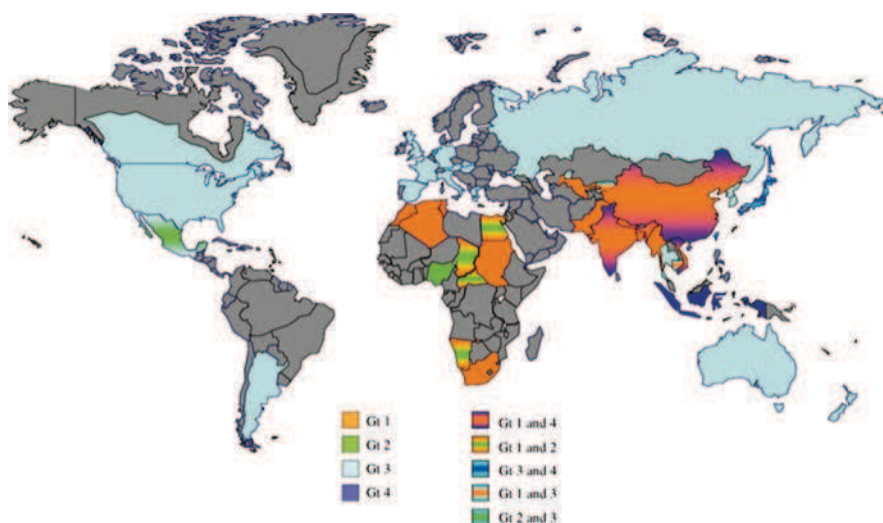


Fig. 1 Geographical distribution of HEV isolates according to genotypes (Gt). HEV Gt 1 and 2: epidemic strains causing human infection. HEV Gt 3 and 4: zoonotic strains isolated from humans and a variety of animals, particularly pigs. In some countries, different genotypes co-circulate in distinct ecological niches: Gt 1 and 4 in China, India, and Vietnam; Gt 1 and 2 in several African countries, including Namibia, Chad, and Sudan; Gt 3 and 4 in Japan; Gt 1 and 3 in Cambodia; Gt 2 and 3 in Mexico (www.eht-forum.org)

Thus the possibility that farm animal species could represent reservoirs for human contamination through food and meat has to be considered seriously. As an example, a recent report indicate that several patients who contracted sporadic acute or fulminant hepatitis E in Japan had a history of consuming grilled or undercooked pig liver 2–8 weeks before the disease onset (Yazaki et al. 2003). Direct transmission of HEV from deer to humans has been reported after eating uncooked meat (Tei et al., 2004). Wild rats have been shown by genetically methods to harbour a strain of HEV (Tsarev et al. 1998). Foods like shellfish have been demonstrated to be potential vehicles for HEV transmission (Smith 2001; Song et al. 2010). Recently pigs and cats infected with HEV, as well as oysters contaminated with HEV have also been shown to be potential risk factors for HEV transmission to humans (Song et al. 2010). In developing countries, zoonotic transmission of HEV is a serious issue, and evolution of virulent strains would have more far-reaching consequences. Lately, the transmission of HEV through irrigated vegetables and fruits has additionally posed a concern of HEV transmission via plants. How and where HEV persists inside plants is not yet known. Fourteen cases of acute HEV infection in three patients receiving liver transplants were identified, in nine receiving kidney transplants, and in two receiving kidney and pancreas transplants. All patients were positive for serum HEV RNA. Chronic hepatitis developed in eight patients, as confirmed by persistently elevated (Kamar et al. 2008).

Hepatitis E Virus Description

HEV is the major etiologic agent of enterically transmitted non-A, non-B hepatitis worldwide. It is a spherical, non-enveloped, single (plus) stranded RNA virus that is approximately 32–34 nm in diameter (Fig. 2). HEV belongs to a genus of HEV-like viruses (unassigned genus). Hepatitis E virus (HEV) is a positive-stranded RNA virus with a 7.2 kb genome that is capped and polyadenylated. The virus is currently unclassified: the organisation of the genome resembles that of the Caliciviridae but sequence analyses suggest it is more closely related to the Togaviridae (Yang and Vyas 1996).



Fig. 2 Schematic presentation of HEV

Hepatitis E virus was cloned in 1991 and the entire 7.5 kb sequence is known. The organization of the genome is distinct from the *Picornaviridae* and the non-structural and structural polypeptides are encoded respectively at the 5' and 3' ends (Wang et al. 2001). HEV resembles the caliciviruses in the size and organization of its genome, as well as the size and morphology of the virion. It has a particle diameter of 32–34 nm, a buoyant density of 1.29 g/ml in KTar/Gly gradient, and is very labile. Serologically related smaller (27–30 nm) particles are often found in faeces of patients with Hepatitis E and are presumed to represent degraded viral particles. HEV has a single-stranded polyadenylated RNA genome consisting of three open reading frames ORF1, ORF2 and ORF3. Of these, ORF2 encodes the major capsid protein of the virus and ORF3 encodes a small protein of unknown function (Fig. 3).

Four genotypes of HEV but only one serotype have been identified. Most of the emerging cases caused by HEV belong to the genotype 3 (Fig. 4) (Borgen et al. 2008).

The Disease

The incubation period following exposure to HEV ranges from 3 to 8 weeks, with a mean of 40 days. The period of communicability is unknown. There are no chronic infections reported. However, recent reports present biochemical, histological and genetic evidence of chronic HEV infection in transplant patients (reviewed by Chandra et al. 2008).

Hepatitis E virus causes acute sporadic and epidemic viral hepatitis. Symptomatic HEV infection is most common in young adults aged 15–40 years. Although HEV

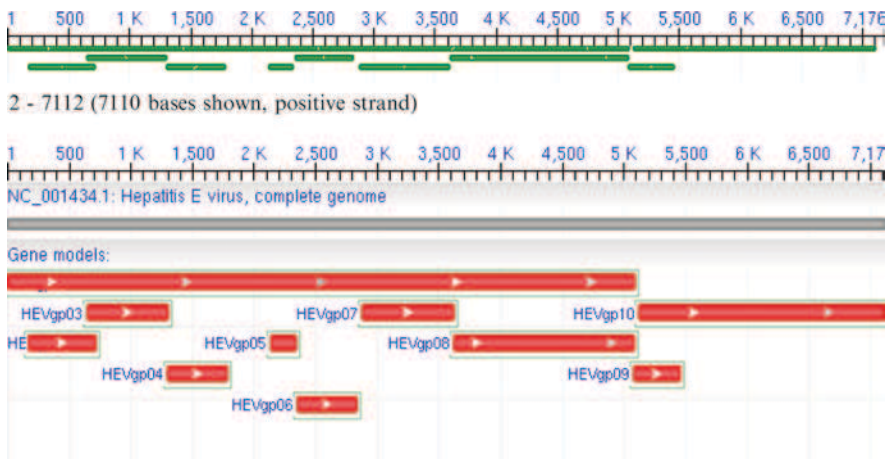


Fig. 3 Hepatitis E virus, complete genome (<http://www.ncbi.nlm.nih.gov/nuccore/9626440?report=graph&from=1&to=7105>)

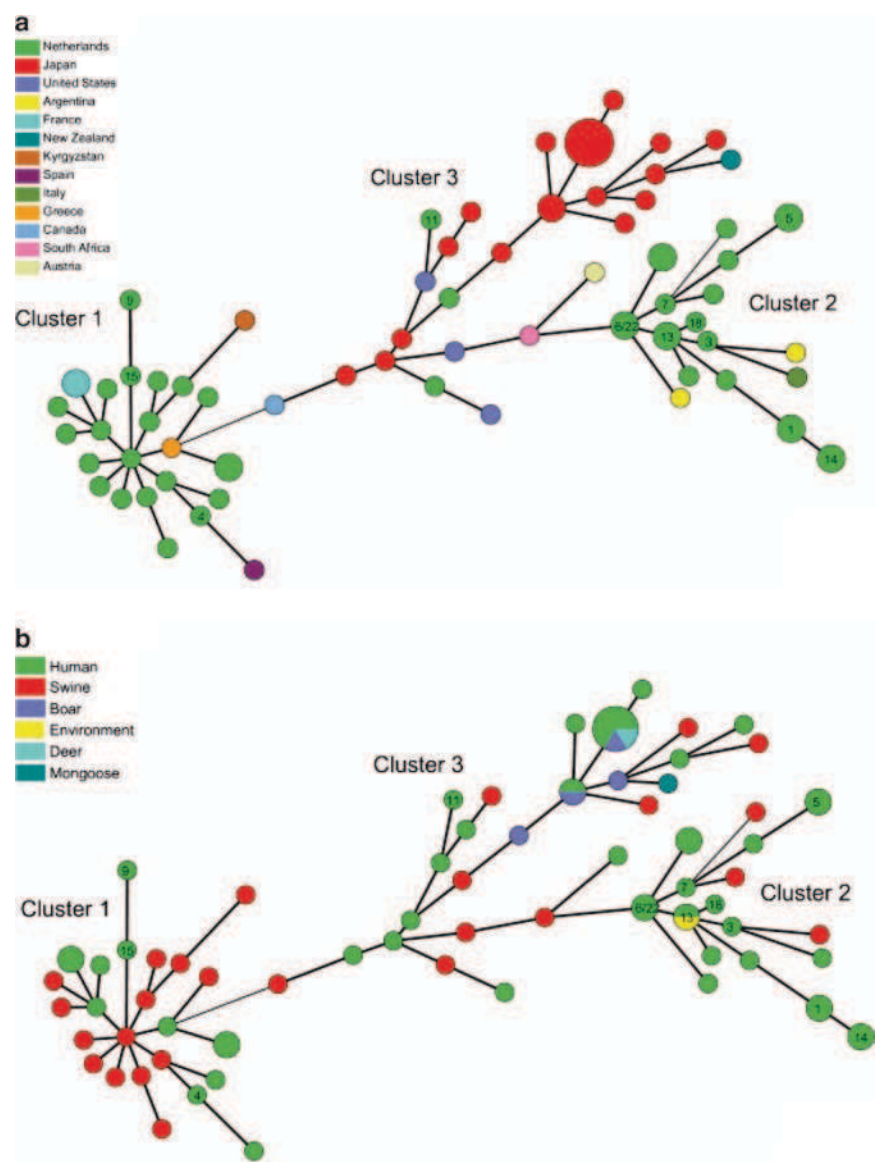


Fig. 4 Genetic relatedness between HEV strains from human and non-human sources and from various countries. Minimal spanning trees of 81 sequences of 148 nucleotides HEV RNA showing genetic distances between genotype three HEV strains from humans and animals. **(a)** Strains labelled by geographical origin. **(b)** Strains labelled by biological origin. The 13 recent Dutch cases are marked with ID numbers inside the coloured circles (adapted from Borgen et al. *BMC Infectious Diseases* 2008 **8**:61 doi:10.1186/1471-2334-8-61)

infection is frequent in children, it is mostly asymptomatic or causes a very mild illness without jaundice (anicteric) that goes undiagnosed. Pregnant women appear to be exceptionally susceptible to severe disease, and excessive mortality has been reported in this group (Schmidt and Wolff 2008).

Typical signs and symptoms of hepatitis include jaundice (yellow discoloration of the skin and sclera of the eyes, dark urine and pale stools), anorexia (loss of appetite), an enlarged, tender liver (hepatomegaly), abdominal pain and tenderness, nausea and vomiting, and fever, although the disease may range in severity from subclinical to fulminant.

Hepatitis caused by HEV is clinically indistinguishable from hepatitis A disease. Symptoms include malaise, anorexia, abdominal pain, arthralgia, and fever, like the general form of hepatitis (Goens and Perdue 2004). The infective dose is not known (Labrique et al. 1999).

The disease is most often seen in young to middle aged adults (15–40 years old). Pregnant women appear to be exceptionally susceptible to severe disease, and excessive mortality has been reported in this group (Schmidt and Wolff 2008).

Diagnosis

Since cases of hepatitis E are not clinically distinguishable from other types of acute viral hepatitis, diagnosis is made by blood tests which detect elevated antibody levels of specific antibodies to hepatitis E in the body or by reverse transcriptase polymerase chain reaction (RT-PCR). Unfortunately, such tests are not widely available.

Hepatitis E should be suspected in outbreaks of waterborne hepatitis occurring in developing countries, especially if the disease is more severe in pregnant women, or if hepatitis A has been excluded. If laboratory tests are not available, epidemiologic evidence can help in establishing a diagnosis (WHO 2005).

Diagnosis of HEV is based on the epidemiological characteristics of the outbreak and by exclusion of hepatitis A and B viruses by serological tests. Confirmation requires identification of the 27–34 nm virus-like particles by immune electron microscopy in faeces of acutely ill patients. Retrospective testing of serum samples from patients involved in various epidemics of hepatitis associated with contamination of water supplies with human faeces indicated that an agent other than HAV (or hepatitis B) was involved. Virus-like particles have been detected in the faeces of infected individuals by immune electron microscopy using convalescent serum. However, such studies have often proved inconclusive, and a large proportion of the excreted virus may be degraded during passage through the gut. The particles have a mean diameter of 32–34 nm. Cross reaction studies between sera and virus in faeces associated with a variety of epidemics in several different countries suggests that a single serotype of virus is involved (WHO 2005).

Sequencing of the HEV genome has allowed the development of a number of specific diagnostic tests (Touze et al. 1999. For example, HEV RNA was detected,

using the polymerase chain reaction (PCR), in faecal samples obtained during a recent epidemic in Kanpur (North India). An enzyme, immunoabsorbent assay, which detects both IgG and IgM anti-HEV, has been developed using a recombinant HEV-glutathione-S-transferase fusion protein and used to detect antibodies in sporadic cases of enterically-transmitted non-A, non-B hepatitis in children in Egypt (Jameel 1999).

Replicative virus has been found in the small intestine, lymph nodes, colon as well as the liver of experimentally infected pigs (Williams et al. 2001). An *in vitro* culture system up to date was not available. Despite this difficulty a number of vaccine candidates are under investigation. HEV has not been isolated from foods. No method is currently available for routine analysis of foods.

Vaccines

At present, no commercially available vaccines exist for the prevention of hepatitis E. However, several studies for the development of an effective vaccine against hepatitis E are in progress. Preliminary but significant progress has been made towards the development of hepatitis E vaccine, using the trpE-C2 fusion protein (Purdy et al. 1993). In limited experiments, three doses of the fusion protein, which represents the carboxyl two-thirds of the putative capsid protein, prevented the development of biochemical evidence of hepatitis after challenge with wild-type virus (Purdy et al. 1993).

Prevention

As almost all HEV infections are spread by the faecal-oral route, good personal hygiene, high quality standards for public water supplies and proper disposal of sanitary waste have resulted in a low prevalence of HEV infections in many well developed societies (WHO 2005).

For travellers to highly endemic areas, the usual elementary food hygiene precautions are recommended. These include avoiding drinking water and/or ice of unknown purity and eating uncooked shellfish, uncooked fruits or vegetables that are not peeled or prepared by the traveller (WHO 2005).

Treatment

Since hepatitis E is a viral disease, and as such, antibiotics are of no value in the treatment of the infection. There is no hyperimmune E globulin available for pre- or post-exposure prophylaxis. HEV infections are usually self-limited, and hospitalization is generally not required. No available therapy is capable of altering the course of acute infection (WHO 2005).

As no specific therapy is capable of altering the course of acute hepatitis E infection, prevention is the most effective approach against the disease. Hospitalization is required for fulminant hepatitis and should be considered for infected pregnant women. However, in our laboratory we obtained promising results with some natural products including probiotics (World Health Organisation 2005).

HEV (an emerging pathogen) interactions with the host intestinal tract

Molecular methods of detecting HEV are well established to study various HEV strains (Kase et al. 2009; Kulkarni et al. 2008; Liu et al. 2009; Reuter et al. 2009; Zhang et al. 2009). More studies are needed to elucidate the genotype of HEV circulating in the world areas and its transmission routes (Taniguchi et al. 2009). However cultural potential of HEV has not yet been fully explored. Propagation and production of HEV in vitro have been attempted in various continuous cell lines (Huang et al. 1999; Kazachkov et al. 1992; Li et al. 1996; Meng et al. 1997a; Wei et al. 2000) and in primary hepatocytes from non-human primates (Kane et al. 1984; Tsarev et al. 1994). However, the lack of an efficient cell-culture system for HEV has greatly hampered detailed analysis of the virus replication cycle in infected cells to resolve many important questions. Recently, Emerson et al. (2006) reported a quantitative cell-culture assay for neutralization tests and thermal stability tests by detecting infected culture cells by immunofluorescence microscopy.

Huang et al. (1999) reported successful isolation and identification of a sporadic HEV strain (G93-2) in human lung carcinoma cell (A549). Huang et al. (1999) made stern recommendations for the establishment of a propagation method for both sporadic and epidemic HEV strains in cell culture systems, the target being to facilitate vaccine research.

No reliable propagation method has been reported for HEV consequently at present infectivity of the virus cannot be reliably studied. Le et al. (2001) reported an attempt to propagate HEV. They passaged HEV in Rhesus monkeys, then, with super-centrifugation they obtained the virus for culture. They then inoculated the HEV culture in various human-derived (including KMB17, A549, BEL7402, and Hela) and non-human primates derived cells (Vero). Sensitivity of cells to HEV was measured by CPE (cytopathic effect), RT-PCR and immunofluorescence. They realized that some cells were sensitive to HEV (namely KMB17, A549 and BEL7402 cells) and the Hela and Vero cells were insensitive. HEV was detectable with in human KMB17 diploid cells by immunofluorescence and RT-PCR. In short their attempt could not provide a protocol to propagate HEV.

Many studies have determined binding sites for HEV on four HEV susceptible human hepatoma cell lines, namely HepG2 (He et al. 2008), Huh7 (Emerson et al. 2004), PLC/PRF5 (Meng et al. 1997; Tanaka et al. 2007) and a lung carcinoma, human (A549) (Huang et al. 1995; Tanaka et al. 2007). It has been determined that a truncated peptide of the HEV structural protein, p239 (aa 368–606), binds to the four cell lines (HepG2, Huh7, PLC/PRF5 and A549). Immunofluorescence

studies performed with two susceptible cell lines, HepG2 and Huh7, showed that binding occurred initially at the cell surface and that the peptide later gained entry to the cell cytoplasm. He et al. (2008) therefore show that p239 can serve as a probe for the HEV receptor, and they also suggest that infection may involve distinct receptor-binding sites on the virus capsid. While efforts have been made to determine the binding of HEV on hepatocytes, no similar reports of binding sites of HEV on enterocytes has been reported.

Recently Lorenzo et al. (2008) reportedly developed a cell culture system for hepatitis E virus (HEV) in hepatocellular carcinoma (HCC) cells, using a genotype 3 HEV (JE03-1760F strain), and noted that the virus was mutating along passages, as previously reported by Tanaka et al. (2007). Lorenzo et al. (2008) noted that passaging was limited by variable adaptation of HEV mutants to growth in vitro, similar to that observed in hepatitis A virus. Attenuation has been noted in the related Hepatitis A Virus along the passages and tends to increase with number of in vitro passages (Karron et al. 1988). Another limitation of this cell model system may be its carcinogenic nature.

The reports available thus far may provide useful information on dynamics of attachment and entry of HEV on/into cells, and mechanisms of loss of viability of HEV due to mutations. Information on the route of infection of HEV from the time it enters an animal (in the gut), to the liver and how it maintains in the intestinal and intestinal cells is still scanty.

Our efforts were and continue to be on establishment of a protocol to propagate HEV using a system composed of several animal cell cultures (enterocytes, monocytes, hepatocytes and so on), as is possible with many other viruses. We used HEV infected pig liver tissue and faecal material, a generous gift from Dr. Frederik Widen, (Swedish University of Agricultural Sciences), to isolate hepatitis E virus. We followed standard isolation procedures for viruses. We tested the virus in cells of porcine small intestinal cell (CLAB, PSI) (Gradisnik et al. 2006), porcine hepatocyte cell (HEP 20), porcine blood macrophages (PoM2) (Gradisnik et al. 2006), all established in our facility in Faculty of Agriculture and Life Sciences, University of Maribor; and those of bovine enterocyte (CIEB), human cancer enterocyte (Caco2), monkey cell (MA104) origin. We determined the presence of HEV using cytopathic effects on different cell lines as stated above, and confirmed with RT-PCR and dot-blot. Propagation was performed with a rotational system of the different cell lines as stated above. We have consequently been able to show that HEV can be maintained and its titre increased using the various cultural treatments and a rotational cell line system.

References

- Atiq M, Shire NJ, Barrett A, Rouster SD, Sherman KE, Shata MT (2009) Hepatitis E virus antibodies in patients with chronic liver disease. *Emerg Infect Dis* 15(3):479–481
- Bihl F, Negro F. 2008. [New aspects of HEV infection] *Rev Med Suisse*. 4(169):1863–1866

- Borgen K, Herremans T, Duizer E, Vennema H, Rutjes S, Bosman A, de Roda Husman AM, Koopmans M (2008) Non-travel related Hepatitis E virus genotype 3 infections in the Netherlands; a case series 2004–2006. *BMC Infect Dis* 8:61
- Cencic A, Borec A (2007) “Microbial Food Safety Standards in Organic Farming – a Review,” by *Agricultura Slovenia Note*. Review article (Keywords: literature reviews, laws and regulations, microbial contamination, food safety, vegetables, fruits (food)) 5(1):5–14. Research affiliation, Slovenia
- Ceylan A, Ertem M, Ilcin E, Ozekinci T (2003) A special risk group for hepatitis E infection: Turkish agricultural workers who use untreated waste water for irrigation. *Epidemiol Infect* 131(1):753–756
- Chandra V, Taneja S, Kalia M, Jameel S (2008) Molecular biology and pathogenesis of hepatitis E virus. *J Biosci* 33(4):451–464
- Chibber RM, Usmani MA, Al-Sibai MH (2004) Should HEV infected mothers breast feed? *Arch Gynecol Obstet* 270(1):15–20
- Clemente-Casares P, Pina S, Buti M, Jardi R, MartIn M, Bofill-Mas S, Girones R (2003) Hepatitis E virus epidemiology in industrialized countries. *Emerg Infect Dis* 9(4):448–454
- Di Bartolo I, Martelli F, Inglese N, Pourshaban M, Caprioli A, Ostanello F, Ruggeri FM (2008) Widespread diffusion of genotype 3 hepatitis E virus among farming swine in Northern Italy. *Vet Microbiol* 132(1–2):47–55
- Emerson SU, Clemente-Casares P, Moiduddin N, Arankelle VA, Torian U, Purcell RH (2006) Putative neutralization epitopes and broad cross-genotype neutralization of *Hepatitis E virus* confirmed by a quantitative cell-culture assay. *J Gen Virol* 87:697–704
- Goens SD, Perdue ML (2004) Hepatitis E viruses in humans and animals, *Animal Health Research Reviews* 5:145–156
- Gradisnik L, Filipic B, de Vaureix C, LeFevre F, La Bonnardiere C, Cencic A (2006) Establishment of a functional cell culture model of the pig small intestine. *ALTEX* 23, 94
- Guo H, Zhou EM, Sun ZF, Meng XJ (2007) Egg whites from eggs of chickens infected experimentally with avian hepatitis E virus contain infectious virus, but evidence of complete vertical transmission is lacking. *J Gen Virol* 88(Pt 5):1532–1537
- He S, Miao J, Zheng Z, Wu T, Xie M, Tang M, Zhang J, Ng MH, Xia N (2008) Putative receptor-binding sites of hepatitis E virus. *J Gen Virol* 89(Pt 1):245–249
- Huang R, Li D, Wei S, Li Q, Yuan X, Geng L, Li X, Liu M (1999) Cell-culture of sporadic *Hepatitis E virus* in China. *Clin Diagn Lab Immunol* 6:729–733
- Huang R, Nakazono N, Ishii K, Li D, Kawamata O, Kawaguchi R, Tsukada Y (1995) Hepatitis E virus (87A strain) propagated in A549 cells. *J Med Virol* 47(4):299–302
- Ijaz S, Vyse AJ, Morgan D, Pebody RG, Tedder RS, Brown D (2009) Indigenous hepatitis E virus infection in England: More common than it seems. *J Clin Virol* 44(4):272–276
- Jameel S (1999) Molecular biology and pathogenesis of hepatitis E virus. *Expet Rev Mol Med* 1:1–16
- Kamar N, Mansuy JM, Cointault O, Selves J, Abravanel F, Danjoux M, Otal P, Esposito L, Durand D, Izopet J, Rostaing L (2008) Hepatitis E virus-related cirrhosis in kidney- and kidney-pancreas-transplant recipients. *Am J Transplant* 8(8):1744–1748
- Kane MA, Bradley DW, Shrestha SM, Maynard JE, Cook EH, Mishra RP, Joshi DD (1984) Epidemic non-A, non-B hepatitis in Nepal. Recovery of a possible etiologic agent and transmission studies in marmosets. *JAMA* 252:3140–3145
- Karron RA, Daemer R, Ticehurst J, D’Hondt E, Popper H, Mihalik K, Phillips J, Feinstone S, Purcell RH (1988) Studies of prototype live hepatitis A virus vaccines in primate models. *J Infect Dis* 157(2):338–345
- Kase JA, Correa MT, Sobsey MD (2009) Detection and molecular characterization of swine hepatitis E virus in North Carolina swine herds and their faecal wastes. *J Water Health* 7(2):344–357

- Kim SE, Kim MY, Kim DG, Song YJ, Jeong HJ, Lee SW, Lee JB, Park SY, Song CS, Oh SJ, Yoo HS, Choi IS (2008) Determination of fecal shedding rates and genotypes of swine hepatitis E virus (HEV) in Korea. *J Vet Med Sci* 70(12):1367–1371
- Krawczynski K, Kamili S, Aggarwal R (2001) Global epidemiology and medical aspects of hepatitis E. *Forum (Genova)* 11(2):166–179
- Kulkarni MA, Arankalle VA (2008) The detection and characterization of hepatitis E virus in pig livers from retail markets of India. *Med Virol* 80(8):1387–1390
- Labrique AB, Thomas DL, Stoszek SK, Nelson KE (1999) Hepatitis E: an emerging infectious disease. *Epidemiol Rev* 21(2):162–179
- Le GY, Wu J, Ma YB, Du RJ, Zhuang JY, Xie TH, Li CH, Dai CB, Sun MS (2001) Propagation of hepatitis E virus in several cell lines including human embryo lung diploid cell KMB17. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao* 23(6):590–593 [abstract, article in chinese, pubmed]
- Legrand-Abravanel F, Mansuy JM, Dubois M, Kamar N, Peron JM, Rostaing L, Izopet J (2009) Hepatitis E virus genotype 3 diversity, France. *Emerg Infect Dis* 15(1):110–114
- Li DR, Huang RT, Pang JJ, Yuan XT, Li XY (1996) Biological feature and genome analysis of HEV isolated by cell-culture. In: Buisson Y, Coursaget P, Kane M (eds) Enterically-transmitted hepatitis viruses, pp. 349–361. Joue-les-Tours, La Simarre, France
- Liu J, Zhang W, Shen Q, Yang S, Huang F, Li P, Guo X, Yang Z, Cui L, Zhu J, Hua X (2009) Prevalence of antibody to hepatitis E virus among pet dogs in the Jiang-Zhe area of China. *Scand J Infect Dis* 41(4):291–295
- Lorenzo FR, Tanaka T, Takahashi H, Ichiyama K, Hoshino Y, Yamada K, Inoue J, Takahashi M, Okamoto H (2008) Mutational events during the primary propagation and consecutive passages of hepatitis E virus strain JE03-1760F in cell culture. *Virus Res* 137(1):86–96
- Maragkoudakis PA, Chingwaru W, Gradisnik L, Tsakalidou E, Cencic A (2008) “Application of lactic acid bacteria on human and farm animal cell lines and protective impact against viruses” PathogenCombat (available online) (http://www.pathogencombat.com/research_results/~media/Adtomic/PatComBilleder/Articles/Research_Results/Petros%20Maragkoudakis.ashx) (accessed on 1st March 2010)
- Meng J, Dubreuil P, Pillot J (1997a) A new PCR-based seroneutralization assay in cell-culture for diagnosis of hepatitis E. *J Clin Microbiol* 35:1373–1377 (Abstract)
- Olsen B, Axelsson-Olsson D, Thelin A, Weiland O (2006) Unexpected high prevalence of IgG-antibodies to hepatitis E virus in Swedish pig farmers and controls. *Scand J Infect Dis* 38(1):55–58
- Peralta B, Casas M, de Deus N, Martín M, Ortuño A, Pérez-Martín E, Pina S, Mateu E (2009) Anti-HEV antibodies in domestic animal species and rodents from Spain using a genotype 3-based ELISA. *Vet Microbiol* (in print)
- Purdy MA, McCaustland KA, Krawczynski K, Spelbring J, Reyes GR, Bradley DW (1993) Preliminary evidence that a trpE-HEV fusion protein protects cynomolgus macaques against challenge with wild-type hepatitis E virus (HEV). *J Med Virol* 41(1):90–94
- Reuter G, Fodor D, Forgach P, Katai A, Szucs G (2009) Characterization and zoonotic potential of endemic hepatitis E virus (HEV) strains in humans and animals in Hungary. *J Clin Virol* 44(4):277–281
- Schlauder GG, Mushahwar IK (2001) Genetic heterogeneity of hepatitis E virus. *J Med Virol* 65:282–292
- Schmidt A, Wolff MH (2008) Hepatitis E infection, Comparative Hepatitis, pp 135–142
- Smith JL (2001) A review of hepatitis E virus. *J Food Prot* 64(4):572–86
- Song YJ, Jeong HJ, Kim YJ, Lee SW, Lee JB, Park SY, Song CS, Park HM, Choi IS (2010) Analysis of complete genome sequences of swine hepatitis E virus and possible risk factors for transmission of HEV to humans in Korea. *J Med Virol* 82(4):583–591
- Stefanidis I, Zervou EK, Rizos C, Syrganis C, Patsidis E, Kyriakopoulos G, Sdrakas L, Tsianias N, Rigopoulou EI, Liakopoulos V, Dalekos GN (2004) Hepatitis E virus antibodies in hemodialysis patients: an epidemiological survey in central Greece. *Int J Artif Organs* 27(10):842–847

- Tanaka E, Takeda N, Tian-Chen L, Orii K, Ichijo T, Matsumoto A, Yoshizawa K, Iijima T, Takayama T, Miyamura T, Kiyosawa K (2001) Seroepidemiological study of hepatitis E virus infection in Japan using a newly developed antibody assay. *J Gastroenterol* 36(5):317–321
- Tanaka T, Takahashi M, Kusano E, Okamoto H (2007) Development and evaluation of an efficient cell-culture system for Hepatitis E virus. *J Gen Virol* 88(Pt 3):903–911
- Taniguchi M, Kim SR, Mishiro S, Takahashi K, Shin MH, Yun H, Park MS, Li ZM, Kim MK, Fang J, Hayashi Y (2009) Epidemiology of hepatitis E in Northeastern China, South Korea and Japan. *J Infect* 58(3):232–237
- Tei S, Kitajima N, Ohara S, Inoue Y, Miki M, Yamatani T, Yamabe H, Mishiro S, Kinoshita Y. (2004) Consumption of uncooked deer meat as a risk factor for hepatitis E virus infection: an age- and sex-matched case-control study. *J Med Virol* 74(1):67–70
- Tsarev SA, Shrestha MP, He J, Scott RM, Vaughn DW, Clayson ET, Gigliotti S, Longer CF, Innis BL (1998) Naturally acquired hepatitis E virus (HEV) infection in Nepalese rodents. *Am J Trop Med Hyg* 59(Suppl):242
- Tsarev SA, Tsareva TS, Emerson SU, Yarbough PO, Legters LJ, Moskal T, Purcell RH (1994) Infectivity titration of a prototype strain of hepatitis E virus in cynomolgus monkeys. *J Med Virol* 43:135–142
- Wei S, Walsh P, Huang R, To SST (2000) 93G, a novel sporadic strain of hepatitis E virus in south China isolated by cell-culture. *J Med Virol* 61:311–318
- Widdowson MA, Jaspers WJM, van der Poel WHM, Verschoor F, de Roda Husman AM, Winter HLJ, Zaaijer HL, Koopmans M (2003) Cluster of cases of acute hepatitis associated with hepatitis E virus infection acquired in The Netherlands. *Clin Infect Dis* 36:29–33
- Williams TP, Kasornrondkua C, Halbur PG, Haqshenas G, Guenette DK, Toth TE, Meng XJ (2001) Evidence of extrahepatic sites of replication of the hepatitis E virus in a swine model. *J Clin Microbiol* 39(9):3040–3046
- World Health Organisation (2005) Hepatitis, Fact sheet N°280. <http://www.who.int/mediacentre/factsheets/fs280/en/> (accessed on 1st March 2010)
- Yazaki Y, Mizuo H, Takahashi M, Nishizawa T, Sasaki N, Gotanda Y, Okamoto H (2003) Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. *J Gen Virol* 84(Pt 9):2351–2357
- Zhang W, Yang S, Shen Q, Liu J, Shan T, Huang F, Ning H, Kang Y, Yang Z, Cui L, Zhu J, Hua X (2009) Isolation and characterization of a genotype 4 Hepatitis E virus strain from an infant in China. *Virol J* 6:24 [Pub Med-in process]

Dengue Virus Diagnostics

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Abstract Dengue fever (DF) is an emerging arboviral disease caused by infection with dengue virus (DENV) which has emerged as the most important vector-borne viral disease in tropical areas and it continues to expand geographically. The four serotypes of DENV that cause human disease are transmitted by *Aedes* mosquitoes. Expansion in geographic distribution of both viruses and mosquito vectors, has led to a current global dengue pandemic. Dengue disease has a wide spectrum of expression: from asymptomatic infection or an influenza-like syndrome to severe disease including dengue hemorrhagic fever (DHF)/ dengue shock syndrome (DSS). Dengue infection diagnosis has come a long way and today, new techniques for detecting the virus and anti- DENV antibody are available. In spite of that there are still problems with those methods and a reliable diagnosis remains a crucial step towards the control of DENV disease in human populations. In this review, different diagnostic methods are described, including routinely used methods, newly developed techniques and commercially available kits. One of the limitations of current diagnostic tools for DENV is the lack of specificity and safety of the antigen employed as part of the available diagnostic tools. Thus there is a

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constant serological effort to develop alternative antigens that will help to improve the detection of anti-dengue antibodies and allow working with non hazardous materials. Several options to deal with this problem is discussed here.

Introduction

Dengue fever (DF) still remains one of the most important public health problems facing the tropical and subtropical areas at the beginning of the twenty-first century. Many of the factors responsible for this dramatic reappearance of epidemic DF are still not well understood. However, it is clear that demographic and societal changes such as population growth, urbanization and modern transportation contribute greatly to the increased incidence and geographical spread of dengue activity (Gubler 2002). Dengue virus (DENV) is a positive-stranded encapsulated RNA virus. The genomic RNA is approximately 11 kb in length and is composed of three structural protein genes and seven nonstructural (NS) protein genes. There are four distinct serotypes of DENV, designated DEN-1, DEN-2, DEN-3 and DEN-4, each causing human disease and are transmitted by *Aedes mosquitoes* (Shu and Huang 2004; Chambers et al. 1990; Holmes and Twiddy 2003; Mukhopadhyay et al. 2005).

Currently there is no available anti-dengue vaccine or specific therapy for the treatment of DENV infection (Sabchareon et al. 2004). Dengue disease has a wide spectrum of expression: from asymptomatic infection or an influenza-like syndrome to severe disease including dengue hemorrhagic fever (DHF)/ dengue shock syndrome (DSS). Moreover, in a small percentage, severe dengue cases occur with unusual manifestations such as hepatitis, encephalopathy or rhabdomyolysis (Mackenzie et al. 2004; Green et al. 1999; Hommel et al. 1998; Murgue et al. 1999). Thus the laboratory tests need to fit not only all these stages and serotypes but also the basis of the clinical syndromes. Therefore, there is a great demand for trustful and fast applications in the detection and differentiation of DENV infection in the acute and convalescent phases of illness. Sensitive and reliable diagnostics, followed by supportive care and symptomatic treatment through fluid replacement are keys to survival in cases of severe dengue infection (AnandaRao et al. 2005). Furthermore, rapid diagnosis may contribute to the control of DF geographical spread with a more accurate public health notification. Many DENV detection techniques were developed in the last decade, while the most routinely used for antibody detection enzyme-linked immunosorbent assays (ELISA) which offer relatively quick and easy methods for diagnosing dengue. Some of them are able to differentiate between primary and secondary infections (Lam and Devine 1998; Palmer et al. 1999; Sang et al. 1998a). Other techniques include dot blot (Cardosa et al. 1995), dipstick (Wu et al. 1997), immune fluorescent antibody assay as well as lateral flow tests, the latter offering the advantage of speed and having the potential to be used in field settings (Lam and Devine 1998; Wu et al. 1997; Lam 1993; Sang et al. 1998b; Vaughn et al. 1998; Vaughn et al. 1999).

High sensitivity, low background, wide dynamic range and relatively inexpensive instrumentation have increased the attraction of chemiluminescent applications during

recent years (Dodeigne and Lejeune 2000). In contrast to the fluorescence applications, luminometry does not suffer from photobleaching and does not need an excitation source or interference filters. Chemiluminescent optic fiber techniques have certain advantages over other methods in that they are simple, flexible and allow for multi-channel and remote sensing (Marks et al. 1997). Fiber-optic biosensors use optical fibers as the transduction element, and rely exclusively on optical transduction mechanisms for detecting target biomolecules. It has been demonstrated that chemiluminescence based optical fiber immunosensors are more sensitive than their analogous colorimetric and chemiluminescent ELISA counterparts (Herrmann et al. 2005; Salama et al. 2004; Sobarzo et al. 2007). The combined advantages of chemiluminescence, optical fibers and immunoassays producing optic fiber based immunosensors (OFIS) led to several studies in the field of diagnostic tools for whole organisms, nucleic acids, IgM and IgG antibodies with high specificity and sensitivity (Herrmann et al. 2005; Sobarzo et al. 2007; Leshem et al. 2004; Konry et al. 2005), in addition to those based on bioluminescence for monitoring genotoxics (Polyak et al. 2000; Polyak et al. 2001).

In summary, dengue is a disease whose accurate diagnosis is difficult when based solely on clinical evidence. Thus, there is a growing demand for techniques that can form the basis of simple, reliable, fast, cost effective, and field operable analytical applications. Such tools could provide timely clinical treatment and epidemic control.

Dengue Viruses

Emerging Flavivirus Diseases

Mosquito-borne members of the genus *Flavivirus* in the family *Flaviviridae* provide some of the most important examples of emerging diseases, as well as one of the earliest documented disease that spread into a new geographic area: Yellow fever virus (YFV) from West Africa into the Americas from the seventeenth century. More recently, the enormous resurgence of DENV in the tropical and subtropical areas of the world, the emergence of West Nile virus (WNV) in North America and the spread of Japanese encephalitis virus (JEV) through much of Asia and into Oceania have been recorded (Mukhopadhyay et al. 2005; Mackenzie et al. 2004).

The Flavivirus genus contains over 70 viruses, most of them arthropod-borne (arboviruses) through mosquitoes or ticks, while some still have no known vector (Mackenzie et al. 2004; Lindenbach 1996). The type species of the genus is YFV, through which the genus and family derive its name (Mukhopadhyay et al. 2005; Mackenzie et al. 2004). Although all flaviviruses are serologically related, they can also be distinctly grouped serologically, the most important of which are DENV and JEV and a less serologically cohesive YFV group (Lindenbach 1996). Flaviviruses are zoonoses that depend on animal species other than humans for their maintenance in nature, with the exception of the dengue viruses. Dengue

viruses have adapted completely to humans and are maintained in large urban areas in the tropics in human-mosquito-human transmission cycles that no longer depend on animal reservoirs (Gubler 2002; Mackenzie et al. 2004).

Epidemiology and Public Health Concerns

Dengue fever is an emerging arboviral disease caused by infection with dengue viruses (Chambers et al. 1990; Holmes and Twiddy 2003; Mukhopadhyay et al. 2005). DENV is endemic in most urban centers of the tropics and subtropic areas since a dramatic increase in urbanization created ideal conditions for increased transmission of mosquito-borne dengue disease (Mackenzie et al. 2004). Dengue is mainly found in Asian and Latin American regions rather than on the African continent; yet poor surveillance has hidden the true prevalence of dengue on this continent (Holmes and Twiddy 2003). World Health Organization (WHO) estimates that there is a 30-fold increase in DENV incidence in the last 50 years and that up to 100 million cases and over 500,000 cases of dengue DHF / DSS occur each year, including about 25,000 fatal cases, mainly in children under the age of 15 (Shu and Huang 2004; Mukhopadhyay et al. 2005; Dussart et al. 2006). For the time being, the global distribution of the mosquito vectors, (genus *Aedes*) is comparable to that of the malaria vector, and an estimated 2.5 billion people live in areas at risk for epidemic transmission (over 100 endemic countries). This dramatic increase in human dengue infections and an expanding geographic distribution of both the viruses and the mosquito vectors have led to a current global dengue pandemic (Gubler 2002; Mackenzie et al. 2004).

Dengue Virus Strains and ‘Immune Protection’

Most people infected with dengue viruses are asymptomatic or develop mild disease, known as DF. The signs and symptoms of DF include high fever, myalgia, retro-orbital headache, joint pain, vomiting, loss of appetite and usually a macular rash. However, a minority of people infected with DENV develop DHF/ DSS. The acute phase of DHF/ DSS begins as with DENV, then suddenly patients develop a severe vascular permeability syndrome that may lead to shock and death (Mackenzie et al. 2004). DF is not usually fatal (case fatality rate 1–5%) when supportive therapy is available. Nevertheless, a significant economic burden is carried by communities in poor health resource settings. The pathogenesis of DHF/DSS is not very well understood, nor are the immune host conditions that enable the severe disease. Despite an increase in health and economic impact, there is currently no available anti-dengue vaccine or specific therapy for the treatment of DENV infection (Sabchareon et al. 2004).

Symptoms of illness generally appear 4–7 days after a mosquito bite. During this incubation period in humans, the amount of virus in the blood (viral load) rises until it is high enough to infect mosquitoes when they feed (Mackenzie et al. 2004). The

ensuing period of infectivity begins shortly before the onset of the clinical symptoms, and generally lasts some 5 days (Yamada et al. 2002; Vaughn et al. 2000). Usually, in a non-immune individual, IgM develops 5–6 days after the primary infection and IgG increases after 7–10 days. During the secondary infection, high levels of IgG are detectable even during the acute phase and they rise over the next two weeks. IgM are lower and in some cases can be absent during a secondary infection. High titers of IgG are a criterion of secondary infection (Kuhn et al. 2002). Dengue infection induces a life-long protective immunity to the homologous serotype but confers only partial and transient protection against subsequent infections by the other three serotypes. Both retrospective and prospective studies have demonstrated that secondary infection by a different dengue serotype or multiple infections with various DENV serotypes are the most significant individual risk factors for DHF/DSS (Guzman et al. 1987; Thein et al. 1997; Phuong et al. 2004; Pancharoen et al. 2001). The hypothesis for the presence of circulating non-neutralizing, cross-reactive antibodies in a previously infected individual enables enhancement of infection, through increased uptake of virus into target cells via Fc receptors (Gollins and Porterfield 1984).

Virus Structure

DENV is a single-stranded positive-sense RNA virus. Like other Flaviviruses the DENV is composed of three structural proteins, designated C (capsid protein), M (membrane) and E (envelope protein), while prM is the intracellular precursor of the M protein (Kuhn et al. 2002). The genomic RNA is translated to give a rise to a large polyprotein precursor, which is cotranslationally processed by host cell and virus-specified proteases to yield the individual viral proteins. The nonstructural (NS) proteins are NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5. There is ~40% amino acid identity among the flaviviruses E proteins. The number and position of potentially glycosylated residues are not conserved among different strains of the same virus and it has been suggested that carbohydrate moieties on the virus surface might modulate specificity of receptor binding (Mukhopadhyay et al. 2005). Due to this high similarity between flavivirus members, it is difficult to develop diagnostic tools without cross reactivity.

Diagnostic Tools for Dengue

There are two main directions for DENV diagnostics: virological and serological. Most laboratories routinely use the isolation of viruses, nucleic acid amplification assays for the detection of genomic sequence and ELISA technologies for antibody determination (Shu and Huang 2004; Gubler 1998; Guzman and Kouri 2002). Table 1 summarizes the advantages and disadvantages of the technique used by the main laboratories in serological and virological dengue diagnostic assays and Table 2 cites some commercial kits available for DENV detection.

Table 1 Advantages and disadvantages of the main techniques used by laboratories in serological and virological dengue diagnostic assays

		Advantages	Disadvantages
Virology	Virus detection using cell culture	• Relatively sensitive • Specific to serotype • Considered to be “Gold standard” for viral detection	• Time consuming (~10–21 days) • Required P3 lab • Expensive
	Genome detection using RT-PCR	• High specificity (also according to the serotype) • High sensitivity • Relatively fast (~1–2 days)	• Relatively expensive (especially in undeveloped countries) • Relatively sophisticated equipment
	Genome detection using Real time RT-PCR	• High sensitivity • High specificity (also according to the serotype) • Relatively fast • Quantitative measurements	• Expensive equipment and reagents • Relatively sophisticated equipment • Not easy to standardize
Serology	IgM detection using MAC-ELISA	• Simple • High throughput method • Relatively fast with in house protocol (1–2 days) • Existence of commercial tests • Reliable • Easy to perform • Considered to be the routine diagnostic test for serological detection	• Lack of specificity
	IgG detection using indirect ELISA	• Simple • High throughput method • Relatively fast with in house protocol (1–2 days) • Reliable • Easy to perform	• Lack of specificity
	HI	• Cheap • Sensitive • Simple, but requires highly skilled technician • Not specific to species • Considered to be “Gold standard” for serological detection	• Lack of specificity • The need for paired samples • Time consuming
	PRNT	• The most sensitive and specific serological test • Detection according to the serotype, but only in primary dengue infection	• Time consuming • Requires highly skilled technician • Required P3 lab

Table 2 Examples of commercial kits for DENV diagnostics

Kit	Method type	Detection time	Percentage of Sensitivity	Percentage of Specificity	Field testing	References
MRL diagnostics	EIA – IgG	4 h	98	100	+	Palmer et al. 1999 ; Groen et al. 2000
PanBio	EIA – IgG	2.5 h	100	100	+	Palmer et al. 1999 ; Groen et al. 2000
PanBio	RIT(rapid test)	7 min	100	92	–	Groen et al. 2000
Progene	Fluorescent (IgM)	2 h	71	89	–	Groen et al. 2000
Integrated diagnostics	Dipstick	1.5 h	96	86	+	Groen et al. 2000
Genelabs	IgM blot	8 h	100	92	–	Groen et al. 2000
PanBio	NS1 antigen detection	3 h ≈	60.4	97.9	–	Dussart et al. 2006
Bio-Rad	NS1 antigen detection	15–30 min	81.5–82.4	100	+	Dussart et al. 2006
Focus diagnostics	Anti DENV IgM capture	6 h	98.6	79.9	+	Diagnostics evaluation series No 3 2009
Omega diagnostics	Indirect IgM detection	4 h	61.5	84.6	+	Diagnostics evaluation series No 3 2009
Pentax	IgM rapid tests	1.5 h	97.7	76.6	+	Diagnostics evaluation series No 3 2009
Zephyr biomedical	IgM and IgG rapid tests	15 min	20.5	86.7	+	Diagnostics evaluation series No 3 2009

Laboratory Diagnosis

Diagnoses of dengue virus infection on the basis of clinical syndromes are not reliable, and should be confirmed by laboratory studies. Since dengue disease has a large spectrum of conditions - from asymptomatic infection to influenza-like syndrome to severe disease including DHF/DSS – the laboratory tests need to fit all of the aforementioned stages. Furthermore, as mentioned above, secondary infection by a different dengue serotype or multiple infections with various DENV serotypes are the most significant individual risk factors for DHF/DSS. Therefore, the need to identify the various DENV serotypes during the course of DENV disease is imperative. Knowing the virus serotype or its corresponding antibody can assist in monitoring people with high risk to develop DHF/DSS. Early diagnosis, followed by supportive care and symptomatic treatment through fluid replacement are the keys to survival in cases of severe dengue infection (AnandaRao et al. 2005). Moreover rapid and accurate dengue diagnosis is important for effective control of dengue outbreaks (De Paula and Fonseca 2004). Accurate and efficient diagnosis of dengue is also important for epidemiological studies and for the clinical management and evaluation of individual patients, particularly if new and specific therapeutic agents develop. Accuracy in diagnosis is also important for pathogenesis studies and vaccine/therapeutic research (Guzman and Kouri 1996). The diagnosis is even more complex because of the co-circulation of other arboviruses in most countries where dengue virus is endemic. For instance, the other arboviruses co-circulating with the DENV are: JEV in South-East Asia, Saint-Louis encephalitis virus (SLEV) and YFV in Latin America, or WNV in the Caribbean (Mackenzie et al. 2004; WHO Organization 2004).

Currently, biological diagnosis of dengue virus is made mainly through: virus isolation via cell culture (Kuberski and Rosen 1977) or molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) (Lanciotti et al. 1992; Harris et al. 1998). Serological tests to detect anti-DENV antibody are made mainly through: ELISA assays, haemagglutination inhibition (HI) techniques (Balmaseda et al. 2003; Innis et al. 1989; Chow and Hsu 1989) and Plaque reduction neutralization test (PRNT)(De Paula and Fonseca 2004; World Health Organization (WHO) 2004).

Virological Diagnosis usually involves cell culture (generally mosquito cell line) with fluorescence-labeled antibodies of various specificities. This technique is relatively sensitive but it takes several days and may not always be successful due to the small amount of viable virus in the sample. Moreover it requires a P3 lab, which usually exists only in a reference laboratory (Shu and Huang 2004; De Paula and Fonseca 2004; World Health Organization (WHO) 2004; Teles et al. 2005; Kao et al. 2005). Despite these disadvantages, this application remains the “gold standard” technique for DENV detection (Gentry et al. 1982; World Health Organization (WHO) 1997). Viral RNA can be detected, according to the serotype, with a high degree of sensitivity by using RT-PCR. RT-PCR is definitely the most sensitive test, since it is able to detect dengue viruses up to the tenth day after the onset of the symptoms (Shu and Huang 2004; De Paula and Fonseca 2004; World Health Organization (WHO) 2004; Teles et al. 2005; Kao et al. 2005). Principle disadvan-

tages are the cost and sophisticated equipment required (AnandaRao et al. 2005; Lemmer et al. 2004).

An isothermal nucleic acid sequence-based amplification (NASBA) assay was optimized to amplify viral RNA of all four dengue virus serotypes by a set of universal primers and to type the amplified products by serotype-specific capture probes. The NASBA assay involved the use of silica to extract viral nucleic acid, which was amplified without thermocycling (Wu et al. 2001a). The main advantage of this assay over a PCR technique is that it is entirely isothermal and is conducted at 41°C. Thus, it would be suitable for epidemiological studies in the field (Wu et al. 2001a).

An increasing number of publications has revealed the use of real-time RT-PCR assays for the detection of DENV in acute-phase serum samples (Callahan et al. 2001; Chen et al. 2001; Drosten et al. 2002; Hounq et al. 2001; Laue et al. 1999; Shu et al. 2003). There are many advantages of this method over regular RT-PCR application, such as rapidity, high sensitivity and specificity, easy standardization and ability to quantify measurements. Currently, there are five main chemistries used for the detection of PCR products during real-time PCR, e.g., DNA binding fluorophores, the 5' endonuclease, adjacent linear and hairpin oligoprobes and the self-fluorescing amplicons (Mackay et al. 2002). The most widely used is the 5' endonuclease chemistry (TaqMan). This is a promising technology with potential to develop multiplex PCR protocols with up to four fluorophores in a single tube (i.e. detection of four different DENV serotypes).

In an attempt to develop a simple, reliable, and universal RT-PCR protocol with systemic detection and differentiation of the various DENV, a real-time quantitative RT-PCR system based on SYBR Green-I DNA dye binding fluorophores was developed (Shu et al. 2003). The rationale is to develop a diagnostic system with an automated platform using real-time PCR equipment and a universal RT-PCR protocol that allows multiple primer sets designed and tested without the need to change the RT-PCR conditions (Shu et al. 2003). The main advantage of the SYBR Green real-time RT-PCR method on the TaqMan assay is simplicity in primer design and capability to use universal RT-PCR protocols suitable for the detection of multiple target sequences (Shu et al. 2003; Kuno 1998).

Viral antigen detection is also possible using ELISA (Kao et al. 2005). Currently, a kit based on sandwich ELISA to test secreted NS1 has been developed (Bio-Rad) (Dussart et al. 2006). It seems that there is a shortage in kits that can distinguish between dengue serotype in the viremia stage. One problem common to all these methods is the narrow time window (~5 days), available for the successful detection of the virus (Yamada et al. 2002; Vaughn et al. 2000). Often, dengue infected patients do not seek immediate medical care as the initial manifestations are usually asymptomatic or mild fever. Thus, in a majority of cases the diagnostic test would have to be based on the identification of anti - dengue antibodies (AnandaRao et al. 2005).

Serological diagnoses have some limitations in areas where other arboviruses are circulating or in individuals previously vaccinated with the yellow fever vaccine or Japanese encephalitis vaccine (cross-reactivity). For many years, the HI was the

standard method used in dengue virus diagnosis due to its high degree of sensitivity and relatively easy execution. The main disadvantages of the HI test are its lack of specificity, the need for paired samples, is time consuming and its inability to identify the infecting virus serotype (De Paula and Fonseca 2004; World Health Organization (WHO) 2004). The Plaque reduction neutralization test (PRNT) is the most sensitive and specific serological test for dengue virus diagnosis. Due to its high specificity, PRNT can be used to identify the infecting serotype in primary dengue infections, since a relatively monotypic response is observed in the patients' serum during the convalescent phase. In secondary and tertiary infections, the determination of the infecting serotype by PRNT is not always reliable. The greatest disadvantages of this method are its high cost, the length of time and expertise necessary to perform it, and the associated technical difficulties (De Paula and Fonseca 2004; World Health Organization (WHO) 2004). Moreover it requires a P3 lab, which usually exists only in a reference laboratory.

Capture ELISAs for IgM (MAC ELISA) and IgG (GAC ELISA) antibodies or IgG detection using indirect ELISA are routinely used for the serological analyses of dengue virus infections, as they are simple and allow large numbers of samples to be tested. A limitation of these tests is that specificity is generally lacking, particularly in the case of secondary infections. Commercial MAC-ELISA kits are available (Groen et al. 2000); recently an envelope and membrane (E/M)-specific IgM capture ELISA kit has become the most powerful assay for the serodiagnosis of DENV infection (Shu and Huang 2004; Groen et al. 2000). Yet most of these kits rely on the use of whole virus antigens (produced in tissue culture or suckling mice brain) for the detection of anti-dengue antibodies in patient sera, and are consequently associated with an inherent biohazard risk. One kit, which has replaced the whole virus antigen with insect cell-expressed dengue envelope protein, eliminates this risk but these kits are relatively expensive due to the high costs associated with antigen production, and consequently unaffordable for use in the economically weaker countries where dengue is mostly prevalent. Apart from this, a major shortcoming of the commercial kits is that they do not differentiate between infections due to dengue and other flaviviruses, and between dengue serotypes (AnandaRao et al. 2005). IgA-specific capture ELISA has also been developed for blood sample (Talarmin et al. 1998) and for saliva (Balmaseda et al. 2003). Several tests are trying to discriminate between primary and secondary dengue virus infection. For this purpose the HI test is the reference test recommended by the WHO (World Health Organization (WHO) 2004) but there is also an ELISA test based on IgM/IgG ratio (World Health Organization (WHO) 2004; Innis et al. 1989; Chow and Hsu 1989).

Today, new techniques for detecting the virus or subviral components and antibodies are in development (De Paula and Fonseca 2004; World Health Organization (WHO) 2004; Teles et al. 2005), although there is still a great need to standardize and improve current dengue diagnostics. Development of more sensitive, specific, rapid and cost effective diagnostic tools, along with field operability for relatively underdeveloped regions of the world, is of increasing importance. As a result, several groups have focused on developing biosensors for detection of DENV using

chip and quartz crystal microbalance or anti-DENV IgG antibodies with vertical cavity surface emitting lasers (Wu et al. 2005; Mateus et al. 2004; Huang et al. 2008a; Baeumner et al. 2002a; Zaytseva et al. 2005a; Huang et al. 2008b; Baeumner et al. 2002b; Zaytseva et al. 2005b). Moreover since working with a virus potentially infective to humans requires extra safety precautions; there is a constant need to develop alternative antigens to replace the whole virus antigen in diagnostic tests (AnandaRao et al. 2005).

Biosensor Technology

The term biosensor generally refers to a small, portable, analytical device based on the combination of recognition biomolecules with an appropriate transducer that is capable of detecting chemical or biological materials selectively and with a high sensitivity. The immobilization layer should guarantee reproducibility, accessibility to the binding site of the target, stabilize the recognition element and, its chemical and/ or physical properties reduce non-specific binding. Biosensors include four components: a recognition module, which can be either biological or biomimetic, a specific binding chemistry, which enables the conjugation of the biological element to the sensor and sometimes helping to reduce a non-specific signal, a transduction module, which transforms the recognition event into a measurable signal and finally, a module of data evaluation. Regarding the recognition module, progression in genetic engineering, protein engineering and polymer chemistry have greatly expanded the availability and quality of the recognition elements. Regarding the transduction module, the signal of the recognition event can be detected usually by electrochemical, optical, magnetic, acoustic or calorimetric transducers (Paddle 1996). Luminometry is characterized by an extremely high sensitivity, a wide dynamic range, a low background, and relatively inexpensive instrumentation (Salama et al. 2004).

Luminescence Based Biosensors

Chemiluminescence measurements are becoming increasingly popular due to their high sensitivity, wide dynamic range and relatively inexpensive instrumentation. Superior sensitivity and low background distinguish luminometry from other analytical methods. Luminometry is up to 100,000 times more sensitive than absorption spectroscopy and more than 1,000 times more sensitive than fluorometry (Salama et al. 2004). A well designed luminometer can detect as little as 0.6 pg of adenosine triphosphate (ATP) or 0.1 fg of luciferase, two common luminescent analytes. Compared to fluorescence, luminometry does not need an excitation source or interference filters, does not cause photobleaching and remains the most

suitable technology to be adapted on a chip. Optical fiber biosensors based on biological or chemical luminescence have already demonstrated their ability to detect biological entities (Herrmann et al. 2005; Leshem et al. 2004; Polyak et al. 2001). In this method the photon reading instrument is based on a photomultiplier tube (PMT). It has been demonstrated that optical fiber biosensors are more sensitive than standard colorimetric and chemiluminescent ELISA based assays. In this work an antigen was used from one of the flaviviruses, West-Nile virus, as a capture for the detection of anti- West-Nile virus IgG (Herrmann et al. 2005). Furthermore samples that were found negative to anti – *Ebola* IgG or anti *hepatitis C* in ELISA were found positive in optical fiber biosensors being false negatives thanks to increased sensitivity of the system (Konry et al. 2005; Petrosova et al. 2007).

Optical fiber sensors are ideal transducers governed by Snell's law. They have the following advantages (Marks et al. 1997): (1) geometric convenience and flexibility; (2) low cost of production; (3) inert and therefore non-hazardous; (4) free of electric interference; (5) being dielectric, protected against atmospheric disturbances; (6) their small volume economizes reagents and enables portability as well as access to difficult areas; (7) robust with high tensile strength; (8) their silica composition enables macromolecular conjugation via silanization; (9) enable solid-phase characterization of the analyte; (10) their potentially long interaction lengths enable remote signal transmission; (11) light transmission is done with minimal loss; (12) high efficiency coupling occurs in the blue region which is ideal for chemiluminescence; (13) optical multiplicity; (14) polyvalence, as an optrode system, so can be adapted easily from one antigen–antibody system to another; (15) amenable to mass production; (16) enable multiple antigen detection via fiber bundles.

A biochemical testing procedure can be divided into four steps: sample preparation, assay, detection and data analysis. Currently, there is considerable effort dedicated to integrate all these steps in a single device, generally referred to as lab-on-a-chip. In the future, introducing this technology may serve specifically to diagnose DENV (and DENV serotypes) and other flavivirus infection agents using a diagnostic Biochip.

A new diagnostic tool was developed based on chemiluminescent optical fiber immunosensor (OFIS), for the detection of anti-DENV Immunoglobulin M (IgM) in human sera samples (Atias et al. 2009). The immunoassay was based on a colorimetric IgM capture enzyme-linked immunosorbent assay (MAC-ELISA), routinely used by the National Reference Center for arboviruses based in French Guiana. The detection of human anti-DENV IgM using colorimetric MAC-ELISA, chemiluminescent MAC-ELISA and chemiluminescent OFIS (Fig. 1) was compared. An internal panel of reference sera was used and 86 sera samples were screened. Compared to standard colorimetric MAC-ELISA, the chemiluminescent OFIS had a lower detection limit, ten times lower than the chemiluminescent MAC-ELISA and 100 times lower than the colorimetric MAC-ELISA. Therefore the colorimetric and chemiluminescent MAC-ELISA's are more suitable for high and intermediate levels of anti-DENV IgM present in serum samples, whereas the chemiluminescent OFIS is also useful at low analyte concentration, with sensitivity and

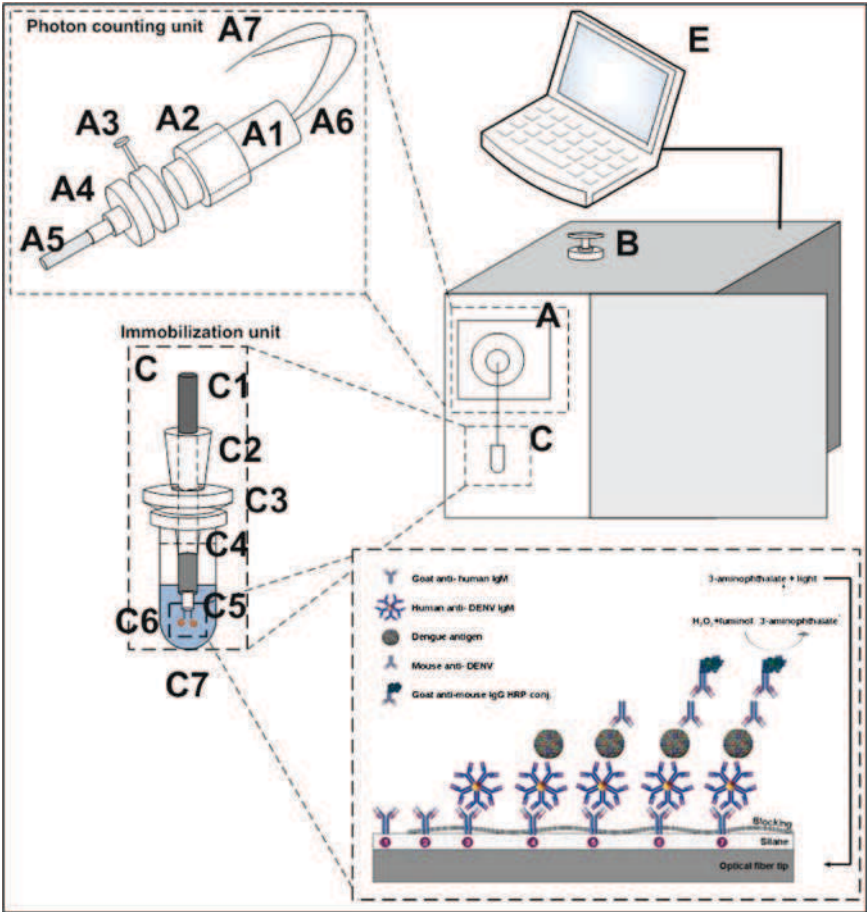


Fig. 1 Descriptive scheme of the experimental setups for dengue virus detection. (A) Photon counting unit. (A1) Hamamatsu HC135-01 PMT Sensor Module. (A2) PMT fixation ring. (A3) Manual shutter (71430, Oriel). (A4) Fiber holder that prevents the movement of the fiber inside the photon counting unit. (A5) Fiber optic. (A6) Connection wire of PMT to computer. (A7) Electricity cable. (B) The outside handle of manual shutter that enables light access to the PMT. (C) Immobilization unit. (C1) Fiber optic. (C2) 100µl pipette tip. (C3) Conical tube cup. (C4) Point of fixation of fiber. (C5) Optical fiber core. (C6) Biorecognition elements according to MAC-ELISA chemiluminescent OFIS (Atias et al. 2009). (C7) Test samples. (E) Connection to computer

specificity of 98.1% and 87.0%, respectively (Atias et al. 2009). Table 3 compares the chemiluminescent OFIS method to the chemiluminescent and colorimetric MAC-ELISA assays. In general, these results support previous studies that demonstrated the advantages of using chemiluminescent methods, especially OFIS, as a useful diagnostic tool for viral detection (Herrmann et al. 2005; Sobarzo et al. 2007). This work, demonstrates for the first time, screening of IgM in serum

Table 3 Comparison of the chemiluminescent OFIS method to the chemiluminescent and colorimetric MAC-ELISA assays. (Atias et al. 2009)

	Chemiluminescent OFIS	Chemiluminescent and colorimetric MAC-ELISA
Lower detection limit	10 times lower than the chemiluminescent MAC-ELISA and 100 times lower than the colorimetric MAC-ELISA, enabling detection of very low analyte concentration.	More suitable for high and intermediate levels of anti-DENV IgM present in sera samples.
Duration of the test	More rapid (~ 3h)	~ 20h
Specificity (compared to routinely used colorimetric MAC-ELISA)	87.0%	95.6% (Chemiluminescent MAC-ELISA)
Sensitivity(compared to routinely used colorimetric MAC-ELISA)	98.1%	100% (Chemiluminescent MAC-ELISA)
Working range	Has a reliable working range at very low anti-DENV IgM concentration with analytical sensitivity enabling a semi-quantitative test.	Has a reliable working range for high and intermediate levels of anti-DENV IgM.
Reproducibility	The reproducibility need to be improved. OFIS is a new methodology probably requiring improvements the chemistry of the binding and homogeneous cutting of the fiber.	Better reproducibility

using chemiluminescent OFIS (Atias et al. 2009). Since IgM is a much larger molecule than IgG, it is difficult to work with in chemiluminescent OFIS, as the assay is based on bottom to top covalent binding and top to bottom detection but not adsorption. However, the main serological routine test in dengue endemic countries is based on anti-DENV IgM detection, so it is important to focus on this kind of diagnostic.

Alternative Antigen

As detailed above, one of the limitations of current diagnostic tools is the lack of specificity and safety of the antigen employed as capture moiety. Thus there is a constant effort in developing alternative antigens that will help to improve the detection of anti-dengue antibodies as detailed below.

Recombinant Protein and Epitopes

The envelope protein (E) plays a key role in numerous aspects of the viral life cycle and viral pathogenesis including virion assembly, membrane fusion, receptor binding, blood cell hemagglutination, and induction of a protective immune response (Kuhn et al. 2002; Hurrelbrink and McMin 2003). The E ectodomain consists of three domains: Domain I, Domain II, and Domain III (E3). The E3 has been proposed to function as the putative receptor-binding domain of DENV (Mukhopadhyay et al. 2005). The E protein is the most immunogenic of all the dengue viral proteins eliciting the first and longest lasting antibodies. Consequently, much research has been spent on trying to find immunodominant epitopes based on E protein (AnandaRao et al. 2005; Megret et al. 1992; Trirawatanapong et al. 1992). The non structural protein NS3 (AnandaRao et al. 2005; Garcia et al. 1997) and especially NS1 (AnandaRao et al. 2005; Garcia et al. 1997; Falconar et al. 1994; Wu et al. 2003) are reported to elicit significant antibody responses, particularly in secondary infections; so they can also be used for this purpose.

Phage Display

“Phage display”, first introduced by George Smith in 1985 (Smith 1985), is a powerful technique that allows expression and presentation of peptides or proteins, including antibodies, on the surface of phage. According to this method a coding domain of interest is fused to that of a bacteriophage coat protein, resulting in phage particles that display the encoded protein as a chimerical one (Marjorie Russel and Clackson 2004; Arap 2005). In general, the DNA that encodes the displayed protein is encapsulated within the same virion, therefore providing a direct link between phenotype and genotype (Marjorie Russel and Clackson 2004; Benhar 2001). This enables rapid amplification and characterization of the desired clone through DNA sequence analysis of the insert.

Phage display has many advantages over other methods for recombinant protein expression including; ease of manipulation, protein folding and ability to affinity mature a collection of recombinant molecules to identify those with the highest affinity (Arap 2005; Cesareni et al. 1999; Petrenko and Vodyanoy 2003). Phage display is not limited to short peptides as it can be used to express polypeptides of a variety of sizes and with varied biological activity, e.g. cytokines, antibodies, receptors, enzymes, DNA binding proteins (Benhar 2001).

A method to map the specific site on the dengue virus envelope protein (E) that interacts with cells and a neutralizer using serially truncated dengue virus type 2 (DENV-2) E was developed (Abd-Jamil et al. 2008). Using the recombinant phage-displayed truncated DENV-2 E, two potential cell receptor attachment sites were located within the DENV-2 ectodomain III (Abd-Jamil et al. 2008). Other phage-displayed peptide libraries were used to generate a serotype-specific monoclonal antibody (mAb), D(2) 16-1 (Ab4), against DENV-2 (Wu et al. 2003). Selected phage

clones reacted specifically with Ab4 and did not react with other mAbs. This mAb and its epitope-based peptide antigen will be useful for serologic diagnosis of DENV-2 infection (Wu et al. 2003). Furthermore, DENV-2 epitope identification makes it feasible to dissect antibody responses to DENV and to address the role of antibodies in the pathogenesis of primary and secondary DENV-2 infections (Wu et al. 2003). Identification of the B-cell epitope of DENV-1 from a random peptide library displayed on phage using a serotype-specific monoclonal antibody (MAb) of DENV-1, 15F3-1 was reported. This epitope-based peptide demonstrated its clinical diagnostic potential when it reacted with a high degree of specificity with serum samples obtained from both DENV-1-infected rabbits and patients (Wu et al. 2001b).

In another application, antibody fragments to the four DENV serotypes were isolated from a human universal naïve library (Cabezas et al. 2008). All phage-displayed antibody fragments were cross-reactive against several strains of distinct genotypes within the corresponding serotype(s). The simple phage selection method on captured virus could be applied in a high throughput way to obtain larger panels of antibody fragments to DENV for multiple applications (Cabezas et al. 2008).

Future Trends

Although Dengue diagnosis has made much progress, since HI and mouse inoculation were the only options available, with development of ELISA using recombinant proteins, there is still a great need to standardize and improve current dengue diagnostics. Development of more sensitive, specific, rapid and cost effective diagnostic tools, along with field operability for relatively underdeveloped regions of the world, is of increasing importance. Special focus is needed to be made on the development of alternative antigen which will improve the specificity and safety of the current diagnostic methods. Emergent technologies based on phage display will benefit diagnostics by producing molecules that are otherwise unobtainable by traditional approaches. Therefore exploitation of the advantages of phage display system together with the ongoing development in the biosensor field will lead to revolutionary diagnostic devices in general and in particular in DENV detection.

References

- Abd-Jamil J, Cheah CY, AbuBakar S (2008) Dengue virus type 2 envelope protein displayed as recombinant phage attachment protein reveals potential cell binding sites. *Protein Engi Design Select* 21(10):605–611
- AnandaRao R, Swaminathan S, Fernando S, Jana AM, Khanna N (2005) A custom-designed recombinant multiepitope protein as a dengue diagnostic reagent. *Protein Expr Purif* 41(1):136–147
- Arap MA (2005) Phage display technology - Applications and innovations. *Gene Mole Biol* 28:1–9

- Atias D, Liebes Y, Chalifa-Caspi V, Bremand L, Lobel L, Marks SR, Dussart P (2009) Chemiluminescent optical fiber immunosensor for the detection of IgM antibody to Dengue virus in humans. *Sensor Actuat B: Chemical* 140:206–215
- Baemumner AJ, Nicole AS, Naomi SS, Joseph R, Eun ML, Richard AM (2002) Biosensor for dengue virus detection: Sensitive, rapid, and serotype specific. *Anal Chem* 74:142–1448
- Baemumner AJ, Schlesinger NA, Slutzki NS, Romano J, Lee EM, Montagna RA (2002b) Biosensor for Dengue virus detection: Sensitive, rapid, and serotype specific. *Anal Chem* 74(6):1442–1448
- Balmaseda A, Guzman MG, Hammond S, Robleto G, Flores C, Tellez Y, Videa E, Saborio S, Perez L, Sandoval E, Rodriguez Y, Harris E (2003) Diagnosis of dengue virus infection by detection of specific immunoglobulin M (IgM) and IgA antibodies in serum and saliva. *Clin Diagn Lab Immunol* 10(2):317–322
- Benhar I (2001) Biotechnological applications of phage and cell display. *Biotechnol Adv* 19(1):1–33
- Cabezas S, Rojas G, Pavon A, Alvarez M, Pupo M, Guillen G, Guzman MG (2008) Selection of phage-displayed human antibody fragments on Dengue virus particles captured by a monoclonal antibody: application to the four serotypes. *J Virol Methods* 147(2):235–243
- Callahan JD, Wu SJ, Dion-Schultz A, Mangold BE, Peruski LF, Watts DM, Porter KR, Murphy GR, Suharyono W, King CC, Hayes CG, Temenak JJ (2001) Development and evaluation of serotype- and group-specific fluorogenic reverse transcriptase PCR (TaqMan) assays for dengue virus. *J Clin Microbiol* 39(11):4119–4124
- Cardosa MJ, Baharudin F, Hamid S, Hooi TP, Nimmanitya S (1995) A nitrocellulose membrane based IgM capture enzyme immunoassay for etiological diagnosis of dengue virus infections. *Clin Diagn Virol* 3(4):343–350
- Cesareni G, Castagnoli L, Cestra G (1999) Phage displayed peptide libraries. *Comb Chem High Throughput Screen* 2(1):1–17
- Chambers TJ, Hahn CS, Galler R, Rice CM (1990) Flavivirus genome organization, expression, and replication. *Annu Rev Microbiol* 44:649–88
- Chen RF, Yeh WT, Yang MY, Yang KD (2001) A model of the real-time correlation of viral titers with immune reactions in antibody-dependent enhancement of dengue-2 infections. *FEMS Immunol Med Microbiol* 30(1):1–7
- Chow L, Hsu ST (1989) MAC-ELISA for the detection of IgM antibodies to dengue type I virus (rapid diagnosis of dengue type I virus infection). *Zhonghua Min Guo Wei Sheng Wu Ji Mian Yi Xue Za Zhi* 22(4):278–285
- De Paula SO, Fonseca BA (2004) Dengue: a review of the laboratory tests a clinician must know to achieve a correct diagnosis. *Braz J Infect Dis* 8(6):390–398
- Diagnostics evaluation series No 3 (2009) Evaluation of commercially available anti dengue virus immunoglobulin M tests. Special programme for research and training in tropical diseases sponsored by WHO, p 1–42. ISBN 978 92 4 159775 3
- Dodeigne LCT, Lejeune R (2000) Chemiluminescence as diagnostic tool. A review. *Talanta* 51(3):415–439
- Drosten C, Gottig S, Schilling S, Asper M, Panning M, Schmitz H, Gunther S (2002) Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. *J Clin Microbiol* 40(7):2323–2330
- Dussart P, Labeau B, Lagathu G, Louis P, Nunes MR, Rodrigues SG, Storck-Herrmann C, Cesaire R, Morvan J, Flamand M, Baril L (2006) Evaluation of an enzyme immunoassay for detection of dengue virus NS1 antigen in human serum. *Clin Vaccine Immunol* 13(11):1185–1189
- Falconar AK, Young PR, Miles MA (1994) Precise location of sequential dengue virus subcomplex and complex B cell epitopes on the nonstructural-1 glycoprotein. *Arch Virol* 137(3–4):315–326
- Garcia G, Vaughn DW, Del Angel RM (1997) Recognition of synthetic oligopeptides from non-structural proteins NS1 and NS3 of dengue-4 virus by sera from dengue virus-infected children. *Am J Trop Med Hyg* 56(4):466–470

- Gentry MK, Henchal EA, McCown JM, Brandt WE, Dalrymple JM (1982) Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. *Am J Trop Med Hyg* 31(3 Pt 1):548–555
- Gollins SW, Porterfield JS (1984) Flavivirus infection enhancement in macrophages: radioactive and biological studies on the effect of antibody on viral fate. *J Gen Virol* 65(Pt 8):1261–1272
- Green S, Vaughn DW, Kalayanaraj S, Nimmannitya S, Suntayakorn S, Nisalak A, Lew R, Innis BL, Kurane I, Rothman AL, Ennis FA (1999) Early immune activation in acute dengue illness is related to development of plasma leakage and disease severity. *J Infect Dis* 179(4):755–762
- Groen J, Koraka P, Velzing J, Copra C, Osterhaus AD (2000) Evaluation of six immunoassays for detection of dengue virus-specific immunoglobulin M and G antibodies. *Clin Diagn Lab Immunol* 7(6):867–871
- Gubler DJ (1998) Dengue and dengue hemorrhagic fever. *Clin Microbiol Rev* 11(3):480–496
- Gubler DJ (2002) Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol* 10(2):100–3
- Guzman MG, Kouri G (1996) Advances in dengue diagnosis. *Clin Diagn Lab Immunol* 3(6):621–627
- Guzman MG, Kouri G (2002) Dengue: an update. *Lancet Infect Dis* 2(1):33–42
- Guzman MG, Kouri G, Martinez E, Bravo J, Riveron R, Soler M, Vazquez S, Morier L (1987) Clinical and serologic study of Cuban children with dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). *Bull Pan Am Health Organ* 21(3):270–279
- Harris E, Roberts TG, Smith L, Selle J, Kramer LD, Valle S, Sandoval E, Balmaseda A (1998) Typing of dengue viruses in clinical specimens and mosquitoes by single-tube multiplex reverse transcriptase PCR. *J Clin Microbiol* 36(9):2634–2639
- Herrmann S, Leshem B, Landes S, Rager-Zisman B, Marks RS (2005) Chemiluminescent optical fiber immunosensor for the detection of anti-West Nile virus IgG. *Talanta* 66(1):6–14
- Holmes EC, Twiddy SS (2003) The origin, emergence and evolutionary genetics of dengue virus. *Infect Genet Evol* 3(1):19–28
- Hommel D, Talarmin A, Deubel V, Reynes JM, Drouet MT, Sarthou JL, Hulin A (1998) Dengue encephalitis in French Guiana. *Res Virol* 149(4):235–238
- Houng HS, Chung-Ming Chen R, Vaughn DW, Kanessa-athan N (2001) Development of a fluorogenic RT-PCR system for quantitative identification of dengue virus serotypes 1–4 using conserved and serotype-specific 3' noncoding sequences. *J Virol Methods* 95(1–2):19–32
- Huang MCY, Carlos FRM, Jonathan EF, Robert B, Brian TC, Connie JC-H (2008) VCSEL Optoelectronic biosensor for detection of infectious diseases. *IEEE Photon Technol Lett* 20(6)
- Huang MCY, Mateus CFR, Foley JE, Beatty R, Cunningham BT, Chang-Hasnain CJ (2008b) VCSEL optoelectronic biosensor for detection of infectious diseases. *IEEE Photon Technol Lett* 20(5–8):443–445
- Hurrelbrink RJ, McMinn PC (2003) Molecular determinants of virulence: the structural and functional basis for flavivirus attenuation. *Adv Virus Res* 60:1–42
- Innis BL, Nisalak A, Nimmannitya S, Kusalerdchariya S, Chongswasdi V, Suntayakorn S, Puttisiri P, Hoke CH (1989) An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *Am J Trop Med Hyg* 40(4):418–427
- Kao CL, King CC, Chao DY, Wu HL, Chang GJ (2005) Laboratory diagnosis of dengue virus infection: current and future perspectives in clinical diagnosis and public health. *J Microbiol Immunol Infect* 38(1):5–16
- Konry T, Novoa A, Shemer-Avni Y, Hanuka N, Cosnier S, Lepellec A, Marks RS (2005) Optical fiber immunosensor based on a poly(pyrrole-benzophenone) film for the detection of antibodies to viral antigen. *Anal Chem* 77(6):1771–1779
- Kuberski TT, Rosen L (1977) A simple technique for the detection of dengue antigen in mosquitoes by immunofluorescence. *Am J Trop Med Hyg* 26(3):533–537
- Kuhn RJ, Zhang W, Rossmann MG, Pletnev SV, Corver J, Lenches E, Jones CT, Mukhopadhyay S, Chipman PR, Strauss EG, Baker TS, Strauss JH (2002) Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell* 108(5):717–725

- Kuno G (1998) Universal diagnostic RT-PCR protocol for arboviruses. *J Virol Methods* 72(1):27–41
- Lam SK (1993) Rapid dengue diagnosis and interpretation. *Malays J Pathol* 15(1):9–12
- Lam SK, Devine PL (1998) Evaluation of capture ELISA and rapid immunochromatographic test for the determination of IgM and IgG antibodies produced during dengue infection. *Clin Diagn Virol* 10(1):75–81
- Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol* 30(3):545–551
- Laue T, Emmerich P, Schmitz H (1999) Detection of dengue virus RNA in patients after primary or secondary dengue infection by using the TaqMan automated amplification system. *J Clin Microbiol* 37(8):2543–2547
- Lemmer K, Donoso Mantke O, Bae HG, Groen J, Drosten C, Niedrig M (2004) External quality control assessment in PCR diagnostics of dengue virus infections. *J Clin Virol* 30(4):291–296
- Leshem B, Sarfati G, Novoa A, Breslav I, Marks RS (2004) Photochemical attachment of biomolecules onto fibre-optics for construction of a chemiluminescent immunosensor. *Luminescence* 19(2):69–77
- B.D. Lindenbach CMR (1996) Flaviviruses (Chapter 33). In: Knipe DM, Fields BN, Howley PM (eds) *Fields virology*. Lippincott-Raven, Philadelphia
- Mackay IM, Arden KE, Nitsche A (2002) Real-time PCR in virology. *Nucleic Acids Res* 30(6):1292–1305
- Mackenzie JS, Gubler DJ, Petersen LR (2004) Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat Med* 10(Suppl 12):S98–109
- Marjorie Russel HBL, Clackson T (2004) Introduction to phage biology and phage display. In: Tim Clackson HBL (Ed) *Phage display a practical approach*, p 1–26. Oxford University Press, New York
- Marks RS, Bassis E, Bychenko A, Levine MM (1997) Chemiluminescent optical fiber immunosensor for detecting cholera antitoxin. *Opt Eng* 36(12):3258–3264
- Mateus CFR, Huang MCY, Chang-Hasnain CJ, Foley JE, Beatty R, Li P, Cunningham BT (2004) Ultra-sensitive immunoassay using VCSEL detection system. *Elect Lett* 40(11)
- Megret F, Hugnot JP, Falconar A, Gentry MK, Morens DM, Murray JM, Schlesinger JJ, Wright PJ, Young P, Van Regenmortel MH et al (1992) Use of recombinant fusion proteins and monoclonal antibodies to define linear and discontinuous antigenic sites on the dengue virus envelope glycoprotein. *Virology* 187(2):480–491
- Mukhopadhyay S, Kuhn RJ, Rossmann MG (2005) A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol* 3(1):13–22
- Murgue B, Deparis X, Chungue E, Cassar O, Roche C (1999) Dengue: an evaluation of dengue severity in French Polynesia based on an analysis of 403 laboratory-confirmed cases. *Trop Med Int Health* 4(11):765–773
- World Health Organization (WHO) (1997) *Dengue haemorrhagic fever: diagnosis, treatment, prevention and control*. Geneva, Switzerland
- World Health Organization (WHO) (2004) *Dengue diagnostics*. Proceedings of an international workshop, Geneva, Switzerland
- Paddle BM (1996) Biosensors for chemical and biological agents of defence interest. *Biosens Bioelectron* 11(11):1079–1113
- Palmer CJ, King SD, Cuadrado RR, Perez E, Baum M, Ager AL (1999) Evaluation of the MRL diagnostics dengue fever virus IgM capture ELISA and the PanBio Rapid Immunochromatographic Test for diagnosis of dengue fever in Jamaica. *J Clin Microbiol* 37(5):1600–1601
- Pancharoen C, Mekmullica J, Thisyakorn U (2001) Primary dengue infection: what are the clinical distinctions from secondary infection? *Southeast Asian J Trop Med Public Health* 32(3):476–480
- Petrenko VA, Vodyanoy VJ (2003) Phage display for detection of biological threat agents. *J Microbiol Methods* 53(2):253–262
- Petrosova TKA, Cosnier S, Trakht I, Lutwama J, Rwaguma E, Chepurnov A, Muhlberger E, Lobel L, Marks R (2007) Development of a highly sensitive, field operable biosensor for serological studies of Ebola virus in central Africa. *Sensor Actuat B* 122(2):578–86

- Phuong CX, Nhan NT, Kneen R, Thuy PT, van Thien C, Nga NT, Thuy TT, Solomon T, Stepniewska K, Wills B (2004) Clinical diagnosis and assessment of severity of confirmed dengue infections in Vietnamese children: is the world health organization classification system helpful? *Am J Trop Med Hyg* 70(2):172–179
- Polyak B, Bassis E, Novodvoretz A, Belkin S, Marks RS (2000) Optical fiber bioluminescent whole-cell microbial biosensors to genotoxics. *Water Sci Technol* 42(1–2):305–311
- Polyak B, Bassis E, Novodvoretz A, Belkin S, Marks RS (2001) Bioluminescent whole cell optical fiber sensor to genotoxics: system optimization. *Sensor Actuat B – Chem* 74(1–3):18–26
- Sabchareon A, Lang J, Chanthavanich P, Yoksan S, Forrat R, Attanath P, Sirivichayakul C, Pengsaa K, Pojjaroen-Anant C, Chambonneau L, Saluzzo JF, Bhamarapravati N (2004) Safety and immunogenicity of a three dose regimen of two tetravalent live-attenuated dengue vaccines in five- to twelve-year-old Thai children. *Pediatr Infect Dis J* 23(2):99–109
- Salama K, Eltoukhy H, Hassibi A, El-Gamal A (2004) Modeling and simulation of luminescence detection platforms. *Biosens Bioelectron* 19(11):1377–1386
- Sang CT, Cuzzubbo AJ, Devine PL (1998a) Evaluation of a commercial capture enzyme-linked immunosorbent assay for detection of immunoglobulin M and G antibodies produced during dengue infection. *Clin Diagn Lab Immunol* 5(1):7–10
- Sang CT, Hoon LS, Cuzzubbo A, Devine P (1998b) Clinical evaluation of a rapid immunochromatographic test for the diagnosis of dengue virus infection. *Clin Diagn Lab Immunol* 5(3):407–409
- Shu PY, Huang JH (2004) Current advances in dengue diagnosis. *Clin Diagn Lab Immunol* 11(4):642–50
- Shu PY, Chang SF, Kuo YC, Yueh YY, Chien LJ, Sue CL, Lin TH, Huang JH (2003) Development of group- and serotype-specific one-step SYBR green I-based real-time reverse transcription-PCR assay for dengue virus. *J Clin Microbiol* 41(6):2408–2416
- Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 228(4705):1315–1317
- Sobarzo A, Paweska JT, Herrmann S, Amir T, Marks RS, Lobel L (2007) Optical fiber immunosensor for the detection of IgG antibody to Rift Valley fever virus in humans. *J Virol Methods* 146(1–2):327–334
- Talarmin A, Labeau B, Lelarge J, Sarthou JL (1998) Immunoglobulin A-specific capture enzyme-linked immunosorbent assay for diagnosis of dengue fever. *J Clin Microbiol* 36(5):1189–1192
- Teles FR, Prazeres DM, Lima-Filho JL (2005) Trends in dengue diagnosis. *Rev Med Virol* 15(5):287–302
- Thein S, Aung MM, Shwe TN, Aye M, Zaw A, Aye K, Aye KM, Aaskov J (1997) Risk factors in dengue shock syndrome. *Am J Trop Med Hyg* 56(5):566–572
- Trirawatanapong T, Chandran B, Putnak R, Padmanabhan R (1992) Mapping of a region of dengue virus type-2 glycoprotein required for binding by a neutralizing monoclonal antibody. *Gene* 116(2):139–150
- Vaughn DW, Nisalak A, Kalayanaroj S, Solomon T, Dung NM, Cuzzubbo A, Devine PL (1998) Evaluation of a rapid immunochromatographic test for diagnosis of dengue virus infection. *J Clin Microbiol* 36(1):234–238
- Vaughn DW, Nisalak A, Solomon T, Kalayanaroj S, Nguyen MD, Kneen R, Cuzzubbo A, Devine PL (1999) Rapid serologic diagnosis of dengue virus infection using a commercial capture ELISA that distinguishes primary and secondary infections. *Am J Trop Med Hyg* 60(4):693–698
- Vaughn DW, Green S, Kalayanaroj S, Innis BL, Nimmanitya S, Suntayakorn S, Endy TP, Raengsakulrach B, Rothman AL, Ennis FA, Nisalak A (2000) Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *J Infect Dis* 181(1):2–9
- Wu SJ, Hanson B, Paxton H, Nisalak A, Vaughn DW, Rossi C, Henchal EA, Porter KR, Watts DM, Hayes CG (1997) Evaluation of a dipstick enzyme-linked immunosorbent assay for detection of antibodies to dengue virus. *Clin Diagn Lab Immunol* 4(4):452–457

- Wu SJ, Lee EM, Putvatana R, Shurtleff RN, Porter KR, Suharyono W, Watts DM, King CC, Murphy GS, Hayes CG, Romano JW (2001a) Detection of dengue viral RNA using a nucleic acid sequence-based amplification assay. *J Clin Microbiol* 39(8):2794–2798
- Wu HC, Huang YL, Chao TT, Jan JT, Huang JL, Chiang HY, King CC, Shaio MF (2001b) Identification of B-cell epitope of dengue virus type 1 and its application in diagnosis of patients. *J Clin Microbiol* 39(3):977–982
- Wu HC, Jung MY, Chiu CY, Chao TT, Lai SC, Jan JT, Shaio MF (2003) Identification of a dengue virus type 2 (DEN-2) serotype-specific B-cell epitope and detection of DEN-2-immunized animal serum samples using an epitope-based peptide antigen. *J Gen Virol* 84(Pt 10):2771–2779
- Wu TZ, Su CC, Chen LK, Yang HH, Tai DF, Peng KC (2005) Piezoelectric immunochip for the detection of dengue fever in viremia phase. *Biosens Bioelectron* 21(5):689–695
- Yamada K, Takasaki T, Nawa M, Kurane I (2002) Virus isolation as one of the diagnostic methods for dengue virus infection. *J Clin Virol* 24(3):203–209
- Zaytseva NV, Montagna RA, Baeumner AJ (2005a) Microfluidic biosensor for the serotype-specific detection of dengue virus RNA. *Anal Chem* 77(23):7520–7527
- Zaytseva NV, Montagna RA, Baeumner AJ (2005b) Microfluidic biosensor for the serotype-specific detection of Dengue virus RNA. *Anal Chem* 77(23):7520–7527

Part IV

Parasites and Fungi

Infectious Forms of Parasites in Food: Man Embedded in Ecosystems

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Abstract Foodborne parasites have usually environmental infectious stages. In developing countries, high concentrations of parasite infectious forms contaminate the environment entailing high prevalence of foodborne parasitic diseases with significant impact on health and economy. Complex interactions among exposure to contaminated food and water, disease, poverty and malnutrition take place. In developed countries, food- or waterborne parasitic infections keep also a considerable importance, as shown by the persistence of infectious water- or foodborne outbreaks and the occurrence of infections due to emergent or reemerging eukaryotic pathogens. Since parasite infections are usually rare in developed areas, a breakdown in prevention measures and specific health staff training often occur. Most emerging or reemerging parasite species are agents of foodborne infections. Considering only parasite “protists”, most emerging or reemerging species can be foodborne, waterborne or contagious as *Giardia*, *Cryptosporidium* species or,

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likely, Microsporidia. These pathogens belong to taxonomically unrelated Eukaryotic groups but they share common features: (a) mostly monoxenous; (b) ubiquitous; (c) challenging taxonomy; (d) unclear notions on transmission, infection source or reservoir; (e) highly resistant infectious life stages; (f) lack of efficient experimental models; (g) uncertain pathogenic power to immunocompetent hosts. Regarding emerging or reemerging helminthes, those transmitted by foodborne route are: *Anisakis simplex*, *Echinococcus granulosus*, *Diphyllbothrium* species, *Metorchis conjunctus*, *Taenia solium* and *Trichinella spiralis*. To consume exotic meals could increase foodborne parasitic risk in Europe. Although common representations of human daily life show a humanity that has in some way “escaped” from ecosystems, men take on their role of top level consumers, and are therefore exposed to the associated highly diversified parasitological risk.

Keywords parasites • fungi • emerging • reemerging • opportunistic • foodborne • cryptosporidiosis • foodborne parasite infections • *Anisakis* • *Trichinella* • eukaryotic pathogens

Foodborne Parasitic Diseases in Developing and Developed Areas

Parasitic diseases are hardly ever considered as public health priorities (except perhaps malaria) and they can more and more frequently be labelled as neglected infectious diseases. However, they constitute the most common infections among the three billion people who live on less than \$2 a day, causing more than half million deaths a year in such populations, leading to long-term infirmities and poverty (Hotez et al. 2007). Among the parasitic diseases, foodborne parasitic infections have high impact. They are recognized as factors of under-development in African countries, interfering further with children’s mental and physical developing. In short, parasitic diseases contribute to deep inequalities between human communities of developed areas and the more vulnerable ones of developing regions (Selgelid 2007; Boury and Dei-Cas 2008).

Foodborne parasites have usually environmental infectious stages. Since sanitary infrastructures are often insufficient in developing areas, parasite infectious forms contaminate the environment at high concentrations. Foodborne parasitic diseases are therefore frequent having strong impact on human or animal health and economy. Complex interactions among exposure to contaminated food and water, disease, poverty and malnutrition take place (Hall 1997). Furthermore, adequately trained healthcare staffs are often little available in these areas.

Interestingly, even in developed areas, where sanitary infrastructures are usually good, food- or waterborne parasitic infections keep also a considerable importance, as shown by the persistence of infectious water- or foodborne outbreaks and the occurrence of infections due to emergent or re-emergent pathogens (Dowell and Levitt 2002). Pathogen emergence or reemergence is favoured by the extension of

vulnerable populations and by environmental changes (see next section). The extension of susceptible populations is mainly due to the increasing of immunocompromised human population groups (subjects severely weakened by cancer, immunosuppressive treatments, AIDS or other conditions) and population ageing. Environmental changes consist mainly in concentration of human or animal food production (often followed by widespread distribution), increased consumption of fresh fruits, vegetables or “ethnic” foods, home-meal replacement, increased use of child-care facilities, increase of international travel and migration, and urban decay (CDC 1994; Dowell and Levitt 2002).

The high efficiency of sanitary infrastructures in developed areas entails paradoxical consequences. As concentration of pathogens in the environment is usually low, the detection/quantification of infectious parasitic forms and risk assessing are difficult. However, foodborne infections keep a strong health and economic impact. In USA, benign gastric or enteric diseases are the second cause of absenteeism with an annual cost of about 20 billion dollars. In this country, foodborne infections cause between two and 9,000 deaths per year (CDC 1994).

Furthermore, some breakdown in public health measures and lack of adequately trained health staffs occurred also in developed areas (CDC 1994). It resulted likely from both the relative scarcity of parasitic diseases in these regions, and the generalised belief that control of major endemics was irreversibly mastered (Selgelid 2007; Boury and Dei-Cas 2008). In addition, these diseases have a falsely “exotic” character since they seem to involve exclusively marginal fractions of population in developed countries (severely weakened patients with AIDS or other immunosuppressing conditions) or poor populations in developing ones. These views had impact on research policies and on the teaching of Parasitology and Medical Mycology in European Medicine Faculties, as it was underlined recently in the tenth European Multicolloquium of Parasitology (EMOP-10, Paris, 2008). Even in papers published in high-impact journals, it is becoming increasingly frequent to see gaps in the basic knowledge of parasite biology, taxonomy, biology of vectors and parasite–host relationships (Dupouy-Camet et al. 2009).

Foodborne Parasitic Diseases as Emerging or Reemerging Infections

The expression emerging infection is currently applied to new or newly identified pathogens or syndromes, which have been recognized over the last 2 decades, or which has resulted in new manifestations of disease follow on genetic changes of already recognized agents (CDC 1995). Re-emerging infection defines a known or previously identified pathogen or syndrome which is increasing in incidence, expanding into new geographic areas, affecting new population groups, or which threatens to increase in the near future (CDC 1995).

Real health impact and costs of foodborne diseases in developing or developed areas are badly known. Only outbreak situations with major public impact are

usually recognized. However, globalization increases the risk of rapid extension of foodborne infection outbreaks to distant regions. Indeed, foodborne diseases are spreading faster and appear to become emerging more rapidly than ever before (Jones et al. 2008).

A recent report of Woolhouse and Gowtage-Sequeria (2005) showed that most emerging or re-emerging pathogenic species are responsible for foodborne infections. Since 1991, the eukaryotic considered as emerging or reemerging species were *Cryptosporidium hominis*, microsporidia, and the trematode *Metorchis conjunctus*, an Opisthorchiidae of North America. The Coccidian *Cyclospora* and the microsporidian *Enterocytozoon bieneusi*, both responsible for diarrhea, were among the emerging pathogens before 1991. All these infections can be contracted by ingestion of contaminated food and also in some cases by consuming contaminated water. Nowadays, among 57 species of recognized protozoan pathogens, 14 (25%) are considered as emerging or re-emerging (Woolhouse and Gowtage-Sequeria 2005). Seven of them cause foodborne or waterborne infections or are transmitted by fecal-oral route: *Cryptosporidium hominis*, *C. parvum*, *Cyclospora cayetanensis*, *Giardia intestinalis*, *Isospora belli*, the tissue coccidian *Toxoplasma gondii* and *Trypanosome cruzi*, a flagellated protist. Foodborne eukaryote microparasites (=unicellular small-sized parasites which display active replication within the host) (Anderson and May 1998) defined as emerging or reemerging after 1984 are shown in Table 1.

Cryptosporidium species and *Giardia intestinalis* are typical waterborne or foodborne parasitic infections. *Cryptosporidium* oocysts or *Giardia* cysts are infectious when excreted with the host feces, whereas *T. gondii* or *Cyclospora cayetanensis* oocysts need to undergo maturation or sporulation in the environment to reach the infective stage. This explains why *Cryptosporidium* or *Giardia* infection can be transmitted by human-to-human direct contact whereas *Toxoplasma* or *Cyclospora* cannot be transmitted in this manner. *Isospora belli* oocysts undergo also environmental maturation (Bonnin et al. 1992). However, as the minimum sporulation time is about 20–24 h, transmission between male homosexuals could be possible (Morakote et al. 1987).

Giardia intestinalis (= *G. duodenalis* or *G. lamblia*) is the commonest intestinal protist worldwide. Prevalence of *Giardia* infection in adults is 2–7.5% in developed areas and 12–30% in developing ones (Favennec et al. 2006; Jiménez et al. 2009). In children, the prevalence is even higher. *G. intestinalis* infection was longtime considered as restricted to humans. However, recent observations indicate that this species can be found in cattle, pets and other mammals. Bovines are frequently infected by *G. intestinalis* genotypes A or E though associated diarrhea is rather rare (Appelbee et al. 2005). In domestic dogs, the prevalence is often >5% and genotypes C and D were the most frequently reported (Berrilli et al. 2004). These animals can however be infected by genotypes A or B, both pathogenic to humans (Favennec et al. 2006). Should domestic or wild mammals be considered as *G. intestinalis* reservoir (=set of biotic or abiotic structures critical to pathogen survival)? Indeed, four *G. intestinalis* life cycles, with infrequent interactions among them, have been identified: dog/cat, livestock, wild and human life cycles. Cross infection

Table 1 Emerging and reemerging foodborne eukaryotic microparasites^a

Taxonomic assignment	Species	Life cycle	Foodborne	Waterborne	Other transmission mechanisms
Metamonadina (Excavata)	<i>Giardia intestinalis</i>	Monoxenous	Yes	Yes	Host-to-host contact
Euglenozoa (Excavata)	<i>Trypanosoma cruzi</i>	Heteroxenous (vectorborne, large wild reservoir)	Yes (food contaminated with triatomine or their dejections)	Rare	Insect vector, blood transfusion, organ transplantation, tranplacental way
Apicomplexa (Alveolata)	<i>Cryptosporidium hominis</i>	Monoxenous (apparently human specific)	Yes	Yes	Human-to-human contact
	<i>Cryptosporidium parvum</i>	Monoxenous (zoonotic)	Yes	Yes	Host-to-host contact
	<i>Cyclospora cayetanensis</i>	Monoxenous	Yes	Yes	–
	<i>Toxoplasma gondii</i>	Heteroxenous (felid definitive hosts; large range of intermediary or paratenic hosts)	Yes	Yes	Blood transfusion, organ transplantation, tranplacental way
	<i>Isospora belli</i>	Monoxenous (human specific)	Yes	Yes	Male homosexual human-to-human transmission (hypothetical)

(continued)

Table 1 (continued)

Taxonomic assignment	Species	Life cycle	Foodborne	Waterborne	Other transmission mechanisms
Microsporidia (Fungi)	<i>Encephalitozoon cuniculi</i>	Monoxenous, with a large mammalian reservoir	Likely	Feasible, though spores detected only rarely in water	Intrauterine transmission (in animals)
	<i>Encephalitozoon intestinalis</i>	Monoxenous, with a large mammalian reservoir	Feasible	Feasible (spores detected in water)	?
	<i>Encephalitozoon hellem</i>	Monoxenous, identified in humans and birds	?	Likely unfeasible	Host-to-host transmission
	<i>Enterocytozoon bieneusi</i>	Monoxenous, with a large mammalian reservoir	Likely (food contaminated with fecal material)	Likely (spores detected in freshwater)	Host-to-host fecal-oral or oral-oral route, male homosexuality, inhalation of aerosols (Mathis et al, 2005)
	<i>Trachypleistophora hominis</i>	Experimental development in mosquito larvae. Infectious by injection to SCID mice	Apparently unfeasible	?	?
	<i>Trachypleistophora anthropophthera</i>	Experimental development on mouse fibroblasts	Unlikely	Unlikely	?
	<i>Pleistophora ronneafiei</i>	Apparently monoxenous, identified in human and fish	?	Feasible (spores detected in freshwater)	?

^aThe term ‘microparasite’ was used in the sense of Anderson and May (1998): small-sized parasites able to replicate usually at very high rates within the host. In the context of the present work, microparasites are exclusively eukaryotic microorganisms (protists and fungi). Most data come from Woolhouse et al. (2006) and Fagherazzi-Pagel (2009).

experiments showed that dogs or beavers can be infected with human-derived *G. intestinalis* isolates, whereas humans can only rarely be infected with non-human *G. intestinalis* isolates. On the whole, non-human mammals could not work as a significant infection source to humans and they would not be reservoirs to *G. intestinalis* human strains. In contrast, in some contexts, humans could constitute a *Giardia* infection source to animals (Graczyk et al. 2002; Favenne et al. 2006).

Emergence of microsporidia (Table 1) is based on the discovery, after 1985 (Desportes et al. 1985), of new microsporidian species infecting humans (*Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *E. hellem*, *Pleistophora ronneafiei*, *Trachypleistophora hominis*, *T. anthrophthera*) along with the development of improved diagnostic methods (especially, molecular techniques) that are revealing an active circulation of highly diversified microsporidian organisms in human and animal populations (Didier 2005). This new insight led the United States Environmental Protection Agency (US-EPA) to include, since June 2008, *E. bieneusi* and *E. intestinalis*, the species with the highest prevalence in humans, on the Second Contaminant Candidate List (CCL 2) (<http://www.epa.gov/safewater/ccl/cclfs.html>). Likewise, microsporidia have been also included on the Centers for Disease Control and Prevention and the National Institutes of Health lists of “Biodefense” Category B Priority Pathogens of concern for waterborne and foodborne transmission (http://www2.niaid.nih.gov/Biodefense/bandc_priority.htm) (Didier 2005), besides other microparasites (*Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Giardia intestinalis*, *Entamoeba histolytica* and *Toxoplasma gondii*).

Trypanosoma cruzi, the agent of American trypanosomiasis or Chagas’ disease, merits a special consideration. This protist is usually transmitted to humans and other mammals by the dejections of blood-sucking triatomine insects, by blood transfusion, organ transplantation or transplacental infection. However, American trypanosomiasis can also be contracted by oral route by consumption of food contaminated with triatomines or their feces (Nóbrega et al. 2009). Apparently, trypomastigotes, epimastigotes and likely, *T. cruzi* amastigotes, are able to infect humans by oral route (Dias et al. 2008). Contaminated home-meals, cane juice, açai (= fruit of açai palm) or other fruit juices as well as soup, broth or even raw or lightly cooked meat of contaminated wild animals can be the infection source. In addition, since contaminated triatomine dejections can keep their infection power for many hours in damp environments, flies or other secondary carriers can in their turn contaminate from such sources and transport the infectious trypanosomes to food. In milk or cane juice at room temperature, the trypanosomes can remain viable for more than 24 h (Dias 2008). Other data (Benchimol-Barbosa 2009) indicate that in açai fruit, *T. cruzi* remains viable for up to 9 h. It has been also found to be viable for up to 12 h at temperatures of 5°C, but is killed after 2 h at –20°C (Neves et al. 2007). Pasteurization techniques seem to be effective for inactivating the parasite. Sanitary managers involved in prevention often overlook oral infection with *T. cruzi*. However, oral infection is often associated with severe acute symptoms and high lethality rates likely due to massive trypanosome penetration in digestive mucosa. Thus, a lethality rate of 12% was reported in the Santa-Catarina (Brazil) outbreak in 2005 (Benchimol-Barbosa 2009), but it could be much higher (Dias et al. 2008).

In the Santa-Catarina outbreak patients deceased on about 12 days after the infection. Most oral outbreaks were described in Brazil and Venezuela but cases have also been reported in Argentina, Colombia and Mexico (Dias et al. 2008).

Characterizing Emerging and Reemerging Microparasites as a Whole

Table 1 (first column) showed the taxonomical assignments of these pathogens. Indeed, the old Kingdom of Protists (or Protoctists) has disappeared from the new phylogenetic classifications of living (Lecointre and Le Guyader 2001). Molecular genetics approaches have shown that unicellular eukaryotic organisms belonging to this Kingdom presented sometimes more phylogenetic affinities with multicellular than with unicellular organisms. Thus, the highest ranks of eukaryotes are the followings: Amoebozoa (unicellular organisms like parasite or free amoebae), Opisthokonta (unicellular and pluricellular organisms like Fungi and Metazoa), Rhizaria (unicellular organisms like Radiolaria and Foraminifera), Archaeplastida (unicellular algae and pluricellular organisms like Plants), Chromalveolata (unicellular organisms like Alveolata and pluricellular ones like Stramenopiles) and Excavata (unicellular organisms, including Metamonadina, Euglenozoa, Parabasalia) (Adl et al. 2005).

Emerging and reemerging eukaryotic microparasites belong to taxonomically unrelated groups of the Eukaryota Division: Metamonadina, Euglenozoa, Apicomplexa and Fungi, since microsporidia (formerly considered as protists) were assigned recently to the group of Fungi. However, though taxonomically unrelated, emerging eukaryotic microparasites have medically important common features:

1. Most are monoxenous (= one host), a feature which is consistent with an easier spreading. Especially, if human is the specific host, he can play the role of reservoir and of infectious source to other humans or to the environment (e.g. *Giardia intestinalis*, *C. hominis*, *Cyclospora cayetanensis*, *Isospora belli*).
2. They are usually ubiquitous: cysts of *Giardia*, oocysts of Apicomplexa coccidians and spores of microsporidia are largely distributed in the environment contaminating food and water. Contamination rates are likely lower in developed than in developing areas. However, infectious parasitic life stages can be detected in marketed autochthonous fruits and comestible vegetables in developed countries in temperate or even cold areas (Robertson and Gjerde 2001; Robertson et al. 2002, 2005), where outbreaks have been reported (Adak et al. 2002; Rocourt et al. 2003; Pönka et al. 2008).
3. Taxonomy is often problematic, making difficult an accurate identification of agents. This is a key concern, especially to both explain the cause of outbreaks or sporadic cases and define suitable prevention measures. There remains uncertainty about the species or genotypes that are able to infect humans (Kaneshiro and Dei-Cas 2009). Emerging eukaryotic microparasites can exhibit wide genetic diversity with significant impact on speciation, pathogenic power or transmission patterns.

Thus, experienced fine analyses of genetic divergence, often at an infra-specific level, have to be used in order to trace these pathogens in host populations and to understand their circulation in the environment. In fact, besides purely academic interest, molecular taxonomy of these pathogens could reveal extremely helpful in the field. Additionally, population genetics methods are often needed in order to assess genetic flow among the genetic variants of microparasites. This last aspect have a double interest: on one hand, population genetics approaches can provide data about potential dissemination of genetic features of medical importance, like virulence, drug susceptibility and transmissibility. On the other hand, such approaches can provide evidence of reproductive isolation between apparently close groups, a kind of information with potential taxonomical involvement.

4. Transmission mechanisms, infection source or reservoir are often unclear. Most outbreaks and sporadic cases remain unexplained. Only a comprehensive understanding of taxonomy and circulation of eukaryotic microparasites in mammal populations can disentangle the natural history of the infections they cause.
5. Infectious stages are usually highly resistant in the environment. Enteric protozoa such as *Giardia* and *Cryptosporidium* are highly resistant in the aquatic environment. They are also resistant to most disinfectants and antiseptics commonly used for water treatment. Contaminated water used to irrigate crops is likely the main source of food contamination. Consistently, many outbreaks of giardiasis and cryptosporidiosis were found associated with potable water, even when processing systems were operated in accordance with conventional standards of water treatment, and while current microbiological standards were met (Leclerc et al. 2004).
6. Efficient in vivo or in vitro models are often lacking, making difficult the assessing of viability or infectious power. Host species specificity or other factors make difficult to set experimental models in laboratories, either for perpetuating parasite isolates or for developing basic biology research. The scarcity of such models has hindered the understanding of the pathophysiological mechanisms of these infections (Kaneshiro and Dei-Cas 2009), and it is, at least partially, one of the reasons why pharmaceutical industry shows a limited interest in the field. The limited availability of effective drugs against these eukaryotic pathogens remains, however, a major concern. For example, no safe drugs to efficiently clear *Cryptosporidium* or *Enterocytozoon bienersi* are available.
7. While emerging eukaryotic microparasites are pathogenic in severely immunocompromised hosts, they are also able to infect those who are immunocompetent. Indeed, most diseases due to emerging or reemerging eukaryotic microparasites (e.g. cryptosporidiosis, microsporidiosis, isosporosis or cerebral toxoplasmosis) have been observed in severely immunosuppressed AIDS patients before the setting up of highly active anti-retroviral therapy (HAART) protocols. After HAART, prevalence of opportunistic parasitic infections decreased in AIDS patients, but “new” clinical presentations of such diseases emerged in either HIV-negative immunocompromised patients (e.g. cryptosporidiosis or microsporidiosis in bone marrow- or solid-organ transplanted patients) or immunocompetent

subjects (e.g. involvement of *Cryptosporidium* or microsporidia in acute diarrhea or in traveler's diarrhea episodes, or asymptomatic carriage of these agents). But only limited data are available on the impact of these infections in immunocompetent hosts (Kaneshiro and Dei-Cas 2009). They are becoming however a public health issue. On one hand, even if healthy hosts develop mild, and often undiagnosed infections, that allows parasite survival and spreading in the population. On the other hand, though usually benign, the infection of immunocompetent hosts can have a significant health, social or economic impact. For instance, *Giardia*, *Cryptosporidium*, *Isospora*, and *Cyclospora* are recognized agents of diarrheal disease (including traveller's diarrhea) in immunocompetent hosts. Acute diarrhea is most commonly caused by gastrointestinal infections, being *Campylobacter*, *Salmonella*, *Cryptosporidium*, and *Giardia* the most common diarrhea agents (WHO 2000). Diarrhea is the ninth leading cause of death worldwide (WHO 2000). About four billion cases each year cause 2.2 million deaths (4% of all deaths). Emerging infections due to eukaryotic microparasites in immunocompromised patients could thus be seen like the tips of icebergs. We need now to explore their submerged part.

The Case of *Cryptosporidium*

The case of *Cryptosporidium* species substantiates at best the common features examined in the precedent section. These atypical coccidians, responsible for severe chronic diarrhea in immunocompromised patients and for acute diarrhea in immunocompetent subjects (Chalmers and Davies 2009), penetrate enterocytes remaining at the microvillous surface level (Fig. 1). This situation is usually qualified as intracellular – extracytoplasmic or, more recently, epicellular (Valigurová et al. 2008), as the parasites develop at the level of the brush border of microvillus. A feeder organelle can be observed in the zone of contact with the host-cell cytoplasm using transmission electron microscopy (TEM) (Valigurová et al. 2008).

The complete development of *Cryptosporidium* occurs in only one host. Infectious forms are sporozoites contained in oocysts that infect the host by oral route. Sporozoites penetrate enterocytes and develop into trophozoites remaining at the brush border level (Valigurová et al. 2008). Trophic forms develop merogonic multiplication that generates at first asexual merozoites and then sexual gamonts whose maturation and nuclear fusion will lead to the production of infectious oocysts. Thick walled oocysts are shed with the host feces, while thin walled oocysts can cause autoinfection, especially in immunocompromised hosts. As thick walled oocysts are immediately infectious, the infection can be transmitted by direct host-to-host contact. As they are highly resistant, they contaminate the environment, water, soil and food, keeping their infectious power for longtime.

Detection of *Cryptosporidium* oocysts in clinical specimens or in environmental samples can be performed using microscopy. Modified Ziehl-Nielsen stain, auramine, fluorescein-labeled specific antibody, differential interference contrast microscopy

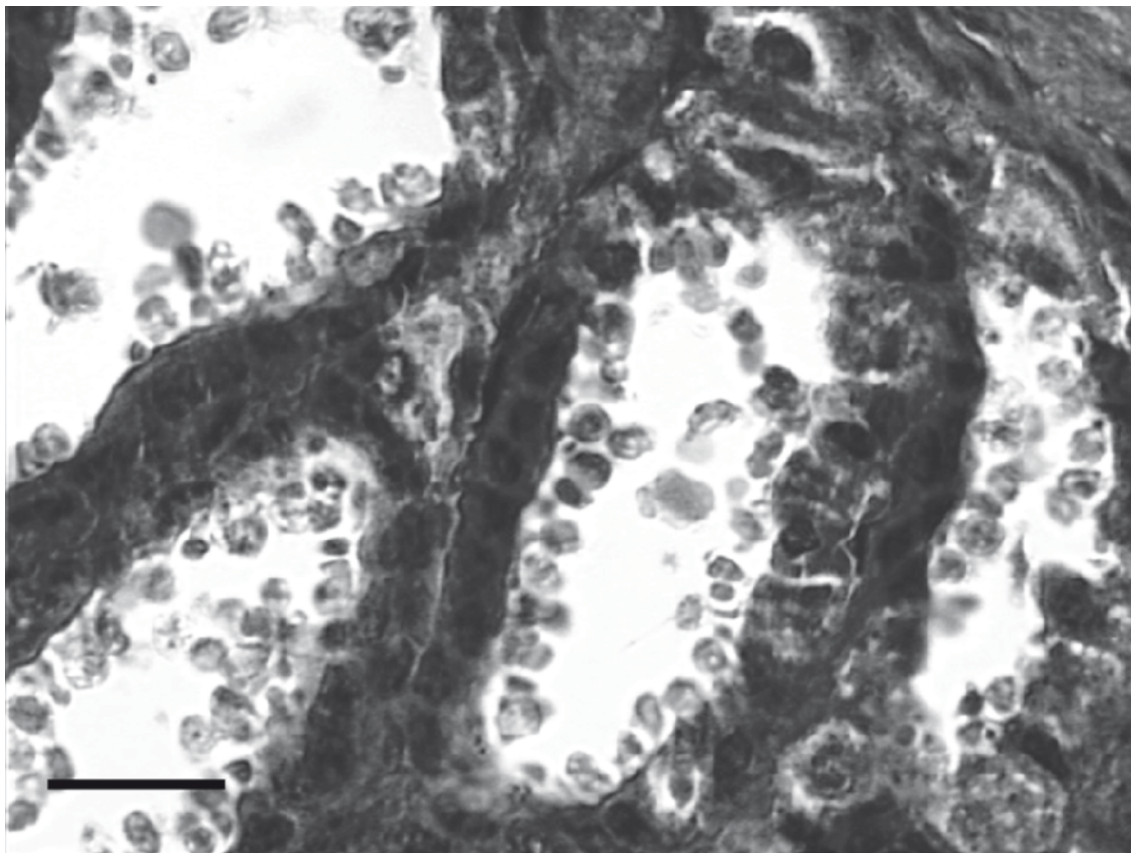


Fig. 1 Development of *Cryptosporidium muris* in the SCID mouse. Stomach section from a SCID mouse euthanatized 46 days post-infection. Gastric glands are filled with numerous parasites at different developmental stages. Hematoxylin & Eosin staining. Bar = 10 μ m

and fluorescent DAPI staining are often used. Immunomagnetic separation (IMS), using magnetic beads coated with anti-*Cryptosporidium* antibody, can be used to purify and concentrate oocysts especially from environmental concentrated samples (water filtration eluates), where oocyst rates are often low (Guyot et al. 2005). In addition, standard in vitro excystation methods to evaluate viability of parasitic samples can be used (Guyot et al. 2000). However, the detection methods we have evoked, did not allow identifying the species.

More than 20 *Cryptosporidium* species were described in members of the five groups of vertebrates. Although this was not always the case, species description should be based on molecular and phenotypic criteria, including oocyst size, host range and life cycle features (Fayer et al. 1997; Xiao et al. 2004; Tzipori and Widmer 2008; Fayer 2009). The following species have been reported in humans until now: *C. hominis*, *C. parvum*, *C. muris*, *C. suis*, *C. felis*, *C. andersoni*, *C. canis*, *C. baileyi* and *C. meleagridis*. As morphology is similar in the diverse *Cryptosporidium* species, molecular methods were developed. In order to identify species, 18S ribosomal DNA is one of the most frequently targeted sequence (Guyot et al. 2001). However, more and more frequently, multilocus sequencing based strategies are used for infraspecific genotyping (Mallon et al. 2003; Certad et al. 2006; Ngouanesavanh et al. 2006; Tanriverdi et al. 2008). These approaches allow to trace genotypes but also to study the genetic structure of *Cryptosporidium* populations, to assess the genetic flow between sub-populations and the structuring role of host species or geography.

The fact that a given *Cryptosporidium* species or variant (genotype) was not reported in humans did not mean it is unable to colonize these hosts. On one hand, in most countries, current diagnosis of cryptosporidiosis is based on detection in stool samples of *Cryptosporidium* oocysts by microscopy or *Cryptosporidium* antigen by either enzyme immunoassay or immunochromatography without molecular determination of the species. On the other hand, in some cases PCR fails to amplify *Cryptosporidium* DNA from samples in which oocysts were seen by microscopy. Unsuccessful DNA extraction, amplification inhibitors, or oocysts that did not belong to the species targeted by the PCR assay (usually *C. hominis* and *C. parvum*) could explain this divergence. It is therefore important to use generic primers at first, and then to proceed to the molecular typing (Guyot et al. 2001).

Cryptosporidium species seem to be frequent parasites in teleostean fishes. Two species, *C. molnari* in dorada (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) and *C. scophthalmi* in turbot (*Scophthalmus maximus*) have been described recently by the Alvarez-Pellitero group (Alvarez-Pellitero and Sitja-Bobadilla 2002; Alvarez-Pellitero et al. 2004; Ryan et al. 2004). In recent still unpublished experiments we showed that *C. molnari* oocysts were unable to infect SCID mice. We do not know, however, if fish *Cryptosporidium* species, whose oocysts could contaminate accidentally raw fish-based food, are infectious to humans.

Infectious *Cryptosporidium* oocysts, *Giardia* cysts and Microsporidia spores were reported in edible aquatic invertebrates (Table 2). The ability of these organisms to recover oocysts or cysts from water, concentrate them and change their biological properties, like infectivity (Li et al. 2006), was also approached (Table 2).

Table 2 Infective forms of pathogenic eukaryotic microparasites in aquatic invertebrates

Species	<i>Crassostrea</i> spp	<i>Ostrea edulis</i>	<i>Dreissena</i> <i>polymorpha</i>	<i>Mytilus</i> <i>galloprovincialis</i> , <i>M. edulis</i>	<i>Ischadium</i> <i>recurvum</i>	<i>Corbicula</i> <i>fluminea</i>	<i>Dosinia exoleta</i> , <i>Ruditapes</i> <i>philippinarum</i> , <i>Venerupis</i> sp, <i>Venus verrucosa</i>	<i>Artemia</i> <i>franciscana</i>
<i>Cryptosporidium parvum</i> or <i>C. hominis</i>	N ^{a,b,c,d} E ^{j,k}	N ^e E ^m	N ^f E ⁿ	N ^{e,g}	N ^h	E ^m	N ^{e,i}	E ^p
<i>Giardia</i>	N ^a E ^{j,k}	—	N ^f E ⁿ	—	—	E ⁿ	—	E ^p
<i>Encephalitozoon intestinalis</i>	E ^j	—	N ^f	—	—	—	—	—
<i>Encephalitozoon hellem</i>	E ^j	—	N ^f	—	—	—	—	—
<i>Enterocytozoon bienersi</i>	E ^j	—	N ^f	—	—	—	—	—
<i>Toxoplasma gondii</i>	E ^l	—	—	E ^o	—	—	—	—
<i>Cyclospora cayentanensis</i>	—	—	—	—	—	E ^q	—	—

N: in nature; E: experimental; — : no report

^a Schets et al. 2007^b Fayer et al. 1998^c Fayer et al. 1999^d Graczyk et al. 2007^e Freire-Santos et al. 2000^f Graczyk et al. 2004^g Li et al. 2006^h Graczyk et al. 1999ⁱ Molini et al. 2007^j Graczyk et al. 2006^k Graczyk et al. 1998a^l Lindsay et al. 2004^m Freire-Santos et al. 2002ⁿ Graczyk et al. 2001^o Arkush et al. 2003^p Méndez-Hermida et al. 2006^q Graczyk et al. 1998b

To our knowledge, no case of human cryptosporidiosis or other infections due to eukaryotic microparasites contracted by shellfish consumption was reported. However, these infections could go unnoticed since parasites are not routinely sought in clinical samples from subjects with gastroenteritis after shellfish eating.

Table 3 shows a non-exhaustive list of documented cases of foodborne cryptosporidiosis sporadic or epidemic human cases.

Cryptosporidium prevalence in the general population is badly known in Europe. A survey in France 10 years ago found 0.4% in healthy adults (AFSSA 2002). Low prevalence was reported also in UK (Wheeler et al. 1999). *Cryptosporidium* was detected in the stools of 2% of patients with acute gastroenteritis in the Netherlands (De Wit et al. 2001). However, important cryptosporidiosis outbreaks have been reported in USA or Europe. The most striking one occurred in Milwaukee, Wisconsin in 1993. It involved more than 400,000 persons and it caused more than 40 deaths (Zhou et al. 2003). Numerous outbreaks occurred in UK and four were reported in France, the first in Sète in 1998, the second in Dracy-le-Fort in 2001, the third in Divonne-les-Bains in 2003, and the last one in Vesoul in 2003 too (Beaudeau et al. 2008). High resistance of oocysts and low infection threshold (1–10 oocysts) explain likely the high frequency of outbreaks (Leclerc et al. 2004).

However, the impact of *Cryptosporidium* species in human health could be still more important. Actually, we have reported recently that *C. parvum* is able to induce intestinal adenocarcinoma in SCID mice (Certad et al. 2007). This is the first demonstration of the ability of an Apicomplexan parasite to induce neoplastic lesions in an epithelium. We do not know if *C. parvum* is able to cause intestinal neoplasia in humans, but a recent survey reported a high frequency of *Cryptosporidium* infection in patients with colorectal cancer (Sulzyc-Bielicka et al. 2007).

Emerging or Reemerging Helminthes

Regarding helminthic species, 10 or 11 species of about 300 helminthes (about 3%) considered as pathogens to humans are emerging or reemerging now (Woolhouse and Gowtage-Sequeria 2005; Fagherazzi-Pagel 2009). Among the emerging/reemerging species, those transmitted by foodborne route are the fluke *Metorchis conjunctus*, the tapeworms *Diphyllobothrium* species, *Echinococcus granulosus*, *E. multilocularis*, *Taenia solium*, and the nematodes *Anisakis simplex* and *Trichinella* species (Table 4).

Emerging or Reemerging Liver Fluke Infections

The trematode *M. conjunctus* is an Opisthorchiidae of the New World (Canada, USA) with a typical digenetic life cycle. Life stages develop through freshwater gastropod molluscs (*Amnicola limosa*) (Woebeser et al. 1983), freshwater fishes, mainly

Table 3 Reported cases of foodborne cryptosporidiosis (non-exhaustive list)^a

Suspected contaminated food	Number of cases	Location	Comments	Reference
Raw goat milk	2	Australia	Unpasteurised milk. Goat was infected by <i>Cryptosporidium</i> sp	Millar et al. 2002
Frozen tripe	1	UK	Oocysts were detected in tripe	Nichols and Thom 1985
Milk	1	Manitoba (Canada)	Consumption of raw milk	Mann et al. 1986
Salad	1	Mexico	Salad provided by a street supplier	Sterling et al. 1986
Milk	22	Mexico	Possible milk borne outbreak	Elsser et al. 1986
Milk and/or sausage	19	UK	Consumption of unpasteurised milk or of sausage (suspicion). Associated <i>Campylobacter</i> outbreak	Casemore et al. 1986
Raw beef meat or raw milk	1	Poland	The wife of the patient had a latent <i>Cryptosporidium</i> infection	Kacprzak et al. 1990
Kefir (fermented milk)	6	Russia	Likely contamination of milk with <i>Cryptosporidium</i> oocysts	Romanova et al. 1992
Apple juice	160	Maine (USA)	Unpasteurised apple juice from fallen apples contaminated by a <i>C. parvum</i> infected calf	Millard et al. 1994
Chicken salad	50	Minnesota (USA)	Caterer handled diapers immediately before preparing the chicken salad	CDC 1996
Milk	50	UK	Impaired pasteurisation device	Gelletlie et al. 1997
Apple juice	31	New York (USA)	Unpasteurized juice from fallen apples. Potential contamination of water used to wash the apples	CDC 1997
Raw onion	54	Washington (USA)	Unwashed salad onions	CDC 1998
University catering	152	Washington DC (USA)	Contamination of food by <i>C. parvum</i> -infected food handler	Quiroz et al. 2000
Apple juice	12	Ohio (USA)	Ozonated apple juice from fallen apples	Blackburn et al. 2006

^aData from AFSSA (2002), Millar et al. (2002), Dawson (2005) and Smith et al. (2007)

Table 4 Emerging and reemerging foodborne helminthes^a

Taxonomic assignment	Species ^a	Life cycle	Foodborne	Waterborne	Other transmission mechanisms
Digenea (Trematoda, Platyhelminthes)	<i>Metorchis conjunctus</i>	Heteroxenous (freshwater mollusc/fish/dog or wild carnivores)	Yes (raw or semiraw freshwater fish)	No	–
Diphyllbothriidae (Cestoda, Platyhelminthes)	<i>Diphyllbothrium latum</i> , <i>D. pacificum</i> , <i>D. denriticum</i> and 12 other Diphyllbothriidae species	Heteroxenous (copepod/fish/human or carnivorous aquatic or terrestrial mammal, fish-eating birds)	Yes (raw or semiraw fish)	?	–
Taenidae (Cestoda, Platyhelminthes)	<i>Echinococcus granulosus</i>	Heteroxenous (mainly dog/sheep)	Yes (food contaminated with <i>E. granulosus</i> eggs)	Yes (water contaminated with <i>E. granulosus</i> eggs)	Direct contact with infested dogs or their dejections
Taenidae (Cestoda, Platyhelminthes)	<i>Echinococcus multilocularis</i>	Heteroxenous (mainly fox/rodents)	Yes (wild berry, fallen fruits or other edible vegetable)	Feasible (water contaminated with <i>E. multilocularis</i> eggs)	Direct contact with infested foxes or their dejections

Taenidae (Cestoda, Platyhelminthes)	<i>Taenia solium</i>	Heteroxenous (human/ pig)	Yes (ingestion of undercooked pork infected with cysticerci leads to taeniasis; ingestion of eggs contaminating food leads to cysticercosis)	Yes (ingestion of water contaminated with <i>T. solium</i> eggs leads to cysticercosis)	Human-to-human fecal-oral transmission of <i>T. solium</i> eggs (or autoinfection) leads to cysticercosis
Anisakidae (Ascaridida, Nematoda)	<i>Anisakis</i> , <i>Pseudoterranova</i> , <i>Contracaecum</i>	Heteroxenous (marine crustacean/fish or squid/fish-eating sea mammal or bird)	Yes (raw or semiraw fish or squid)	?	–
Trichinellidae (Trichocephalida, Nematoda)	<i>Trichinella spiralis</i> , <i>Trichinella</i> spp	Heteroxenous (predator or scavenger behavior, cannibalism)	Yes (raw or undercooked meat of pig, horse, wild boar and carnivorous or omnivorous wild mammals or reptiles)	No	–

^a Most data come from Woolhouse et al. (2006) and Fagherazzi-Pagel (2009)

Catostomids (suckers), Cyprinids (minnows, carps), or Percids (perches), and dogs, cats, other carnivorous mammals (wolf, fox, coyote, raccoon, muskrat and some mustelids) and occasionally man. Worms reach sexual maturity in liver, gall bladder, and bile ducts of mammal hosts. Embryonated eggs are released through the bile duct to the intestine and then eliminated with feces. Ingested by snail host, miracidia emerge from eggs in the digestive tract, and move to the mollusc's liver to form a sporocyst. The sporocyst generates rediae which produce numerous cercariae. Cercaria leaves the snail and swim in search of a fish to penetrate and encyst as metacercariae in the fish muscles. Metacercaria is infectious by oral route to definitive mammal host. Man contracts the infection by ingesting raw, undercooked, marinated or smoked fish infected with encysted metacercariae. These larvae are released in the host duodenum becoming young flukes (*adolescarias*), which gain the intra-hepatic biliary duct. Infections with a few worms may cause no symptoms. At least one outbreak of hepatic distomatosis due to *M. conjunctus* was reported in North America (MacLean et al. 1996). Common infection source was raw fish (sashimi) prepared from the white sucker, *Catostomus commersoni*, caught in a river north of Montreal (Canada). Adult *M. conjunctus* worms were recovered from golden hamsters infected with metacercariae collected from uneaten fish. Nineteen subjects presented upper abdominal pain, low grade fever, high blood eosinophil counts and raised liver enzymes. Opisthorchiid-like eggs were identified in the stools and symptoms responded rapidly to praziquantel (MacLean et al. 1996).

For the last years, a well known Old-World Opisthorchiidae, *Opisthorchis felineus*, has caused four outbreaks of opisthorchiidosis in Italy (Armignacco et al. 2008). The last ones in August and November 2007. The August outbreak was the most important. It involved more than 30 persons who consumed marinated fish filets of tench (*Tinca tinca*) and of white fish (*Coregonus* sp.) from Lake Bolsena (Viterbo Province, central Italy). Fish had been frozen at -10°C for 3 days before marinating. Fecal samples of 20 subjects showed Opisthorchiidae eggs and 11 of these patients had fever, nausea, abdominal pain, myalgia, associated with eosinophilia and specific serum IgG antibody to *O. felineus*. Praziquantel was an effective treatment for almost all patients, and albendazole revealed also apparently effective (Armignacco et al. 2008).

***Diphyllobothrium* Infection: A Changing Landscape**

Regarding *Diphyllobothrium* tapeworms, an increased number of human cases of diphyllobothriasis due to *Diphyllobothrium latum* was recorded in Europe: 48 new cases in France and Switzerland in 2001–2002, contracted by consuming freshwater fish from Léman lake (Dupouy-Camet and Peduzzi 2004; AFSSA 2004). Also, the consumption of seawater fish contaminated with plerocercoid larvae of marine *Diphyllobothrium* species (Chai et al. 2005) led to human diphyllobothriasis. For instance, *D. pacificum* (from sea-lions) infects humans in coastal zones of Chile,

Peru, Ecuador and Japan. Interestingly, diphyllobothriasis due to marine *Diphyllobothrium* species was recently reported in Western Europe (Clavel et al. 1997; Yera et al. 2006).

Inventory of the *Diphyllobothrium* species important to human health has been summarized elsewhere (Chai et al. 2005; Scholz et al. 2009). Occurrence of this zoonosis is linked to the frequent practice of consuming raw or marinated fish infected with larval plerocercoids. This epidemiological feature of diphyllobothriasis appears as a consequence of the complex life cycles of these cestodes. Life cycles are not fully elucidated for all *Diphyllobothrium* species but it is known they require at least three hosts for completion (Rausch and Adams 2000), while additional or paratenic hosts may also intervene. At the adult stage, the tapeworm inhabits the gastrointestinal tract of a variety of fish-eating mammals or birds. Parasite eggs hatch in water after several days of maturation. The released motile embryos (named coracidium) are in general ingested by a zooplanktonic crustacean copepod and develop into proceroid larvae. Freshwater, anadromous or marine fish that serve as secondary intermediate hosts may ingest infected copepods. Then, the second larval stage (plerocercoid larvae) may develop in various fish tissues depending on the tapeworm species (Dick et al. 2001). The plerocercoid larvae represent the infective form to fish-eating mammals or birds. Once the fish consumed by the final (or definitive) host, the tapeworm develops rapidly in the intestine.

About 20 million people are infected worldwide (Chai et al. 2005) and the parasites are found usually associated with cold freshwaters of Europe, North America and Asia although some cases have also been reported in South America. Due to the frequent mild intensity of its symptoms, diphyllobothriasis is not systematically reported, which renders the prevalence map still fragmented. Globally, the incidence of human infections has declined in recent years particularly in North America and Europe especially in former high prevalence Baltic and Scandinavian countries (Dick et al. 2001; Dupouy-Camet and Peduzzi 2004; Scholz et al. 2009). But some authors also indicated from an analysis of 20 years of diphyllobothriasis case records and surveys in Europe that it persists in several regions such as Switzerland or Italian and French Alpine lake areas (Dupouy-Camet and Peduzzi 2004). The infection has shown to reemerge in some countries such as Russia, South Korea, Japan and South America (reviewed by Scholz et al. 2009). *Diphyllobothrium* is in turn considered as one of the emerging food-borne parasites (Table 4) (Dorny et al. 2009). Factors such as dumping of improperly-treated wastewater into lakes, yachtsmen who also fish, and a potential animal reservoir contribute to the maintenance of the tapeworm life cycle (Dupouy-Camet and Peduzzi 2004). Raw fish consumption as a part of a healthier diet becomes increasingly popular. Sushi, sashimi, carpaccio and ceviche can be prepared from local or imported species thus leading to the emergence of diphyllobothriasis or other fishborne diseases. Especially, an outbreak of human diphyllobothriasis was reported lately in Brazil where the fish broad tapeworm was rare (Sampaio et al. 2005). The origin of contamination was attributed to the consumption of either raw imported salmon produced by the aquaculture industry in southern Chile or an indigenous Brazilian fish, *Centropomus undecimalis* (Cabello 2007; Sampaio et al. 2005). Food market

globalization as well as promptness of the international transport of refrigerated fish leads to the occurrence of human infections due to 'exotic' species such as *Diphyllbothrium nihonkaiense*. This species, once restricted to Japan is now believed to be distributed in Far East Russia up to the Kamchatka peninsula and along the Pacific coast of North America (Arizono et al. 2009). *D. nihonkaiense* has been recently identified as the causative agent of human diphyllbothriasis contracted following consumption of wild raw salmon imported in Europe probably from the Pacific coast of North America (Yera et al. 2006; Wicht et al. 2007; Shimizu et al. 2008; Paugam et al. 2009). The epidemiology of diphyllbothriasis is drastically evolving from rural to urban settings where efficient transportation systems provide fresh or frozen fish to meet the increasing consumer desire of fish-based healthier diets. Because of the permissive specificity of *Diphyllbothrium* species for their definitive hosts, animal reservoir is wide and contributes to the maintenance of natural life cycles even when human diphyllbothriasis is controlled. Natural life cycles of *Diphyllbothrium* species could nevertheless be modified by environmental changes such as El Niño-Southern Oscillation (ENSO) in the Eastern Pacific that affected fish populations and also the primary definitive host of *D. pacificum*, the sea lion (reviewed in Chai et al. 2005). Global warming or other human-related activities such as the introduction of fish species in new aquatic ecosystems may modify the intermediate or definitive host populations leading to emergence or reemergence of this fishborne zoonosis.

Emerging or Reemerging Cyclophyllidean Larval Cestoda

Echinococcus infection (Table 4) is acquired through ingestion of parasite eggs shed with the feces of carnivore final hosts (mainly dogs for *E. granulosus* and foxes for *E. multilocularis*). Eggs may contaminate water, edible vegetables including fruits causing therefore a foodborne infection. The infection may also be acquired by direct contact with definitive hosts (risk to persons that have dogs as pets, use dogs like tool of work, or persons that have direct contact with dogs or foxes like veterinarians or hunters). Four species of *Echinococcus* are recognized pathogens to humans: *E. granulosus*, agent of hydatid disease or cystic echinococcosis, *E. multilocularis*, agent of alveolar echinococcosis (invasive pseudoneoplastic lesions and metastasis), *E. oligarthrus*, from South American wild felids and *E. vogeli* from the South American dog *Speothos venaticus*, both causing polycystic echinococcosis.

Recent data suggest that *E. granulosus* and *E. multilocularis* echinococcosis are reemerging. Regarding *E. granulosus*, reemergence could be due to interruption or unsetting of effective control programs, which are mainly based on limitation of dog population, current treatment of dogs with cestodicidal drugs, home slaughter interdiction and supportive health education. Reemergence is being recorded in central Asia (Kazakhstan, Turkmenistan, Uzbekistan, Tajikistan and Kyrgyzstan), some Chinese regions (Eckert et al. 2002), and in some South American countries, especially in Peru (Dorny et al. 2009). Even in Europe, high prevalence rates were

reported from Yugoslavia, Romania, Bulgaria and Greece. In some Balkan countries the deterioration of the economic situation entailed the cessation of control measures, which led to the reemergence of echinococcosis (Eckert et al. 2002).

Echinococcus multilocularis is typically restricted to Palearctic and Nearctic biogeographic regions including central Europe, most of northern and central Eurasia and North America (Canada and USA). In the 1980s it was currently accepted that endemic regions of central Europe were restricted to four European countries: Austria, France, Germany and Switzerland. However, after 2000, new data indicate that *E. multilocularis* has a much wider geographic range, including at least 12 European countries (Eckert et al. 2002): Austria, Belgium, Czech Republic, Denmark, France, Germany, Liechtenstein, Luxembourg, Poland, the Slovak Republic, the Netherlands and Switzerland. In addition, metacestodes of *E. multilocularis* were found in rodents on the Norwegian Islands of Svalbard (Barent's Sea) (Slettbakk and Karlsen 1999). The increasing parasite prevalence in European red foxes (1–60%), growing of fox populations and progressive spread of foxes to cities have been evoked as risk factors contributing to both the widening of the *E. multilocularis* distribution area and the present reemergence of alveolar echinococcosis in Europe (Eckert et al. 2002; Dorny et al. 2009).

Two other *Echinococcus* species, both present in South and Central America, are responsible for polycystic echinococcosis in humans and are likely emerging at present: (a) *E. oligarthrus* that utilizes wild felids as definitive hosts, and rodents, especially agouti (*Dasyprocta* sp), as intermediate hosts; (b) *E. vogeli*, with bush dogs (*Speothos venaticus*) and rodents, especially paca (*Cuniculus paca*), as definitive and intermediate hosts, respectively. The two species, were considered like anecdotic for longtime. However, more than 100 cases of polycystic echinococcosis from 12 American countries have been documented before 2007, and prevalence rates are increasing at present (Tappe et al. 2008). Furthermore, *E. vogeli* infection can evolve in humans exhibiting invasive growth and external budding like alveolar echinococcosis (Tappe et al. 2008). *E. oligarthrus* infection is usually more benign.

Finally, *Taenia solium* cysticercosis, another disease caused by a larval tapeworm, is also reemerging (Sato et al. 2006; Kraft 2007) (Table 4). Man is the sole specific host of adult *T. solium*, a 3–5-m tapeworm that dwell in his gut. Men are therefore the unique source of infectious eggs. Adult tapeworm sheds proglottids loaded of eggs into human feces. Eggs may thus contaminate water, food, including pig food supply. In addition, pigs, which are intermediate hosts, are often coprophagous and may contract massive infections by eating directly ovigerous proglottids from human stools. Ingested eggs release embryos that pass through the intestinal wall, enter the bloodstream, lodge in various tissues, especially striated (cardiac or skeletal) muscle, and develop into cysticerci, which are vesicular larvae containing an invaginated scolex (=cephalic part of the tapeworm). When humans ingest infected raw or undercooked pork, the scolex evaginates, attaches to intestinal epithelium using suckers and hooks, and grows into adult tapeworm.

Indeed, *T. solium* eggs may also infect humans, who may develop therefore foodborne or waterborne cysticercosis, or, alternatively, they may infect humans directly by human-to-human fecal-oral transmission or by autoinfection (Table 4).

In humans, cysticerci lodge preferentially in muscle and nervous system, including eyes. Neurocysticercosis is a leading cause of adult-onset seizures worldwide and more than 1,000 new cases are diagnosed in the United States each year (Kraft 2007).

Intensification of pig breeding has led to eradication of autochthonous *T. solium* infection in most industrialized countries (Dorny et al. 2009). In contrast, the infection is endemic in many developing or poor countries where pigs have access to human feces. In addition, cysticercosis is reemerging in some areas. New data revealing previously unknown human and porcine cysticercosis endemic areas (Sato et al. 2006) could contribute to reemergence. However, significant factors are, likely, increased travelling and migration, which favor extension of the infection to non-endemic areas, and expansion of smallholder pig production in underdeveloped regions (Dorny et al. 2009).

Anisakiasis: A Widening Spectrum of the Disease

In Western Europe Diphyllbothriasis and Anisakiasis (Audicana et al. 2002; Audicana and Kennedy 2008; Dei-Cas and Verrez-Bagnis 2009) represent major fishborne risk. Reemerging Diphyllbothriasis was examined in a precedent section. Anisakiasis is a digestive infection resulting usually from the ingestion of raw or undercooked fish or cephalopods contaminated with third-stage larvae of ascarid nematodes of *Anisakis*, *Contracaecum* or *Pseudoterranova* (= *Phocanema*) genera, whose definitive hosts are whales (*Anisakis*), pinnipeds (*Pseudoterranova*) and piscivorous birds (*Contracaecum*), respectively. In definitive hosts, Anisakidae female worms shed undeveloped eggs in host feces. Eggs embryonate and hatch in water releasing free-swimming larvae that are ingested by crustaceans such as euphausiaceans, decapods, copepods and amphipods, where they localize within the haemocoel (Chai et al. 2005; Dei-Cas 1996). Definitive avian or mammalian host may acquire the infection directly by ingesting infected crustaceans. However, infected crustaceans are more frequently ingested by teleostean fish or cephalopod molluscs. Predators of infected fish, cephalopods or, likely, other aquatic vertebrates can in turn get infected and play the role of paratenic or transport hosts. Anisakidae larvae (10–30 mm × 1 mm) penetrate then their intestine and invade their tissues being often the source of infection for definitive bird or mammal hosts or for humans, who constitute dead-end hosts. In fish hosts, anisakid larvae can be found inside the gastro-intestinal tract, in the pleuro-peritoneal cavity, liver, gonads or embedded in muscles (Mudry et al. 1986). These larvae are infectious either to definitive hosts (piscivorous birds or mammals) or to other fish-eating vertebrates such as larger fishes, sea turtles (Burke and Rodgers 1982) or even crocodiles, which could be potential paratenic hosts (Magnino et al. 2009). Indeed, *Contracaecum* larvae have been identified in crocodilians (Goldberg et al. 1991; Acha and Szyfres 1994; Moravec 2001) and Anisakid larvae have been also reported in the green sea turtle (*Chelonia mydas*) (Burke and Rodgers 1982).

In humans, anisakid larvae can penetrate deeply the gastric or intestinal wall. The infection with these roundworms may remain asymptomatic or cause symptoms of variable importance (digestive hemorrhage, intestinal obstruction, eosinophilic granuloma, allergic symptoms) according to larval localization, number of larvae and intensity of the tissue response. In some cases, the larvae migrate across this wall to the peritoneal cavity and invade adjacent organs (liver, pancreas) or, more rarely, distant organs (Acha and Szyfres 1994; Dei-Cas 1996; Mudry et al. 1986). The infection can be asymptomatic or, alternatively, cause clinical signs more or less severe depending on both the number of larvae and the intensity of the inflammatory response. Recent works showed that human disease associated with allergic reactions against anisakid larvae also occurs (Daschner et al. 2005), even when larvae are dead. Thus, anisakid larvae might represent a neglected cause of allergic diseases in Europe.

In short, consumption of fish parasitized with Anisakidae larvae can cause both direct gastrointestinal lesions provoked by infective larvae (typical anisakiasis), and allergic symptoms (rhinoconjunctivitis, urticaria–angioedema, anaphylaxis, and asthma) often falsely attributed to allergy to fish flesh (Kasuya et al. 1990). In recent years, *Anisakis simplex* has been recognized as an important foodborne allergen source, especially in Spain (Ibarrola et al. 2008). The parasite larvae die after freezing or cooking, but the tolerance of sensitized subjects to eating frozen and/or cooked fish remains a matter of controversy because some worm allergens are thermo-resistant (Audicana et al. 2002; Audicana and Kennedy 2008).

Interestingly, *A. simplex* allergy was reported in eight chicken consumer subjects who had totally avoided fish (Armentia et al. 2006). All eight patients presented positive prick and challenges to *A. simplex*, and the sera of 6/8 reacted against sera of chickens fed with fishmeal used as antigen, but not with sera from chickens fed only with cereals. The use of fish-derived food in farming, associated to the concentration of feed or food production, could therefore contribute to the spreading of *Anisakis* allergy.

Diagnosis of anisakiasis is usually uneasy and often only endoscopy or surgical treatment is effective (Dei-Cas 1996). Anisakiasis prevalence is higher in cold or temperate areas or in regions where people eat currently raw fish. Thus, 95% of world cases, that is 1,000 cases per year, occur in Japan (Butt et al. 2004). In the Netherlands, the incidence was also high (300 cases per year) before the enforcement of a rule imposing -20°C freezing (>24 h) of edible fish (Dei-Cas 1996). Sporadic cases are currently reported in Western Europe. In France, anisakiasis is rare or underestimated: only 55 cases reported till 1995 (Bourée et al. 1995). Cases would be apparently restricted to fishing port neighboring areas (Hubert et al. 1989). However, parasite rates may be very high in edible fishes (about 80% in herring, 100% in whiting, 62% in Atlantic mackerel, 88% in European hake, and 86% in Ocean perch) (Mudry et al. 1986; Huang 1988). Recent unpublished observations (EDC) confirmed these data. The European Union regulations on the freezing of fish to kill nematode specify “a temperature below -20°C in all parts of the product for at least 24 h”. The requirements of the U.S. Food and Drug Administration are even stricter, imposing storage at -20°C for 7 days or at -35°C for at least 15 h.

The present reemergence of anisakiasis could be due, at least in part, to the emergence of new patterns of production, preparation and consumption of food (evoked above in the section devoted to Diphyllbothriasis), and to the revelation of the involvement of *Anisakis* thermo-stable antigens in allergy. The growing consuming of “exotic” food, especially of food based on raw, marinated or undercooked fish could contribute to the emergence of both typical anisakiasis and *Anisakis*-induced allergy.

Emergence and Reemergence of Trichinellosis

Trichinella nematodes have been detected in all continents except Antarctica (Pozio 2007a). Natural *Trichinella* infections have been reported in more than 100 species of mammals, seven avian species, two crocodilian and one saurian species (Pozio and Zarlenga 2005). At present, 12 taxa with eight species and four genotypes are recognized in the genus *Trichinella*: (a) *Trichinella spiralis* (T1); (b) *T. nativa* (T2) and its related genotype *Trichinella* T6; (c) *T. britovi* (T3) and its related genotype *Trichinella* T8; (d) *T. pseudospiralis* (T4); (e) *T. murrelli* (T5) and its related genotype *Trichinella* T9; (f) *T. nelsoni* (T7); (g) *T. papuae* (T10); (h) *T. zimbabwensis* (T11) (Pozio 2007b). *Trichinella* T12 is a new genotype recently detected in a puma (*Puma concolor*) from Patagonia, Argentina (Krivokapich et al. 2007). On the basis of genetic and biological features, *Trichinella* species and genotypes are classified into two clades, with the main criterion being the presence of either a thick (encapsulated) or a very thin (nonencapsulated) collagen membrane enveloping the parasite-infected muscle cell (Zarlenga et al. 2006). One clade comprises the species that encapsulate in host muscle tissue of mammals (T1 to T9, and T12), while the other one includes the species that do not encapsulate after muscle cell invasion. The latter occurs in birds (T4) and reptiles (T10 and T11) (Pozio and Murrell 2006).

The life cycle of this ovo-viviparous nematode is typically associated to predator or scavenger behavior or, more rarely, to cannibalism (Table 4). After ingesting the meat of an infected host, first-stage larvae are released in the digestive tract of the new host following gastric digestion. Subsequently larvae reach the small intestine where they molt four times in 30–40 h, to develop into the adult stage (Smyth 1994; Gagliardo et al. 2002). Male worms (1.5 mm) and ovo-viviparous females (3.5 mm) sink then in the intestinal mucosa and copulate within hours of the final ecdysis. Eggs hatch in utero, and female worms begin to release first stage larvae 4 days postinfection (Gagliardo et al. 2002). One female can generate 1,500 larvae (Smyth 1994). Newborn larvae migrate into lymphatic and blood vessels, penetrate into striated muscle cells by using a stilettto apparatus and lytic enzymes, and develop to infective muscle first stage larvae in about 15 days post infection. Basically anaerobic metabolism of larvae favors their survival in decaying tissues (Despommier 1990) until they are ingested by a new host. Predatory, scavenger and cannibal host behaviors ensure the circulation of *Trichinella* species and genotypes in ecosystems.

Humans are highly susceptible to infection with several *Trichinella* species and genotypes. Thus, although trichinellosis cases have not been reported for all species so far, all trichinellae are considered to be pathogenic for humans (Magnino et al. 2009). Whereas infections with few *Trichinella* larvae can remain asymptomatic, higher larval burdens can cause gastrointestinal symptoms at first, followed by general symptoms such as fever, facial edema, myositis, associated with eosinophilia, all changes that are directly related to the development of parasite life cycle stages in the human host (Dupouy-Camet 2006). Human trichinellosis can be a serious disease, particularly in elderly patients in whom neurological or cardiovascular complications can lead to death.

The situation of human trichinellosis in Europe was examined recently (Dupouy-Camet 2006). Horse meat has been identified as the main source of infection with more than 3,300 cases and 14 outbreaks reported for the last 30 years (Dupouy-Camet 2006). However, outbreaks due to wild boar meat are still reported in France, Spain and Poland. In addition, outbreaks due to infected pork have been reported in Spain, Germany, Latvia and Lithuania. Infected pigs were detected in Corsica in 2004, and small outbreaks of pork-related trichinellosis occurred in Sardinia in 2005. Infected foxes have been found in Ireland. A survey by the International Commission on Trichinellosis (<http://www.med.unipi.it/ict/ICT%202004%20human%20survey.htm>) identified more than 1,100 human cases in Europe in 2004, with 984 cases being reported from Serbia, Croatia, Romania and Bulgaria, four countries in which the disease has reemerged in recent years (Dupouy-Camet 2006).

In addition to the typical *Trichinella* infection sources (wild boar, pork, horse, bear), reptile meat is emerging as a new potential infection source (Magnino et al. 2009). The increasing demand of reptile meat (e.g. from terrapins, crocodiles, caimans, alligators and iguanas) in some regions, including Europe, has resulted in the development of breeding programs in more than 30 countries in North, Central and South America, Africa, Europe, Asia and Australia (Magnino et al. 2009). Reptiles can be carriers of a variety of parasitic agents, including *Trichinella* species, and their meat can become contaminated depending on the housing, feeding, and hygienic practices under which they are reared or slaughtered (Magnino et al. 2009). Last years, two non-encapsulated *Trichinella* species have been described in crocodiles: *T. papuae* (Pozio et al. 1999) in saltwater crocodile (*Crocodylus porosus*) from Papua New Guinea and Australia, and *T. zimbabwensis* (Pozio et al. 2002) in Nile crocodile (*Crocodylus niloticus*) from Africa. *T. papuae* and *T. zimbabwensis* can complete their life cycle in crocodilian hosts, which have been found parasitized by *Trichinella* either in the wild or in captive breeding farms. *T. zimbabwensis* can infect primates, and *T. papuae* was identified in wild pigs from Papua New Guinea (Owen et al. 2005; Pozio et al. 2005; Pozio and Zarlenga 2005). *T. zimbabwensis* and *T. papuae* can easily infect mammals experimentally. Other reptile species, especially caimans (*Caiman sclerops*) and varans (*Varanus exanthemicus*), and to a lower degree turtles (*Pelomedusa subrufa*) and pythons (*Python molurus bivittatus*) are susceptible to *T. zimbabwensis* and *T. papuae* (Pozio et al. 2004). No data about

T. zimbabweensis or *T. papuae* human infection are available. However, one human trichinellosis outbreak due to consumption of reptile meat from a monitor lizard (*Varanus nebulosus*) has been documented in Thailand (Pozio 2007a). In addition, a 10% prevalence of serum anti-*Trichinella* IgG antibody was detected in the inhabitants of Morehead District of Papua New Guinea, where wild-pig meat, potentially infected with *T. papuae*, represents the main source of protein (Owen et al. 2005; Pozio and Zarlenga 2005). Although persons reported muscular or joint pain, the infection was apparently no severe, suggesting that seropositive people are exposed to recurring infections with very few larvae (Owen et al. 2005).

Concluding Remarks

On the whole, which are the infectious forms of foodborne parasites? Main infectious stages (to humans) of coccidian parasites are either highly resistant oocysts (monoxenous species), like *Cryptosporidium*, or tissue zoites (heteroxenous species), contained in tissue cysts, like those of *Toxoplasma*. Other parasitic Protozoa develop resistant cystic forms in their life cycle, like *Giardia*.

Regarding Metazoan parasites, a large range of highly diversified, specialized, infectious stages is noticed: Mesocercaria and Metacercaria in Digenetic Trematodes like *Alaria* or *Fasciola*, respectively, several types of Cestode larvae able to develop into adult tapeworms in humans, cestode eggs that develop into larval cestode in human tissues (cysticercosis, echinococcoses), and largely ubiquitous highly resistant nematode eggs (geohelminthes) and larvae able to contaminate food. More rarely, humans can develop acanthocephalan or pentastomid infections through the ingestion of larval stages of these metazoan parasites.

Interestingly, larval platyhelminthes or larval nematodes can be present in highly diverse biological groups, in other words, they are able to “pass through” the ecosystems, from producers (green plants) to primary, secondary and top-level consumers, which accumulate thus parasitic metazoan infectious stages in their tissues. Humans may behave as consumers of first order (eating green vegetables) or second order (eating livestock or seafood), but, substantially, they are top-level consumers, being able to consume even other top-level consumers (like sharks or crocodiles).

Therefore, though common representations of human daily life show a human that has somehow “escaped” from ecosystems (human as the user of an illusory “nature”, inexhaustible source of matter and energy), men take on their role of top level consumers, and are therefore exposed to the associated, highly diversified parasitological risk. Interestingly, from the analysis of foodborne parasitic risk emerged the image of a man fully integrated into trophic networks, entirely dependent on the flow of matter and energy of ecosystems, sharing advantages, risks and pathologies with non-human organisms (Boury and Dei-Cas 2008).

References

- Acha PN, Szyfres B (1994) Zoonoses and communicable diseases common to man and 1176 animals, Science Publication No 503, 2nd ed. Pan American Health Organization, Washington 1177 DC
- Adak GK, Long SM, O'Brien SJ (2002) Trends in indigenous foodborne disease and deaths, England and Wales: 1992 to 2000. *Gut* 51:832–841
- Adl SM, Simpson AG, Farmer MA, Andersen RA, Anderson OR, Barta JR, Bowser SS, Brugerolle G, Fensome RA, Fredericq S, James TY, Karpov S, Kugrens P, Krug J, Lane CE, Lewis LA, Lodge J, Lynn DH, Mann DG, McCourt RM, Mendoza L, Moestrup O, Mozley-Standridge SE, Nerad TA, Shearer CA, Smirnov AV, Spiegel FW, Taylor MF (2005) The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J Eukaryot Microbiol* 52:399–451
- AFSSA (2002) Rapport sur les: évaluation scientifique des risques associés à *Cryptosporidium* sp. Rapport du groupe de travail: "Infections à protozoaires liées aux aliments et à l'eau". http://www.sante.gouv.fr/html/dossiers/etud_impact/crypto_ei637.pdf
- AFSSA (2004) Évaluation des risques nutritionnels et sanitaires. Priorités 2004/2007. Rapport, juillet 2004. http://www.academie-medecine.fr/UserFiles/File/rapports_thematiques/nutrition/Afssa_evaluation_risques_nutritionnels_et_sanitaires_priorit_s_2004_2007.pdf
- Alvarez-Pellitero P, Sitja-Bobadilla A (2002) *Cryptosporidium molnari* n. sp. (Apicomplexa: Cryptosporidiidae) infecting two marine fish species, *Sparus aurata* L. and *Dicentrarchus labrax* L. *Int J Parasitol* 32:1007–1021
- Alvarez-Pellitero P, Quiroga MI, Sitja-Bobadilla A, Redondo MJ, Palenzuela O, Padrós F, Vázquez S, Nieto JM (2004) *Cryptosporidium scophthalmi* n. sp. (Apicomplexa: Cryptosporidiidae) from cultured turbot *Scophthalmus maximus*. Light and electron microscope description and histopathological study. *Dis Aquat Organ* 62:133–145
- Anderson RM, May RM (1998) Infectious diseases of humans. Dynamics and control. Oxford University Press, Oxford
- Appelbee AJ, Thompson RC, Olson ME (2005) *Giardia* and *Cryptosporidium* in mammalian wildlife-current status and future needs. *Trends Parasitol* 21:370–376
- Arizono N, Yamada M, Nakamura-Uchiyama F, Ohnishi K (2009) Diphyllorhynchiasis associated with eating raw pacific salmon. *Emerg Infect Dis* 15:866–870
- Arkush KD, Miller MA, Leutenegger CM, Gardner IA, Packham AE, Heckerroth AR, Tenter AM, Barr KC, Conrad PA (2003) Molecular and bioassay-based detection of *Toxoplasma gondii* oocyst uptake by mussels (*Mytilus galloprovincialis*). *Int J Parasitol* 33:1087–1097
- Armentia A, Martin-Gil FJ, Pascual C, Martín-Esteban M, Callejo A, Martínez C (2006) *Anisakis simplex* allergy after eating chicken meat. *J Investig Allergol Clin Immunol* 16:258–263
- Armignacco O, Caterini L, Marucci G, Ferri F, Bernardini G, Natalini Raponi G, Ludovisi A, Bossù T, Gomez Morales MA, Pozio E (2008) Human illnesses caused by *Opisthorchis felinus* flukes, Italy. *Emerg Infect Dis* 14:1902–1905
- Audicana M, Kennedy MW (2008) *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. *Clin Microbiol Rev* 21:360–379
- Audicana MT, Ansotegui JJ, de Corres LF, Kennedy MW (2002) *Anisakis simplex*: dangerous—dead and alive? *Trends Parasitol* 18:20–25
- Beaudeau P, de Valk H, Vaillant V, Mannschott C, Tillier C, Mouly D, Ledrans M (2008) Lessons learned from ten investigations of waterborne gastroenteritis outbreaks, France, 1998–2006. *J Water Health* 6:491–503
- Benchimol-Barbosa PR (2009) Further comments on oral transmission of Chagas' disease in Brazil: epidemiology, geographical distribution and viability of the infective parasite. *Int J Cardiol* 8. doi:10.1016/j.ijcard.2008.11.124
- Berrilli F, Di Cave D, De Liberato C, Franco A, Scaramozzino P, Orecchia P (2004) Genotype characterization of *Giardia duodenalis* isolates from domestic and farm animals by SSU-rRNA gene sequencing. *Vet Parasitol* 122:193–199

- Blackburn BG, Mazurek JM, Hlavsa M, Park J, Tillapaw M, Parrish M, Salehi E, Franks W, Koch E, Smith F, Xiao L, Arrowood M, Hill V, da Silva A, Johnston S, Jones JL (2006) Cryptosporidiosis associated with ozonated apple cider. *Emerg Infect Dis* 12:684–686
- Bonnin A, Dei-Cas E, Camerlynck P (1992) *Cryptosporidium* and *Isospora*. In: Myint S, Cann A (eds) Molecular and cell biology of opportunistic infections in AIDS. Chapman & Hall, London
- Bourée P, Paugam A, Petithory JC (1995) Anisakidosis: report of 25 cases and review of the literature. *Comp Immunol Microbiol Infect Dis* 18:75–84
- Boury D, Dei-Cas E (2008) Current bioethical issues in parasitology. *Parasite* 15:489–494
- Burke JB, Rodgers LJ (1982) Gastric ulceration associated with larval nematodes (*Anisakis* sp. type I) in pen reared green turtles (*Chelonia mydas*) from Torres Strait. *J Wildlife Dis* 18:41–46
- Butt AA, Aldridge KE, Sanders CV (2004) Infections related to the ingestion of seafood. Part II: parasitic infections and food safety. *Lancet Infect Dis* 4:294–300
- Cabello FC (2007) Acuicultura y salud pública. Emergencia de difilobotriasis en Chile y en el mundo. *Rev Med Chil* 135:1064–1071
- Casemore DP, Jessop EG, Douce D, Jackson FB (1986) *Cryptosporidium* plus *Campylobacter*: an outbreak in a semi-rural population. *J Hyg* 96:95–105
- Centers for Disease Control (CDC) and Prevention (1994) Addressing emerging infectious disease threats: a prevention strategy for the United States. US Department of Health and Human Services, Public Health Service, Atlanta, Georgia
- Centers for Disease Control (CDC) and Prevention (1995) Infectious disease – a global health threat. Report of the National Science and Technology Council Committee on International Science, Engineering, and Technology Working Group on Emerging and Re-emerging Infectious Diseases. Center for Disease Control and Prevention, White House
- Centers for Disease Control (CDC) and Prevention (1996) Foodborne outbreak of diarrheal illness associated with *Cryptosporidium parvum* – Minnesota 1995. *MMWR* 45:783–784
- Centers for Disease Control (CDC) and Prevention (1997) Outbreaks of *Escherichia coli* O157:H7 infection and cryptosporidiosis associated with drinking unpasteurized apple cider – Connecticut and New York, October 1996. *MMWR* 46:4–8
- Centers for Disease Control (CDC) and Prevention (1998) Foodborne outbreak of cryptosporidiosis – Spokane, Washington, 1997. *MMWR-CDC* 47:565–567
- Certad G, Ngouanesavanh TM, Hernan A, Rojas E, Contreras R, Pocaterra L, Nuñez L, Dei-Cas E, Guyot K (2006) First molecular data on Cryptosporidiosis in Venezuela. *J Eukaryot Microbiol* 53(S1):30–32
- Certad G, Ngouanesavanh TM, Guyot K, Gantois N, Chassat T, Mouray A, Fleurisse L, Pinon A, Cailliez JC, Dei-Cas E, Creusy C (2007) *Cryptosporidium parvum*, a potential cause of colic adenocarcinoma. *Infect Agents Cancer* 2(1):22. doi:10.1186/1750-9378-2-22
- Chai J-Y, Darwin Murrell K, Lymbery AJ (2005) Fish-borne parasitic zoonoses: status and issues. *Int J Parasitol* 35:1233–1254
- Chalmers RM, Davies AP (2009) Minireview: clinical cryptosporidiosis. *Exp Parasitol*. doi:10.1016/j.exppara.2009.02.003
- Clavel A, Bargues MD, Castillo FJ, Rubio MD, Mas-Coma S (1997) Diplogonoporiasis presumably introduced into Spain: first confirmed case of human infection acquired outside the Far East. *Am J Trop Med Hyg* 57:317–320
- Daschner A, Vega de la Osada F, Pascual CY (2005) Allergy and parasites reevaluated: wide-scale induction of chronic urticaria by the ubiquitous fish-nematode *Anisakis simplex* in an endemic region. *Allergol Immunopathol (Madr)* 33:31–37
- Dawson D (2005) Foodborne protozoan parasites. *Int J Food Microbiol* 103:207–227
- De Wit MA, Koopmans MP, Kortbeek LM, Van Leeuwen NJ, Bartelds AI, Van Duynhoven YT (2001) Gastroenteritis in sentinel general practices, The Netherlands. *Emerg Infect Dis* 7:82–91
- Dei-Cas E (1996) *Larva migrans*. Encyclopédie Médico-Chirurgicale (Paris, France), Maladies Infectieuses, 8-518-A-10, 16 p

- Dei-Cas E, Verrez-Bagnis V (2009) Les parasites des poissons. In: Les produits aquatiques: caractéristiques, transformation, valorisation. Éditions Lavoisier (in press)
- Despommier DD (1990) *Trichinella spiralis*: the worm that would be virus. *Parasitol Today* 6:193–196
- Desportes I, Le Charpentier Y, Galian A, Bernard F, Cochand-Priollet B, Lavergne A, Ravisse P, Modigliani R (1985) Occurrence of a new microsporidan: *Enterocytozoon bieneusi* n.g., n. sp., in the enterocytes of a human patient with AIDS. *J Protozool* 32:250–254
- Dias JP, Bastos C, Araújo E, Mascarenhas AV, Netto EM, Grassi F, Silva M, Tatto E, Mendonça J, Araújo RF, Shikanai-Yasuda MA, Aras R (2008) Acute Chagas disease outbreak associated with oral transmission. *Rev Soc Bras Med Trop* 41:296–300
- Dias JP (2008) Notes about *Trypanosoma cruzi* and bio-ecology characteristics with agents of the transmission by meals. *Rev Soc Bras Med Trop* 39:370–375
- Dick TA, Nelson PA, Choudhury A (2001) Diphyllorhynchiasis: update on human cases, foci, patterns and sources of human infections and future considerations. *Southeast Asian J Trop Med Public Health* 32(Suppl 2):59–76
- Didier ES (2005) Microsporidiosis: an emerging and opportunistic infection in humans and animals. *Acta Trop* 94:61–76
- Dorny P, Praet N, Deckers N, Gabriel S (2009) Emerging food-borne parasites. *Vet Parasitol* 163:196–206
- Dowell SF, Levitt AM (2002) Protecting the nation health in an era of globalization. CDC's Global Infectious Disease Strategy. CDC report, Atlanta, Georgia
- Dupouy-Camet J (2006) Trichinellosis: still a concern for Europe. *Euro Surveill* 11:5
- Dupouy-Camet J, Peduzzi R (2004) Current situation of human diphyllorhynchiasis in Europe. *Euro Surveill* 9:31–35
- Dupouy-Camet J, Olesen OF, Dei-Cas E, Loiseau PM, Mas-Coma S (2009) Perspectives for parasitology and parasitology networks in Europe. *Trends Parasitol* 25:293–295
- Eckert J, Gemmell MA, Meslin FX, Pawłowski ZS (2002) WHO/OIE manual on echinococcosis in humans and animals: a public health problem of global concern. World Organisation for Animal Health (Office International des Epizooties) and World Health Organization, Paris. <http://whqlibdoc.who.int/publications/2001/929044522X.pdf>
- Elsser KA, Moricz M, Proctor EM (1986) *Cryptosporidium* infections: a laboratory survey. *CMAJ* 135:211–213
- Fagherazzi-Pagel H (2009) Maladies émergentes et réémergentes chez l'homme. Dossier de synthèse. CNRS/INIST, Paris
- Favennec L, Magne D, Chochillon C, Gargala G, Gobert JG (2006) Infections intestinales humaines à *Giardia duodenalis*. EMC (Elsevier SAS, Paris), Maladies infectieuses, 8-515-A-10
- Fayer R (2009) Taxonomy and species delimitation in *Cryptosporidium*. *Exp Parasitol*. doi:10.1016/j.exppara.2009.03.005
- Fayer R, Speer CA, Dubey JP (1997) The general biology of *Cryptosporidium*. In Fayer RE (ed) *Cryptosporidium* and cryptosporidiosis. CRC Press, Inc., Boca Raton, FL, pp 1–41
- Fayer R, Graczyk TK, Lewis EJ, Trout JM, Farley CA (1998) Survival of infectious *Cryptosporidium parvum* oocysts in seawater and eastern oysters (*Crassostrea virginica*) in the Chesapeake BAY. *Appl Environ Microbiol* 64:1070–1074
- Fayer R, Lewis EJ, Trout JM, Graczyk TK, Jenkins MC, Higgins J, Xiao L, Lal AA (1999) *Cryptosporidium parvum* in oysters from commercial harvesting sites in the Chesapeake bay. *Emerg Infect Dis* 5:706–710
- Freire-Santos F, Oteiza-Lopez AM, Vergara-Castiblanco CA, Ares-Mazas E, Alvarez-Suarez E, Garcia-Martin O (2000) Detection of *Cryptosporidium* oocysts in bivalve molluscs destined for human consumption. *Parasitology* 86:853–854
- Freire-Santos F, Gomez-Couso H, Ortega-Inarrea MR, Castro-Hermida JA, Oteiza-Lopez AM, Garcia-Martin O, Ares-Mazas ME (2002) Survival of *Cryptosporidium parvum* oocysts recovered from experimentally contaminated oysters (*Ostrea edulis*) and clams (*Tapes decussatus*). *Parasitol Res* 88:130–133

- Gagliardo LF, McVay CS, Appleton JA (2002) Molting, ecdysis, and reproduction of *Trichinella spiralis* are supported in vitro by intestinal epithelial cells. *Infect Immun* 70:1853–1859
- Gelletlie R, Stuart J, Soltanpoor N, Armstrong R, Nichols G (1997) Cryptosporidiosis associated with school milk. *Lancet* 350:1005–1006
- Goldberg SR, Bursey CR, Aquino-Shuster AL (1991) Gastric nematodes of the Paraguayan caiman, *Caiman yacare* (Alligatoridae). *J Parasitol* 77:1009–1011
- Graczyk TK, Farley CA, Fayer R, Lewis EJ, Trout JM (1998a) Detection of *Cryptosporidium* oocysts and *Giardia* cysts in the tissues of eastern oysters (*Crassostrea virginica*) carrying principal oyster infectious diseases. *J Parasitol* 84:1039–1042
- Graczyk TK, Ortega YR, Conn DB (1998b) Recovery of waterborne oocysts of *Cyclospora cayentanensis* by Asian freshwater clams (*Corbicula fluminea*). *Am J Trop Med Hyg* 59:928–932
- Graczyk TK, Fayer R, Lewis EJ, Trout JM, Farley CA (1999) *Cryptosporidium* oocysts in Bent mussels (*Ischadium recurvum*) in the Chesapeake Bay. *Parasitol Res* 85:518–21
- Graczyk TK, Marcogliese DJ, de Lafontaine Y, Da Silva AJ, Mhangami-Ruwende B, Pieniazek NJ (2001) *Cryptosporidium parvum* oocysts in zebra mussels (*Dreissena polymorpha*): evidence from the St Lawrence river. *Parasitol Res* 87:231–234
- Graczyk TK, Bosco-Nizeyi J, Ssebide B, Thompson RC, Read C, Cranfield MR (2002) Anthroponotic *Giardia duodenalis* genotype (assemblage) a infections in habitats of free-ranging human-habituated gorillas, Uganda. *J Parasitol* 88:905–909
- Graczyk TK, Conn DB, Lucy F, Minchin D, Tamang L, Moura LN, DaSilva AJ (2004) Human waterborne parasites in zebra mussels (*Dreissena polymorpha*) from the Shannon river drainage area, Ireland. *Parasitol Res* 93:385–391
- Graczyk TK, Girouard AS, Tamang L, Nappier SP, Schwab KJ (2006) Recovery, bioaccumulation, and inactivation of human waterborne pathogens by the Chesapeake bay nonnative oyster, *Crassostrea ariakensis*. *Appl Environ Microbiol* 72:3390–3395
- Graczyk TK, Lewis EJ, Glass G, Dasilva AJ, Tamang L, Girouard AS, Curriero FC (2007) Quantitative assessment of viable *Cryptosporidium parvum* load in commercial oysters (*Crassostrea virginica*) in the Chesapeake bay. *Parasitol Res* 100:247–253
- Guyot K, Gireaudot-Liepmann MF, Cabon A, Riveau-Ricard I, Lange M, Delattre JM, Dei-Cas E (2000) Influence of US Epa 1622 method successive steps on the viability of *Cryptosporidium* oocysts. *Water Sci Technol* 41:189–196
- Guyot K, Follet-Dumoulin A, Lelièvre E, Sarfati C, Rabodonirina M, Nevez G, Cailliez JC, Camus D, Dei-Cas E (2001) Molecular characterization of *Cryptosporidium* obtained from humans in France. *J Clin Microbiol* 39:3472–3480
- Guyot K, Ngouanesavanh TM, Dei-Cas E (2005) Strategies for detecting pathogenic protists in water: the point on *Cryptosporidium*. *Eur J Water Quality* 36:51–70
- Hall R (1997) Foodborne illness: implications for the future. *Emerg Infect Dis* 3:355–359
- Hotez PJ, Molyneux D, Fenwick A, Kumarsan J, Ehlich S, Sachs JD, Savioli L (2007) Control of neglected tropical diseases. *N Engl J Med* 357:1018–1027
- Huang WY (1988) Anisakides et anisakidoses humaines. Deuxième partie: Enquête sur les anisakides de poissons commerciaux du marché Parisien. *Ann Parasitol Hum Comp* 63:197–208
- Hubert B, Bacou J, Belveze H (1989) Epidemiology of human anisakiasis: incidence and sources in France. *Am J Trop Med Hyg* 40:301–303
- Ibarrola I, Arilla MC, Herrero MD, Esteban MI, Martínez A, Asturias JA (2008) Expression of a recombinant protein immunochemically equivalent to the major *Anisakis simplex* allergen Ani s 1. *J Investig Allergol Clin Immunol* 18:78–83
- Jiménez JC, Pinon A, Dive D, Capron M, Dei-Cas E, Convit J (2009) Antibody response in infected children with *Giardia intestinalis* before and after treatment with Secnidazole. *Am J Trop Med Hyg* 80:11–15
- Jones KE, Patel N, Levy MA, Storeygard A, Balk D, Gittleman JL, Daszak P (2008) Global trends in emerging infectious diseases. *Nature* 451:990–993
- Kacprzak E, Kurczewska M, Stefaniak J (1990) 2 cases of cryptosporidiosis in adults. *Przegl Epidemiol* 44:245–248

- Kaneshiro E, Dei-Cas E (2009) Why the international workshops on opportunistic protists? *Eukaryot Cell* 8:426–428
- Kasuya S, Hamano H, Izumi S (1990) Mackerel-induced urticaria and Anisakis. *Lancet* 335:665
- Kraft R (2007) Cysticercosis: an emerging parasitic disease. *Am Fam Physician* 76:91–96
- Krivokapich S, Gonzalez Prous C, Gatti G, Molina V, Matarasso H, Guarnera E (2007) Molecular evidence for a novel encapsulated genotype of *Trichinella* from Patagonia. *Proceedings of Argentina XII International Conference on Trichinellosis, Plitvice Lakes, Croatia*, 25–30 September, p 13
- Leclerc H, Schwartzbrod L, Dei-Cas E (2004) Microbial agents associated with waterborne diseases. In: Cloete TE, et al (eds) *Microbial waterborne pathogens*. IWA Publishing, London, pp 1–55
- Lecoindre G, Le Guyader H (2001) *Classification phylogénétique du vivant*. Belin, Paris
- Li X, Guyot K, Dei-Cas E, Mallard JP, Ballet JJ, Brasseur P (2006) *Cryptosporidium* oocysts in mussels (*Mytilus edulis*) from Normandy (France). *Int J Food Microbiol* 108:321–325
- Lindsay DS, Collins MV, Mitchell SM, Wetch CN, Rosypal AC, Flick GJ, Zajac AM, Lindquist A, Dubey JP (2004) Survival of *Toxoplasma gondii* oocysts in Eastern oysters (*Crassostrea virginica*). *J Parasitol* 90:1054–1057
- MacLean JD, Arthur JR, Ward BJ, Gyorkos TW, Curtis MA, Kokoskin E (1996) Common-source outbreak of acute infection due to the North American liver fluke *Metorchis conjunctus*. *Lancet* 347(8995):154–158
- Magnino S, Colin P, Dei-Cas E, Madsen M, McLauchlin J, Nöckler K, Prieto-Maradona M, Tsigarida E, Vanopdenbosch E, Van Peteghem C (2009) Biological risks associated with consumption of reptile products. *Int J Food Microbiol* 134:163–175
- Mallon ME, MacLeod A, Wastling JM, Smith H, Tait A (2003) Multilocus genotyping of *Cryptosporidium parvum* type 2: population genetics and sub-structuring. *Infect Genet Evol* 3:207–218
- Mann ED, Sekla LH, Nayar GP, Koschik C (1986) Infection with *Cryptosporidium spp.* in humans and cattle in Manitoba. *Can J Vet Res* 50:174–178
- Mathis A, Weber R, Deplazes P (2005) Zoonotic potential of the microsporidia. *Clin Microbiol Rev* 18(3):423–445
- Méndez-Hermida F, Gomez-Couso H, Ares-Mazas E (2006) *Artemia* is capable of spreading oocysts of *Cryptosporidium* and the cysts of *Giardia*. *J Eukaryot Microbiol* 53:432–434
- Millar BC, Finn M, Xiao L, Lowery CJ, Dooley JSG, Moore JE (2002) *Cryptosporidium* in food-stuffs – an emerging aetiological route of human foodborne illness. *Trends Food Sci Technol* 13:168–187
- Millard PS, Gensheimer KF, Addiss DG, Sosin DM, Beckett GA, Houck-Jankoski A, Hudson A (1994) An outbreak of cryptosporidiosis from fresh-pressed apple cider. *JAMA* 272:1592–1596
- Molini U, Traversa D, Ceschia G, Iorio R, Boffo L, Zentilin A, Capelli G, Giangaspero A (2007) Temporal occurrence of *Cryptosporidium* in the Manila clam *Ruditapes philippinarum* in northern Adriatic Italian lagoons. *J Food Prot* 70:494–499
- Morakote N, Muangyimpong Y, Somboon P, Khamboonruang C (1987) Acute human isosporiasis in Thailand: a case report. *Southeast Asian J Trop Med Public Health* 18:107–111
- Moravec F (2001) Some helminth parasites from Morelet's crocodile, *Crocodylus moreletii*, from Yucatan, Mexico. *Folia Parasitol (Praha)* 48:47–62
- Mudry J, Lefebvre P, Dei-Cas E, Vernes A, Poirriez J, Debat M, Marti R, Binot P, Cortot A (1986) Anisakiase humaine: 5 cas dans le Nord de la France. *Gastroenterol Clin Biol* 10:83–87
- Neves AL, Gomes FS, Freitas AM, Almeida RN, Valente VC, Valente AS. [Estudo Experimental da Viabilidade do Trypanossoma Cruzi no Açai e Infecção em Camundongos]. In 59th Annual Meeting of Sociedade Brasileira para o Progresso da Ciência (SBPC); 2007. <http://www.servicos.sbpcnet.org.br/sbpc/59ra/senior/livroeletronico/resumos/R7591-1.html>.
- Ngouanesavanh TM, Guyot K, Certad G, Le Fichoux Y, Chartier C, Verdier RI, Cailliez JC, Camus D, Dei-Cas E, Bañuls AL (2006) *Cryptosporidium* population genetics: evidence of clonality in isolates from France and Haiti. *J Eukaryot Microbiol* 53(S1):33–36

- Nichols G, Thom BT (1985) Food poisoning caused by *Cryptosporidium*: a load of tripe. *Commun Dis Rep* 17:3
- Nóbrega AA, Garcia MH, Tatto E, Obara MT, Costa E, Sobel J, Araujo WN (2009) Oral transmission of Chagas disease by consumption of açaí palm fruit, Brazil. *Emerg Infect Dis* 15:653–655
- Owen IL, Gomez Morales MA, Pezzotti P, Pozio E (2005) *Trichinella* infection in a hunting population of Papua New Guinea suggests an ancient relationship between *Trichinella* and human beings. *Trans R Soc Trop Med Hyg* 99:618–624
- Paugam A, Yera H, Poirier P, Lebuissou A, Dupouy-Camet J (2009) Infection à *Dipyllobothrium nihonkaiense*: un nouveau risque associé à la consommation de saumon. *Presse Med* 38:675–677
- Pönkä A, Kotilainen P, Rimhanen-Finne R, Hokkanen P, Hänninen ML, Kaarna A, Meri T, Kuusi M (2008) A foodborne outbreak due to *Cryptosporidium parvum* in Helsinki. *Euro Surveill* 14(28). pii: 19269
- Pozio E (2007a) World distribution of *Trichinella* spp infections in animals and humans. *Vet Parasitol* 149:3–21
- Pozio E (2007b) Taxonomy, biology and epidemiology of *Trichinella* parasites. In: Dupouy-Camet J, Murrell KD (eds) FAO/WHO/OIE guidelines for the surveillance, management, prevention and control of trichinellosis. World Organisation for Animal Health Press, Paris, pp 1–35
- Pozio E, Murrell KD (2006) Systematics and epidemiology of *Trichinella*. *Adv Parasitol* 63:367–439
- Pozio E, Zarlenga DS (2005) Recent advances on the taxonomy, systematics and epidemiology of *Trichinella*. *Int J Parasitol* 35:1191–1204
- Pozio E, Owen IL, La Rosa G, Sacchi L, Rossi P, Corona S (1999) *Trichinella papuae* n.sp. (Nematoda), a new non-encapsulated species from domestic and sylvatic swine of Papua New Guinea. *Int J Parasitol* 29:1825–1839
- Pozio E, Foggin CM, Marucci G, La Rosa G, Sacchi L, Corona S, Rossi P, Mukaratirwa S (2002) *Trichinella zimbabwensis* n.sp. (Nematoda), a new non-encapsulated species from crocodiles (*Crocodylus niloticus*) in Zimbabwe also infecting mammals. *Int J Parasitol* 32:1787–1799
- Pozio E, Marucci G, Casulli A, Sacchi L, Mukaratirwa S, Foggin CM, La Rosa G (2004) *Trichinella papuae* and *Trichinella zimbabwensis* induce infection in experimentally infected varans, caimans, pythons and turtles. *Parasitology* 128:333–342
- Pozio E, Owen IL, Marucci G, La Rosa G (2005) Inappropriate feeding practice favors the transmission of *Trichinella papuae* from wild pigs to saltwater crocodiles in Papua New Guinea. *Vet Parasitol* 127:245–251
- Quiroz ES, Bern C, MacArthur JR, Xiao L, Fletcher M, Arrowood MJ, Shay DK, Levy ME, Glass RI, Lal A (2000) An outbreak of cryptosporidiosis linked to a foodhandler. *J Infect Dis* 181:695–700
- Rausch RL, Adams AM (2000) Natural transfer of helminthes of marine origin to freshwater fishes, with observations on the *Dipyllobothrium alascense*. *J Parasitol* 86:319–327
- Robertson LJ, Gjerde B (2001) Occurrence of parasites on fruits and vegetables in Norway. *J Food Prot* 64:1793–1798
- Robertson LJ, Johannessen GS, Gjerde BK, Loncarevic S (2002) Microbiological analysis of seed sprouts in Norway. *Int J Food Microbiol* 75:119–126
- Robertson LJ, Greig JD, Gjerde B, Fazil A (2005) The potential for acquiring cryptosporidiosis or giardiasis from consumption of mung bean sprouts in Norway: a preliminary step-wise risk assessment. *Int J Food Microbiol* 98:291–300
- Rocourt J, Moy G, Vierk K, Schlundt J (2003) The present state of foodborne disease in OECD countries. WHO Document Production Services, Geneva, Switzerland
- Romanova TV, Shkarin VV, Khazenson LB (1992) Group cryptosporidiosis morbidity in children. *Med Parazitol* 3:50–52
- Ryan U, O'Hara A, Xiao L (2004) Molecular and biological characterization of a *Cryptosporidium molnari*-like isolate from a guppy (*Poecilia reticulata*). *Appl Environ Microbiol* 70:3761–3765
- Sampaio JL, de Andrade VP, Lucas Mda C, Fung L, Gagliardi SM, Santos SR, Mendes CM, Eduardo MB, Dick T (2005) Dipyllobothriasis, Brazil. *Emerg Infect Dis* 11:1598–1600

- Sato MO, Cavalcante TV, Sako Y, Nakao M, Yamasaki H, Yatsuda AP, Nakaya K, Ito A (2006) Short report: Evidence and potential for transmission of human and swine *Taenia solium* cysticercosis in the Piracuruca region, Piauí, Brazil. *Am J Trop Med Hyg* 75(5):933–935
- Schets FM, van den Berg HH, Engels GB, Lodder WJ, de Roda Husman AM (2007) *Cryptosporidium* and *Giardia* in commercial and noncommercial oysters (*Crassostrea gigas*) and water from the Oosterschelde, The Netherlands. *Int J Food Microbiol* 113:189–194
- Scholz T, Garcia HH, Kuchta R, Wicht B (2009) Update on the human broad tapeworm (genus *Diphyllobothrium*), including clinical relevance. *Clin Microbiol Rev* 22:146–160
- Selgelid MJ (2007) Ethics and drug resistance. *Bioethics* 21:218–229
- Shimizu H, Kawakatsu H, Shimizu T, Yamada M, Tegoshi T, Uchikawa R, Arizono N (2008) Diphyllobothriasis *nihonkaiense*: possibly acquired in Switzerland from imported Pacific salmon. *Intern Med* 47:1359–1362
- Slettback T, Karlsen E (1999) Svalbard og tidligere funn av *Echinococcus multilocularis* pa mus. Orientering til pressen, 15 October, Statens Dyrehelsetilsyn, Fylkesveterinaeren for Troms og Finmark, Harstad (Norway). (Quoted by Eckert et al. 2002)
- Smith HV, Cacciò SM, Cook N, Nichols RA, Tait A (2007) *Cryptosporidium* and *Giardia* as foodborne zoonoses. *Vet Parasitol* 149:29–40
- Smyth JD (1994) Introduction to animal parasitology. Cambridge University Press, New York
- Sterling CR, Seegar K, Sinclair NA (1986) *Cryptosporidium* as a causative agent of traveler's diarrhea [letter]. *J Infect Dis* 153:380–381
- Sulzyc-Bielicka V, Kuzna-Grygiel W, Kolodziejczyk L, Bielicki D, Kladny J, Stepien-Korzonek M, Telatynska-Smieszek B (2007) Cryptosporidiosis in patients with colorectal cancer. *J Parasitol* 93:722–724
- Tanriverdi S, Grinberg A, Chalmers RM, Hunter PR, Petrovic Z, Akiyoshi DE, London E, Zhang L, Tzipori S, Tumwine JK, Widmer G (2008) Inferences about the global population structures of *Cryptosporidium parvum* and *Cryptosporidium hominis*. *Appl Environ Microbiol* 74:7227–7234
- Tappe D, Stich A, Frosch M (2008) Emergence of polycystic neotropical echinococcosis. *Emerg Infect Dis* 14:292–297
- Tzipori S, Widmer G (2008) A hundred-year retrospective on cryptosporidiosis. *Trends Parasitol* 24:184–189
- Valigurová A, Jirků M, Koudela B, Gelnar M, Modrý D, Slapeta J (2008) Cryptosporidia: epicellular parasites embraced by the host cell membrane. *Int J Parasitol* 38:913–922
- Wheeler JG, Sethi D, Cowden JM, Wall PG, Rodrigues LC, Tompkins DS, Hudson MJ, Roderick PJ (1999) Study of infectious intestinal disease in England: rates in the community, presenting to general practice, and reported to national surveillance. The infectious intestinal disease study executive. *BMJ* 318:1046–1050
- WHO (2000) Global water supply and sanitation assessment report. WHO and UNICEF. WHO/UNICEF/WSSCC (2000) – ISBN 92 4 156202 1). Pg 2, Box. 1.2
- Wicht B, de Marval F, Peduzzi R (2007) *Diphyllobothrium nihonkaiense* (Yamane et al., 1986) in Switzerland: first molecular evidence and case reports. *Parasitol Int* 56:195–199
- Woebeser G, Runge W, Stewart RR (1983) *Metorchis conjunctus* (Cobbold, 1860) infection in wolves (*Canis lupus*), with pancreatic involvement in two animals. *J Wild Dis* 19:353–356
- Woolhouse ME, Gowtage-Sequeria S (2005) Host range and emerging and reemerging pathogens. *Emerg Infect Dis* 11:1842–1847
- Woolhouse M, Gowtage-Sequeria S, Evans B (2006) T16: quantitative analysis of the characteristics of emerging and reemerging human pathogens. UK Government's Foresight project, Infectious Diseases: preparing for the future. <http://www.foresight.gov.uk/Infectious%20Diseases/t16.pdf>
- Xiao L, Fayer R, Ryan U, Upton SJ (2004) *Cryptosporidium* taxonomy: recent advances and implications for public health. *Clin Microbiol Rev* 17:72–97
- Yera H, Estran C, Delaunay P, Gari-Toussaint M, Dupouy-Camet J, Marty P (2006) Putative *Diphyllobothrium nihonkaiense* acquired from a Pacific salmon (*Oncorhynchus keta*) eaten in France; genomic identification and case report. *Parasitol Int* 55:45–49

- Zarlenga DS, Rosenthal B, La Rosa G, Pozio E, Hoberg EP (2006) An old genus learns new tricks: late tertiary colonization and speciation of *Trichinella* nematodes among Eutheria. *Proc Nat Acad Sci USA* 103:7354–7359
- Zhou L, Singh A, Jiang J, Xiao L (2003) Molecular surveillance of *Cryptosporidium* spp. in raw wastewater in Milwaukee: implications for understanding outbreak occurrence and transmission dynamics. *J Clin Microbiol* 41:5254–5257

Unusual Developmental Pattern of Expression of Enzymes Involved in DNA Biosynthesis in *Trichinella spiralis* and *Trichinella pseudospiralis*

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Abstract All species in the genus *Trichinella*, between them *T. spiralis* and *T. pseudospiralis*, have been successful in colonizing striated skeletal muscle tissue and remain infective in this niche for months to years. *Trichinella spiralis* causes trichinellosis, a serious disease in man and other mammals. Mating of adult worms (developing from infective larvae, deriving from digested infected meat) occurs in a non membrane-bound portion of columnar epithelium of the host's small intestine. The fertilized females enter the intestinal wall and release to the bloodstream the newborn larvae. Each of these penetrates host's skeletal muscle cell and lives in its modified portion, the nurse cell, surrounded by a collagen capsule around which a circulatory rete develops. The nurse cell development, initiated by *T. spiralis* infection, is associated with a variety of changes, including cell cycle re-entry and induction of DNA synthesis, followed by the apparent G2/M arrest of the infected cell in the cell cycle. Similar changes appear to be caused by *T. pseudospiralis* infection, albeit the nurse cell complexes are not encapsulated by collagenous fibres and the larvae may move between muscle cells.

Thymidylate (dTMP) is formed intracellularly either de novo, in a process of the C(5) methylation of 2'-deoxyuridylate (dUMP), catalyzed by the enzyme thymidylate synthase (TS), or as a product of thymidine salvage via phosphorylation, catalyzed by the enzyme thymidine kinase. The dUMP methylation reaction involves a concerted transfer and reduction of the one-carbon group of

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N^{5,10}-methylenetetrahydrofolate, with concomitant production of thymidylate and dihydrofolate. The coenzyme tetrahydrofolate is regenerated *via* dihydrofolate reduction by the enzyme dihydrofolate reductase (DHFR). One of the sources of TS substrate, dUMP, is dUTP hydrolysis in a pyrophosphatase reaction catalyzed by the enzyme dUTPase. TS and dUTPase induction is known to be associated with cell proliferation. Thymidylate synthesis inhibition by drugs targeted at either TS or DHFR is taken advantage of in chemotherapy.

TS, DHFR and dUTPase were found to be persistently expressed at a high and constant level, comparable to that found in regenerating rat liver, in crude extracts from adult worms of *Trichinella spiralis*, as well as from developmentally arrested muscle larvae of both *Trichinella spiralis* (isolated 1–24 months after infection) and *Trichinella pseudospiralis* (isolated 5.5–13 months after infection). The results obtained with *Trichinella pseudospiralis* muscle larvae isolated with the use of pepsin did not differ from those obtained when pepsin was not used. Moreover, *T. spiralis* muscle larvae (*T. pseudospiralis* larvae were not tested) contained also high level, comparable with that found in mouse leukemia L1210 cells, of DNA polymerase α , a key enzyme of the eukaryotic replication complex, its expression also known to be associated with cell proliferation.

Immunofluorescent detection of TS protein was done with the use of monoclonal antibodies, developed by *in vivo* immunization of Balb/c mice with homogeneous recombinant rat hepatoma TS protein as an antigen. The specific anti-rat TS antibodies recognized also *T. spiralis* TS, as indicated by cross-reactivity on Western blot. Localization of the enzyme was based on analysis of pictures collected by confocal microscopy. Two types of *T. spiralis* muscle larvae preparations were studied: muscle larvae isolated from mouse muscles by a procedure destroying nurse cells and muscle larvae remaining in nurse cells, isolated as an intact nurse cell preparation.

The results revealed reproducible TS localization patterns, reflected by strong fluorescence emitted by cells of both female and male gonad primordium, as well as from the regions around stichocyte nuclei.

High expression in *Trichnella* muscle larva of thymidylate synthase, and certain other enzymes involved in DNA biosynthesis, was found also in *Caenorhabditis dauer* larva and appears to be connected with their cells being arrested in the cell cycle.

Keywords *Trichinella spiralis* • *Trichinella pseudospiralis* • Thymidylate synthase • Dihydrofolate reductase, dUTPase • Pyrimidine salvage

Introduction

Thymidylate is formed intracellularly either *de novo*, in a process of N^{5,10}-methylenetetrahydrofolate-dependent C(5) methylation of 2'-deoxyuridylate (dUMP), catalyzed by the enzyme thymidylate synthase (EC 2.1.1.45), or as a product

of thymidine salvage *via* phosphorylation, catalyzed by the enzyme thymidine kinase (EC 2.7.1.75). The dUMP methylation reaction involves a concerted transfer and reduction of the one-carbon group (at the aldehyde oxidation level) of N⁵, 10-methylenetetrahydrofolate, with concomitant production of thymidylate and dihydrofolate (Carreras and Santi 1995). The coenzyme tetrahydrofolate is regenerated *via* dihydrofolate reduction by the enzyme dihydrofolate reductase (EC 1.5.1.3) (Young 1994). One of the sources of thymidylate synthase substrate, dUMP, is dUTP hydrolysis in a pyrophosphatase reaction catalysed by the enzyme dUTPase (EC 3.6.1.23). Thymidylate synthase (Friedkin 1973; Santi and Danenberg 1984) and dUTPase (Hokari et al. 1987; Vilpo 1983; also citations therein) induction is known to be associated with cell proliferation. Thymidylate synthesis inhibition by drugs targeted at either thymidylate synthase (Heidelberger et al. 1983; Rathod 1997; Georgopapadakou and Walsh 1996; Takemura and Jackman 1997) or dihydrofolate reductase (Schweitzer et al. 1990; Rosowsky 1992) is taken advantage of in antibacterial, anticancer, antiviral, antifungal and antiprotozoan chemotherapy.

All species of parasitic nematodes in the genus *Trichinella*, between them *T. spiralis* and *T. pseudospiralis*, have been successful in colonizing striated skeletal muscle tissue and can occupy this niche for months to years (Despommier 1998). *T. spiralis* causes trichinellosis, a serious disease in man and other mammals.

Trichinella spiralis muscle larva lives in a modified portion of host's skeletal muscle cell, the nurse cell, surrounded by a collagen capsule around which a circulatory rete develops (Despommier 1993). The nurse cell development, initiated by *T. spiralis* infection, is associated with a variety of changes, including cell cycle re-entry and induction of DNA synthesis, followed by the apparent G2/M arrest of the infected cell in the cell cycle (Jasmer 1995). Similar changes appear to be caused by *T. pseudospiralis* infection, albeit the nurse cell complexes are not encapsulated by collagenous fibres and the larvae may move between muscle cells (Ko et al. 1994). An outbreak of human trichinellosis caused by this species has been reported (Jongwutiwes et al. 1998).

An earlier observation of surprisingly high thymidylate synthase specific activity in crude extracts from muscle larvae and from adult worms of *T. spiralis* (Dąbrowska et al. 1996) prompted us to further studies of this phenomenon. As thymidylate synthase is expressed in association with cell proliferation and cell cycle (Santi and Danenberg 1984; Pestalozzi et al. 1995), its high specific activity found in non-growing muscle larvae of the parasite were surprising. Therefore activities of other enzymes involved in DNA biosynthesis, dihydrofolate reductase, dUTPase, thymidine kinase, ribonucleotide reductase (EC 1.17.4.1) and DNA polymerase α (EC 2.7.7.7), also known to be expressed in association with cell proliferation (Hokari et al. 1987; Vilpo 1983; Békési et al. 2004; Kit 1976; Blakley 1984; Johnson et al. 1978; Wahl et al. 1988; Wong et al. 1988; Takemura et al. 2001; Engstrom et al. 1985; Björklund et al. 1990) were encompassed in the study. Besides, localization of thymidylate synthase in different developmental forms of the parasite was studied, using immunofluorescent detection of the enzyme protein. Of obvious interest was whether the phenomenon, observed in *T. spiralis* muscle larvae and potentially exploitable as a target in antiparasitic chemotherapy, is (i) specific for *T. spiralis* or

may be observed in another species of intracellular parasite, *T. pseudospiralis* and (ii) specific for intracellular parasitic life or is common for developmentally arrested life cycle stages of other, including free-living, nematodes. In order to test the latter possibility, specific activity developmental pattern of enzymes involved in thymidylate biosynthesis was studied for *Caenorhabditis elegans*. The life cycle of this free-living nematode, being a model for parasitic nematodes (Bürglin et al. 1998), also involves a developmentally arrested stage, dauer larva, corresponding to *T. spiralis* muscle larva (Bürglin et al. 1998).

The results indicate an unusual enzyme expression pattern in developmentally arrested larvae, pointing to a possibility of a global cell cycle arrest and characteristic not only for both *Trichinella* species, but also for *C. elegans*. They also indicate a convenient model for further studies of the underlying mechanism of this unusual regulatory pattern.

Materials and Methods

Materials Protease Inhibitor Cocktail Tablets (Complete, Mini) were purchased from Roche Diagnostics GmbH (Mannheim, Germany). (6RS, α S)Tetrahydrofolic acid, thymidine, dUMP, FdUMP, tiophilic adsorbent (T Reisin), ISO-2 kit, as well as antibodies, anti-mouse IgM (μ -chain-specific)-HRP conjugate and anti-mouse IgG -HRP conjugate, and TMB were from Sigma (St. Louis, MO). Goat anti-mouse IgG (H + L) – HRP conjugate and goat anti-mouse Amplified Opti-4CN Detection Kit were obtained from Bio-Rad. DEAE-cellulose (DE-52) from Whatman (Maidstone, England), [5-³H]dUMP (15 Ci/mmol) and [methyl-³H] thymidine (64, 7 Ci/mmol) were from Moravек Biochemicals (Brea, CA) and other reagents were of analytical grade. pBluescript SK⁻ vector and *E. coli* DHF α F' strain were from Stratagene, TX61, a derivative of the *E. coli* BL21(DE3) strain with the endogenous thymidylate synthase gene inactivated by transposon-mediated insertion, was a gift from Dr. W.S. Dallas (Glaxo Wellcome, UK).

Biological Material *Trichinella spiralis* human isolate H2 from the Instituto Superiore di Sanita, Rome, Italy, ref. no. MHOM/PO/88/ISS67 (T1), and *T. pseudospiralis*, isolated by Garkavi (1972) and since 1976 passaged and maintained by Cabaj (1986) at the Institute of Parasitology of the Polish Academy of Sciences (Warsaw, Poland), were maintained in Swiss adult mice and infective larvae were isolated from mouse infected carcasses by pepsin-HCl digestion (Gould 1970) for 1 h at 37°C. Larvae were washed with PBS and used within 30 min after isolation to infect mice or in enzyme studies. Adult worms of both sexes were recovered from isolated small intestines of mice, infected 6 days previously (Takada and Tada 1988).

T. spiralis infective muscle larvae were obtained from mouse carcasses (3 months following infection) with the use of pepsin-HCl digestion (Gould 1970). The larvae were separated from undigested particles of host tissues by passing through three layers of cotton gauze, followed by repeated sedimentation in phosphate-buffered saline (PBS).

In order to isolate *T. spiralis* premature muscle larvae, a mixed population of larvae at various periods of development was obtained by homogenization in PBS (with peptic digestion omitted) of infected mouse muscles isolated at day 20 postinfection. The homogenate was incubated 2 h at 37°C to allow the release of larvae from the tissue. The released larvae were separated by sedimentation and washing with PBS.

T. spiralis adult forms and newborn larvae were isolated according to Wranicz et al. (1998).

Intact nurse cells were obtained from the diaphragms of mice infected with *T. spiralis*, excised at day 30 postinfection. Nurse cells were isolated by restricted digestion (its progress controlled under binocular), by 1% trypsin in PBS buffer containing 0.02% EDTA. Digestion was stopped by washing with PBS containing Ca^{++} and Mg^{++} ions.

C. elegans wild type strain N2, variety Bristol, was used, with stocks maintained at 25°C on agar plates streaked with *E. coli* strain OP50 (Brenner 1974). Worms to be used for determination of specific activity or purification of thymidylate synthase were grown with addition of *E. coli* strain TX61⁻ as the bacterial food. In other cultures the OP50 strain (a gift from the Caenorhabditis Genetic Center, University of Minnesota-St. Paul) was used. In order to obtain large number of worms, 1 L liquid culture in the complete S medium (Lewis and Fleming 1995) at 25°C with forced aeration was performed. *C. elegans* cultures were synchronized and worms at specific developmental stages obtained according to earlier described procedures (Lewis and Fleming 1995). Briefly, dauer larvae were obtained by adding the stock culture to 1 L of the S medium containing 50 g of bacteria and growing, unless otherwise indicated, for 2 weeks at 25°C. Dauer larvae, constituting 67–100% of the resulting worm population, were purified by sedimentation at $1 \times g$ and either stored frozen at –30°C or used (after culturing in the S medium containing bacteria) as a source of worms growing to adulthood. The adult worms were cleaned by sedimentation and flotation on 30% (w/v) sucrose in water and either stored at –30°C or treated with alkaline hypochlorite solution to obtain embryos. Embryos were cleaned in the M9 buffer (cf. Lewis and Fleming 1995) by centrifugation at $150 \times g/5$ min and either stored frozen at –30°C or suspended in the buffer M9 and allowed to develop to starvation-arrested L1 larvae (17–24 h at 25°C). The L1 larvae were cleaned in the M9 buffer by centrifugation at $150 \times g/5$ min and either stored frozen at –30°C or allowed to resume synchronous growth in the S medium at 25°C (after reintroduction to bacterial food) to develop to L3 larvae. Those were cleaned by centrifugation at $100 \times g/5$ min and stored frozen at –30°C.

Preparation of Crude Extracts The parasite larvae or adult forms were homogenized by grinding with sand in a mortar (placed on ice) with three to five volumes of ice-cold 0.05 M phosphate buffer, pH 7.5, containing 0.1 M KCl and 0.01 M 2-mercaptoethanol, except for assays of thymidine kinase activity, when phosphate buffer was substituted by Tris-HCl buffer at the same concentration and pH. The suspension was sonicated and centrifuged to obtain crude extract.

C. elegans worms were homogenized by grinding with quartz sand in a mortar (placed on ice) with three to five volumes of homogenizing buffer and centrifuged

(20,000 $\times$ g/20 min), and the supernatant (crude extract) was saved. For assays of thymidylate synthase and dUTPase activities the worms were homogenized in ice-cold 0.05 M phosphate buffer, pH 7.5, containing 0.1 M KCl and 0.01 M 2-mercaptoethanol. For assays of thymidine kinase activity the worms were homogenized in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M KCl, 2 mM EDTA, protease inhibitor cocktail (Complete, Mini tablets from Roche; concentration corresponding to one tablet dissolved in 10 ml) and 0.01 M 2-mercaptoethanol. For assays of dihydrofolate reductase the worms were homogenized in 0.05 M Tris-HCl buffer, pH 7.0, containing 0.1 M KCl and 0.01 M 2-mercaptoethanol.

L5178Y cells were grown and extract prepared as previously described for L1210 cells (Rode et al. 1980), except that fetal calf serum was used instead of horse serum.

Purified Enzyme Preparations Previously described *T. spiralis* endogenous (from muscle larvae (Dąbrowska et al. 1996) and recombinant (Dąbrowska et al. 2004), and rat endogenous (from regenerating liver (Rode et al. 1990)) and recombinant (Cieśla et al. 1995a) thymidylate synthase preparations were used.

Enzyme Assays Activities of thymidylate synthase (Rode et al. 1990), thymidine kinase (Tsukamoto et al. 1991) and dihydrofolate reductase (Mathews et al. 1963) were assayed according to previously published procedures. dUTP-ase activity was determined by coupling with the thymidylate synthase-catalyzed reaction and measuring tritium released from [^3H]dUMP (Gołos and Rode 1999). The activity unit was defined as the enzyme amount required to convert 1 μmol of substrate per 1 min at 37°C.

The general DNA polymerase activity was assayed with the use of activated DNA matrix (Kaiserman and Benbow 1987) in a reaction mixture (total volume 40 μl), containing 10 mM Tris-HCl (pH 8), 10 mM MgCl_2 , 50 μM each dNTP, 10 mM 2-mercaptoethanol, ^3H -dTTP (200000 dpm/nmol), 10 mM NaF, 2 mM sodium pyrophosphate and activated DNA (660 $\mu\text{g/ml}$). DNA polymerase α activity was determined with the use of poly(dT)-oligo(rA) matrix (preferred by polymerase α) according to Zierler et al. (1985), in a 40 μl reaction mixture containing 10 mM Tris-HCl (pH 8), 0.1 mM EDTA, 6 mM MgCl_2 , 200 $\mu\text{g/ml}$ BSA, 1 mM dithiothreitol, 25 mM NaCl, 25 mM KCl, 10% glicerol, 100 μM ^3H -dATP (2,00,000 dpm/nmol), 10 mM NaF, 2 mM sodium pyrophosphate and 200 μM poly(dT)-50 μM oligo(rA₁₀). In both DNA polymerase assays reaction was initiated by adding crude extract (8–15 μl in 30 μl of homogenization buffer), run for 15 min at 30°C and stopped by transferring a 25 μl sample of the reaction mixture on a circle of 3 MM Whatman filter paper that was immediately immersed in 5% TCA containing 1% $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$. The collected circles were washed twice in 5% TCA, later twice in ethanol, and finally twice in diethyl ether. Following drying, each circle was put into a scintillation vial, scintillation cocktail added and radioactivity determined (Bollum 1959). Controls contained 30 μl homogenization buffer and no crude extract.

Ribonucleotide reductase activity was assayed based on CDP reduction, using a modified method of Jong et al. (1998), with the [^{14}C]CDP reduction product determined as radioactivity incorporated into DNA in a series of two coupled reactions, catalyzed by nucleoside diphosphate kinase and DNA polymerase (Klenow fragment). A 40 μl reaction mixture contained 50 mM Hepes pH 7.2, 10 mM dithiothreitol,

4 mM magnesium acetate, 4 mM ATP and 20 μ M [^{14} C] CDP (52 mCi/mmol) and the reaction was started by adding crude extract (10–30 μ l, with homogenization buffer filling up to 30 μ l; controls contained homogenization buffer alone), run for 7–20 min at 37°C and stopped by immersing samples for 5 min in a boiling water bath. Following centrifugation (10,000 $\times$ g/5 min), to a 25 μ l sample of each supernatant nucleoside diphosphate kinase (from baker's yeast; 1.5 U) and ATP (4 mM final concentration) were added and samples incubated for 30 min at 37°C. Next 37 μ g of fragmented DNA (120–3,000 nucleotides; Roche) was added and the reaction started by adding buffer containing random sequence primers (300 ng/ μ l), each dNTP (50 μ M), Klenow polymerase reaction buffer and Klenow polymerase (3 U). Following 60 min incubation at 30°C, a 20 μ l sample of the reaction mixture was transferred onto a circle of DE-81 filter paper (Whatman). After drying, the collected filters underwent triple washing in 5% Na₂HPO₄ (each time 5 min), double washing in H₂O, single washing in 95% ethanol and again drying. Each dry filter was put into a scintillation vial, eluting solution (200 μ l), containing 0.2 M KCl and 0.1 M HCl was added and, following scintillation cocktail addition, radioactivity determined. In final calculations of ribonucleotide reductase activity, the yield was considered of incorporation of nucleotides into DNA by the Klenow fragment under the above reaction conditions, determined at 30% level based on [^{3} H]dATP incorporation into fragmented DNA.

RNA Purification Total RNA was isolated from *T. spiralis* muscle larvae, adult forms and newborn larvae, and *C. elegans* adult worms, and L1, L3 and dauer larvae, using TRIZOL ultra pure reagent from GibcoBRL, according to manufacturer's protocol.

Northern Blot Analysis Total RNA was size-fractionated on 1% agarose-formaldehyde gel then subjected to Northern transfer onto Hybond-N⁺ nylon membranes (Amersham) and hybridized with one of the following specific probes: (i) 921 bp long cDNA fragment, corresponding to *T. spiralis* thymidylate synthase coding region (cf. Dąbrowska et al. 2004), (ii) 610 bp long cDNA fragment corresponding to a part (452 bp) of the *T. spiralis* histone H3.3 coding region (used to follow the expression of mRNA for a constitutive protein of the *Trichinella* nuclei; cf. Dąbrowska et al. 2004), (iii) 647 bp long cDNA fragment, corresponding to a part of *C. elegans* thymidylate synthase coding region or (iv) a reference probe – 612 bp long *T. spiralis* rDNA fragment (Kuratli et al. 1999). All procedures were performed according to Sambrook et al. (1989) with hybridization and autoradiography of each blot carried out three times in a sequence, each time with a different probe, at 68°C in the presence of 10% dextran sulfate. The probes were radioactively labelled with the use of Prime-a-Gene Labeling system (Promega). Autoradiographic results were assessed using ImageQuant program.

Thymidylate Synthase Protein Level Immunoblot Analysis After PAGE/SDS (1 μ g of purified recombinant thymidylate synthase or 50 μ g of crude extract protein per lane), proteins were transferred to PVDF membrane. Thymidylate synthase was detected by treatment with polyclonal antibodies raised in rabbits against purified recombinant (Wińska et al. 2005) thymidylate synthase (antibodies were purified by means of affinity chromatography on the same recombinant thymidylate synthase,

immobilized on CNBr-Sepharose in the reaction using 2 mg of the recombinant thymidylate synthase protein per 1 ml of CNBr-Sepharose). Anti-rabbit IgG coupled with HRP (Sigma) were used as secondary antibodies and colour was developed with the DAB/metal enhancer reagent (Bio-Rad). Density of bands was measured using Scion Image program.

Anti-thymidylate Synthase Monoclonal Antibodies These were generated with purified recombinant rat hepatoma thymidylate synthase (Cieřła et al. 1995a) used as an antigen. Antibodies were developed by immunization of Balb/c mice in vivo with the antigen (6 µg/mouse) in five steps: (1) with complete Freund adjuvant (1:1) subcutaneously and intramuscularly, (2–3) antigen in PBS intraperitoneally biweekly and (5) antigen in PBS intravenously (booster). Splenocytes were fused (3 days following the booster) with mouse myeloma SP2/0 HGPRT cells (standard method with 50% polyethylene glycol and 5% DMSO) and seeded in HAT medium on plates coated with macrophage feeder layer. Isotyping of immunoglobulins was done with the use of ISO-2 kit (Sigma) and anti-mouse IgM (µ-chain-specific)-HRP conjugate antibodies (Sigma). Color reaction was developed using TMB (Sigma) as a substrate. Specificity of antibodies was evaluated by ELISA method. Multiwell plates (Maxisorp NUNC) were coated with antigen (0.5 µg protein/well) in bicarbonate buffer pH 9.6. Goat anti-mouse IgG- HRP conjugate or goat anti-mouse IgM (µ-chain-specific)-HRP conjugate (Sigma) were used as secondary antibodies. Isolated clones resulted in 52 hybridoma lines producing anti-recombinant rat hepatoma thymidylate synthase antibodies (IgM or IgG type). Chosen antibodies were purified by precipitation with ammonium sulfate (50% saturation) and used after dialysis of the precipitate against PBS and dilution (10^2 – 10^3 -fold) for immunoblotting. For immunohistology antibody IgG 2a type, produced by clones 27-1, 27-7 and III-40, were additionally purified by thiophilic adsorption chromatography (Knudsen et al. 1992), with antibodies bound on the column from 1.2 M ammonium sulfate containing 50 mM sodium acetate pH 5.2, the column washed with 0.8 M ammonium sulfate containing 50 mM sodium acetate pH 5.2, and antibodies eluted with 50 mM Tris-HCL pH 9.0.

Immunoblot Analysis of Cross-Reactivity of Monoclonal Anti-rat Hepatoma Thymidylate Synthase Antibodies After PAGE/SDS (12.5% polyacrylamide, 100 µg of crude extract protein or 1 µg of purified endogenous/recombinant thymidylate synthase protein per lane), proteins were transferred to PVDF membrane. Thymidylate synthase was detected by treatment with a mixture of monoclonal antibodies (produced by clones 27-1, 27-7 and III-40 and mixed at 1:1:1 ratio) and goat anti-mouse IgG (H + L) – HRP conjugate (BioRad) as a secondary antibody. Color reactions were developed using goat anti-mouse Amplified Opti-4CN Detection Kit (Bio-Rad).

Preparation of T. spiralis Specimens for Immuno-Localization Studies of Premature and Mature Muscle Larvae and Intact Nurse Cells This procedure was adapted from that used for *C. elegans* (Miller and Shakes 1995).

Fixation of larvae, adult worms or intact nurse cells, suspended in cold PBS, was done by adding cold $\times 2$ Ruvkun fixation buffer, diluted with 2% formaldehyde to $\times 1$ final concentration. Frozen samples were stored at -80°C . After thawing,

glutaraldehyde was added to a final concentration of 1%, samples were incubated for 1 h and washed three times in TBST buffer.

Permealization was done by incubating samples overnight at 37°C in TBST containing 1% β -mercaptoethanol, washing twice in borate buffer containing 0.01% Triton X100, next incubating 15 min in the same buffer containing 10 mM dithiotreitol, washing again in the buffer alone and incubating 15 min in the same buffer containing 0.3% H_2O_2 . The fixed samples were washed in TBST buffer, suspended in TBST buffer containing 3% BSA and 0.005% sodium azide and stored at 4°C.

Photobleaching of *T. spiralis* preparations was performed, as the worms, as well as cytoplasm of nurse cells, emit a strong autofluorescence. In order to reduce it, the corresponding samples, protected against UV, were exposed to solar radiation for over 2 weeks. The process was monitored by confocal microscopy.

Immunohistology A solution of BSA (3%) in PBST was used to prevent a nonspecific antibody staining. Thymidylate synthase protein localization was determined by an indirect immunofluorescence staining, with mouse monoclonal IgG 2a (clones 27-1, 27-7 and III-40, purified by thiophilic adsorption chromatography and mixed at 1:1:1 ratio), generated against recombinant rat hepatoma thymidylate synthase protein and cross-specific to native *T. spiralis* enzyme, used as a primary antibody and polyclonal goat, anti-mouse IgG -TRITC conjugate applied as a secondary antibody. Optimal concentration of the primary antibody and incubation time were determined empirically.

Confocal Microscopy The specimens were mounted as suspended in 50% glycerol in PBS and examined by a scanning confocal laser system (LSM 510 – Zeiss, laser HeNe1 543 nm, LP 560 nm).

Protein Assay The procedure of Spector [1978] was used, with bovine serum albumin as a standard

Statistically Evaluated Results These are presented as means $\pm$ S.E.M. or means $\pm$ % difference between the mean and each of the two results, followed by the number of experiments (N) in parentheses.

Results

Enzyme Activities Involved in dTMP Biosynthesis

Thymidylate synthase specific activity, determined in crude extracts, was found to be expressed at a constant and surprisingly high level both in adult worms and in muscle larvae of *T. spiralis*, with monitoring continued as long as until 2 years after infection (Table 1). Mean specific activity was 0.094 ± 0.005 nmol/min/mg (N = 27), comparable with that found in adult worms ($0.10 \pm 16\%$ nmol/min/mg protein; N = 2) and regenerating rat liver (0.096 nmol/min/mg protein; cf. Rode et al. 1990).

Table 1 Specific activities of selected enzymes involved in thymidylate de novo biosynthesis in *T. spiralis* and *T. pseudospiralis* muscle larvae, *T. spiralis* adult forms, *C. elegans* adult forms, and L1, L3 and dauer larvae. For both *Trichinella* species time between infection and isolation is indicated

Developmental stage (with the parasite time between infection and isolation given)	Enzyme specific activity [nmol/min/mg protein]		
	Thymidylate synthase	Dihydrofolate reductase	dUTPase
<i>T. spiralis</i>			
Adult forms			
6 days	0.058 ± 27% (2) ^a	36.0 ± 64%	NT ^b
Muscle larvae ^c			
30 days	0.092 ± 0.013 (8)	7.60 ± 1.44 (5)	0.25 (1)
6 weeks	0.125 (1)	26.5 (1)	0.17 (1)
8 weeks	0.105 ± 12% (2)	8.5 ± 44% (2)	0.32 (1)
10 weeks	0.130 (1)	7.6 (1)	0.60 (1)
12 weeks	0.112 (1)	22.8 (1)	1.43 (1)
13 weeks	0.083 (1)	3.4 (1)	NT
4 months	0.113 (1)	8.0 (1)	NT
5 months ^d	0.070 (1)	NT	NT
6 months	0.072 ± 0.007 (4)	20.2 ± 8.6 (3)	0.81 ± 0.40 (3)
7 months	0.080 (1)	16.7 (1)	0.80 (1)
9 months	0.093 ± 53% (2)	12.8 ± 22%	1.0 ± 89% (2)
12 months	0.123 (1)	28.5 (1)	0.93 (1)
15 months	0.087 (1)	20.0 (1)	NT
16 months	0.121 (1)	16.2 (1)	NT
24 months	0.088 (1)	NT	0.23 (1)
1–24 months	0.094 ± 0.005 (27)	13.9 ± 2.0 (21)	0.68 ± 0.16 (14)
<i>T. pseudospiralis</i>			
Muscle larvae ^c			
5.5 months	0.100 (1)	36.0 (1)	1.7 (1)
6.5 months	0.098 (1)	8.1 (1)	0.10 (1)
11 months	0.128 (1)	15.0 (1)	1.7 (1)
13 months ^e	0.100 (1)	36.0 (1)	1.7 (1)
5.5–13 months	0.107 ± 0.007 (4)	23.8 ± 7.20 (4)	1.3 ± 0.4 (4)
<i>C. elegans</i>			
Adult forms			
L1 larvae	0.12 ± 17% (2)	7.4 ± 12% (2)	3.9 ± 7.7% (2)
L3 larvae	0.11 ± 45% (2)	13.2 ± 27% (2)	2.7 (1)
L3 larvae	0.40 ± 0.15 (3)	7.8 ± 6.4% (2)	4.2 ± 1.35 (3)
Dauer larvae	0.10 ± 0.03 (3)	14.0 ± 4.3% (2)	0.83 ± 0.15 (3)
Dauer larvae (4 weeks at 25°C) ^f	0.10 ± 0.00 (3)	NT	NT
Dauer larvae (6 weeks at 25°C) ^f	0.09 (1)	NT	NT

^aFor statistical evaluation of results see Section [Materials and Methods](#)

^bNot tested

^cIsolation of muscle larvae involved 1 h pepsin-HCl digestion, except in the two following cases (marked^{d-e})

^dDigestion (7 min) underwent homogenized boneless muscle tissue

^eNo digestion was applied as larvae were allowed to migrate from small boneless muscle pieces to the bottom part (warmed up to 37°C) of a glass reservoir during incubation in Hank's balanced salt solution

^fThe culture prepared to obtain dauer larvae was continued for the time indicated

High thymidylate synthase level was accompanied (Table 1) by high specific activities of other enzymes involved in thymidylate biosynthesis, dihydrofolate reductase (14 ± 2 nmol/min/mg protein; $N = 21$) and dUTPase (0.68 ± 0.16 nmol/min/mg protein; $N = 14$), again comparable with the corresponding activities (67 nmol/min/mg protein for dihydrofolate reductase and 0.6–1.5 nmol/min/mg protein for dUTPase) found in regenerating rat liver crude extracts.

The three enzyme activities were expressed also at similarly high and rather constant levels in *T. pseudospiralis* muscle larvae, isolated between 5.5 and 13 months after infection, crude extract specific activities being 0.11 ± 0.01 nmol/mg protein ($N = 4$), 23.8 ± 7.2 nmol/min/mg protein ($N = 4$) and 1.3 ± 0.4 nmol/min/mg protein ($N = 4$) for thymidylate synthase, dihydrofolate reductase and dUTPase, respectively (Table 1). Interestingly, the results obtained with the larvae isolated with the use of pepsin did not differ from those obtained when pepsin was not used (Table 1).

In *C. elegans* development, the specific activities of the three enzymes involved in dTMP biosynthesis, thymidylate synthase, dUTPase and dihydrofolate reductase, were high in adult forms, L1, L3 and dauer larvae (Table 1). Thymidylate synthase activity was not lowered when dauer larvae remained up to 6 weeks in the culture (Table 1). When measured in an extract from dauer larvae that were obtained from a starved stock culture based on their climbing behaviour (Riddle and Albert 1997), thymidylate synthase activity was found to be similarly high (0.18 nmol/min/mg protein; $N = 1$).

General DNA Polymerase and DNA Polymerase α Activities

While the general DNA polymerase activity found in *T. spiralis* muscle larvae crude extract was $0.7 \pm 0\%$ nmol/h/mg protein ($N = 2$), the corresponding activity assayed under conditions preferred by DNA polymerase α was $4.6 \pm 74\%$ nmol/h/mg protein ($N = 2$). As a reference, the two sets of assay conditions were used with crude extracts from mouse leukemia L1210 cells (harvested in the logarithmic phase of growth) and the corresponding activities determined as 1.4 nmol/h/mg protein ($N = 1$) and 3.7 nmol/h/mg protein ($N = 1$), respectively.

Both the general DNA polymerase and DNA polymerase α activities were found in all *C. elegans* developmental forms studied. The general DNA polymerase activity was determined in starved L1 (0.27 nmol/h/mg protein; $N = 1$), L3 ($0.48 \pm 21\%$ nmol/h/mg protein; $N = 2$) and dauer (0.33 ± 0.08 nmol/h/mg protein; $N = 5$) larvae, and adults (0.4 ± 0.06 nmol/h/mg protein; $N = 4$), as well as in a mixed population of developing larvae ($0.5 \pm 20\%$ nmol/h/mg protein; $N = 2$). DNA polymerase α activity was assayed in starved L1 ($0.48 \pm 4\%$ nmol/h/mg protein; $N = 2$), L3 ($3.2 \pm 31\%$ nmol/h/mg protein; $N = 2$) and dauer (0.74 ± 0.34 nmol/h/mg protein; $N = 5$) larvae, and adults (2.8 ± 0.38 nmol/h/mg protein; $N = 4$), as well as in a mixed population of developing larvae ($1.3 \pm 0\%$ nmol/h/mg protein; $N = 2$).

Ribonucleotide Reductase Activity

Ribonucleotide reductase activity, comparable to that (0.7 nmol/h/mg protein; N = 1) determined in murine leukemia L1210 cells (harvested in the logarithmic phase of growth) was found in extracts from *T. spiralis* muscle larvae (0.2 nmol/h/mg protein; N = 1), as well as from all *C. elegans* developmental forms studied, including dauer larvae (0.47 ± 0.07 nmol/h/mg protein; N = 3), mixed population of developing larvae ($0.64 \pm 6\%$ nmol/h/mg protein; N = 2) and adults (0.7 ± 0.06 nmol/h/mg protein; N = 3).

Thymidine Kinase Activity

No detectable thymidine (dThd) kinase activity could be found in crude extracts of *T. spiralis* muscle larvae isolated 1–24 months after infection and of *T. pseudospiralis* muscle larvae isolated 6–13 months after infection (including extracts of larvae isolated without pepsin), although L1210 cell crude extract, prepared as previously described (Rode et al. 1990) and used as a positive control, showed enzyme specific activity (with dThd as a substrate) of 0.02 nmol/min/mg. On the other hand, in all *C. elegans* developmental forms studied, including adults, and L1, L3 and dauer larvae, thymidine kinase activity was high, its levels determined as 0.032 ± 0.006 (3), 0.019 ± 0.009 (3), $0.016 \pm 25\%$ (2) and 0.013 ± 0.005 (3), respectively.

Expression of Thymidylate Synthase mRNA, Histone H3-Like mRNA and 18S rRNA in Muscle Larvae, Adult Forms and Newborn Larvae of T. spiralis

The results of Northern blot analyses of *T. spiralis* total RNA are presented in Fig. 1. Thymidylate synthase mRNA, relative to 18S rRNA, level was found to be maintained at a constant level in *T. spiralis* muscle larvae, adult forms and newborn larvae (Fig. 1a–b). In contrast, *T. spiralis* histone H3-like mRNA level, also relative to 18S rRNA, was different at those three developmental stages (Fig. 1a and c), with the highest level observed in newborn larvae and the lowest in non-growing muscle larvae.

Expression of Thymidylate Synthase mRNA and 18S rRNA in Adult Worms, Embryos and L1, L3 and Dauer Larvae of C. elegans

The results of Northern blot analyses of *C. elegans* total RNA are presented in Fig. 1d–e. It should be noted that previously obtained 612 bp long *T. spiralis*

rDNA fragment (Dąbrowska et al. 2004; cf. GeneBank Accession no. AY497012) was used as a reference probe, as the corresponding *C. elegans* rDNA sequence (GeneBank Accession no. AY268117) is over 80% identical. Thymidylate synthase mRNA, relative to 18S rRNA, level was found to be constant in *C. elegans* adult worms, embryos and L1 and L3 larvae, only several-fold higher than that in dauer larvae. On the other hand, thymidylate synthase mRNA level in dauer larvae was not lowered when the larvae remained up to 6 weeks in the culture (Fig. 1d–e).

Specificity of Anti-thymidylate Synthase Monoclonal Antibodies Binding on Western blots

The monoclonal antibodies, generated with the rat hepatoma enzyme protein and used in immunoblot analysis, reacted not only with blotted highly purified preparations of rat thymidylate synthase, both endogenous (Fig. 2, lane 4) and recombinant (Fig. 2, lane 5), but also showed a strong cross-specificity towards *T. spiralis* endogenous thymidylate synthase purified from muscle larvae (Fig. 2, lane 3), allowing to detect the enzyme protein in the parasite muscle larvae crude extracts (Fig. 2, lane 2) and pointing to a possibility of using the antibodies to determine the distribution of thymidylate synthase epitopes in the parasite tissues. Interestingly, only a feeble cross-specificity was apparent when purified recombinant (expressed in bacterial cells) *T. spiralis* thymidylate synthase (Fig. 2, lane 2) and mouse tumour cells crude extract (Fig. 2, lane 6) were studied.

Specificity of Anti-thymidylate Synthase Monoclonal Antibodies Binding In Situ

In order to test whether the immunofluorescent signal observed in confocal microscopy reflects monoclonal antibody specific binding to thymidylate synthase epitopes, control reactions were done, including (i) incubation of the primary antibody with an excess of purified recombinant rat hepatoma thymidylate synthase protein preceding incubation of worms with this antibody, (ii) omission of the primary antibody treatment of worms (to test possible non-specific binding of the secondary antibody), (iii) treatment of worms with serum of *T. spiralis*-infected mice (containing antibodies specific to cuticular antigens), in place of the primary antibody (a positive activity control of the secondary antibody). The results confirmed the immunostaining to be thymidylate synthase protein-specific.

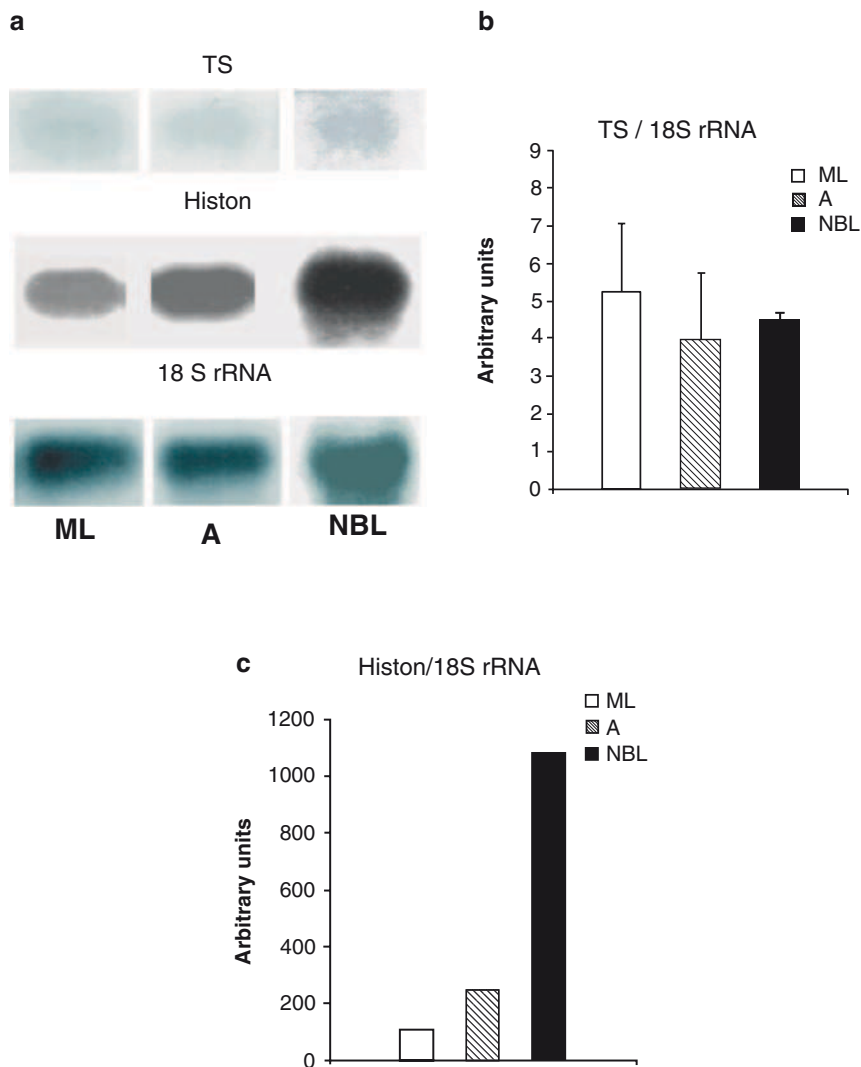


Fig. 1 (a) Thymidylate synthase and histone H.3-like mRNA, and 18S rRNA, bands in *T. spiralis* muscle larvae (ML), adult forms (A) and newborn larvae (NBL). (b) Thymidylate synthase mRNA level in *T. spiralis* muscle larvae (ML), adult forms (A) and newborn larvae (NBL) related to 18S rRNA level. (c) Histone H.3-like mRNA level in *T. spiralis* muscle larvae (ML), adult forms (A) and newborn larvae (NBL) related to 18S rRNA level. Densitometric results in B and C are presented as mean result of three separate experiments $\pm$ SD for ML and (A), and as mean result of two separate experiments for NBL $\pm$ % difference from each of the two results.

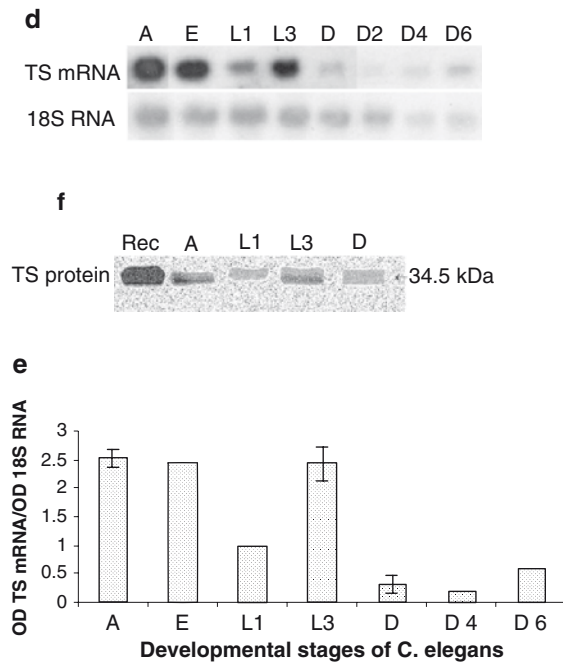


Fig. 1 (continued) (d) Thymidylate synthase (TS) mRNA and 18S rRNA bands in *C. elegans* adult forms (A), embryos (E), L1, L3 and dauer larvae (L1, L3 and D, respectively), and dauer larvae isolated from cultures prolonged up to 4 and 6 weeks at 25°C (D4 and D6, respectively). (e) Thymidylate synthase mRNA level in *C. elegans* adult forms (A), embryos (E), L1, L3 and dauer larvae (L1, L3 and D, respectively), and dauer larvae isolated from cultures prolonged up to 4 and 6 weeks at 25°C (D4 and D6, respectively), related to 18S rRNA level. Densitometric results are expressed as arbitrary units. Results for A, L3 and D are presented as mean result of N separate experiments $\pm$ SD (N = 3 for A and L3, and N = 4 for D) and those for E and L1 as mean result of two separate experiments, differing from each of the two results by no more than 2% (E) or 13% (L1). N = 1 for D4 and D6. (f) Thymidylate synthase protein level in *C. elegans* adult forms (A), and L1, L3 and dauer larvae (L1, L3 and D, respectively). Immunoblot analysis with purified *C. elegans* recombinant thymidylate synthase preparation (Rec) used as a positive control. After PAGE/SDS (1 μ g of RecTS or 50 μ g of crude extract protein per lane), proteins were transferred into PVDF membrane, thymidylate synthase was detected by treatment with polyclonal antibodies raised against the recombinant enzyme. Anti-rabbit IgG coupled with HRP (Sigma) were used as secondary antibodies and color was developed with the DAB/metal enhancer reagent (Bio-Rad). Density of bands were measured using Scion Image program. The study was repeated twice with virtually identical results

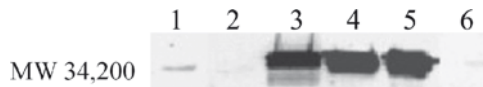


Fig. 2 Immunoblot analysis of cross-reactivity of monoclonal antibody generated against rat hepatoma thymidylate synthase. *T. spiralis* muscle larvae crude extract (lane 1; 100 µg protein), purified recombinant (lane 2; 1 µg protein) or purified muscle larvae endogenous (lane 3; 1 µg protein) thymidylate synthase, rat purified regenerating liver endogenous (lane 4; 1 µg protein) or rat recombinant (lane 5; 1 µg protein) thymidylate synthase and L5178Y crude extract (lane 6; 100 µg protein) were separated by SDS polyacrylamide (12%) gel electrophoresis and proteins were transferred into PVDF membrane. Thymidylate synthase was detected by treatment with monoclonal antibody and goat anti-mouse IgG (H + L) – HRP conjugate (BioRad) as a secondary antibody

Localization of Thymidylate Synthase Protein in T. spiralis Developmental Forms

Localization of thymidylate synthase protein was followed in selected *T. spiralis* developmental forms, including (i) juvenile muscle larvae, (ii) infective muscle larvae (isolated either alone or in the intact nurse cell complex), (iii) adult worms and (iv) embryos developing within adult worms.

In infective muscle larvae of both sexes, whether isolated alone or as an intact nurse cell-larva complex, strong fluorescence signals were observed. They were associated mainly with cells of gonad and uterus primordia, and stichocyte. Strong positive reactions were also observed in the stichocyte nuclei vicinity, as well as in the canalicular tree of stichocytes and in lumen of esophagus of adult worms (not shown).

In silico analysis of magnified pictures of gonad and uterus primordia of infective muscle larvae revealed a positive immunostaining mainly in the cytoplasmic region. A similar pattern of immunofluorescence staining was apparent in gonad tissues, egg cells and developing embryos. In contrast, in stichosomes of juvenile larvae at 13 days postinfection, as well as those of infective muscle larvae and adult worms, the presence of thymidylate synthase protein was indicated mainly around nuclei. The immunofluorescence patterns were similar in both egg cells and semen (in semen receptacle) seen in female adult worms, with semen nuclei region being less intensely stained (not shown).

Discussion

Persistent expression of the three enzyme activities involved in thymidylate de novo biosynthesis in muscle larvae (≥ 1 month after infection) of both *Trichinella* species is surprising. In view of the identical result obtained with the larvae of *T. pseudospiralis* isolated without the use of pepsin, a possibility that muscle larvae, exposed to

pepsin-HCl, may have developed metabolic and enzyme expression patterns characteristic for adults (cf. Stewart 1983; Dąbrowska et al. 1996) appears to be negligible. High level of the enzyme proteins was additionally documented by successful attempts to purify and study properties of two of the *T. spiralis* muscle larvae enzymes, including thymidylate synthase (Dąbrowska et al. 1996) and dihydrofolate reductase. The latter, partially purified (up to specific activity of 0.7 $\mu\text{mol}/\text{min}/\text{mg}$ protein at 37°C) with the use of affinity chromatography on immobilized MTX (Kaufman 1974), showed the native molecular weight of 21,600, K_m values 18.9 μM and 2.1 μM for NADPH and dihydrofolate, respectively, and was inhibited by both urea and guanidine hydrochloride (Szczygłowska A and Rode W, 2000; unpublished).

Of particular interest is the presence in developmentally arrested muscle larvae of high activities of enzymes known to be associated with cell proliferation, i.e. thymidylate synthase (Friedkin 1973; Santi and Danenberg 1984) and dUTPase (Hokari et al. 1987; Vilpo 1983; also citations therein), as well as ribonucleotide reductase (Engstrom et al. 1985; Björklund et al. 1990) and DNA polymerase (Wahl et al. 1988; Wong et al. 1988). Neither proliferation nor DNA biosynthesis may be expected to occur, at least not to any significant extent, at that developmental stage, considering the reported substantial decrease of thymidine label incorporation into larval DNA of *T. spiralis* 38 days after infection (Stewart and Read 1973). In this context, high thymidylate synthase levels have been demonstrated to be associated not only with proliferation (Friedkin 1973; Santi and Danenberg 1984) but also with DNA replication alone, observed during polyploidization in silk gland cells of the silkworm, *Bombyx mori* (Rode and Szymanowska 1976) or with a state of readiness for DNA replication, as in unfertilized eggs (Carpenter 1973; Rode and Szymanowska 1976; Yasumasu et al. 1979). Recently, animal oocytes of many species were shown to undergo cell cycle arrest, usually in the first meiotic prophase, before fertilization (Sagata 1996). Hence, high thymidylate synthase levels observed in unfertilized eggs may be connected with oocytes arrested in the cell cycle, as induction of this enzyme in cells was shown to be due to leaving quiescence (cell cycle G_0 phase) (Pestalozzi et al. 1995). The latter assumption leads to a hypothetical explanation of high levels of thymidylate synthase, and other enzymes involved in dTMP biosynthesis, observed in *Trichinella* muscle larvae as being connected with their cells being arrested in the cell cycle.

The latter prompted us to further investigate the developmental pattern of *T. spiralis* thymidylate synthase gene expression by studying the corresponding mRNA levels. In parallel *T. spiralis* histone H.3-like mRNA levels were determined and all results related to 18S rRNA expression levels, used as a reference. No differences between thymidylate synthase mRNA levels, related to 18S rRNA, were found in the muscle larvae, adults and newborn larvae. On the other hand, *T. spiralis* histone H3-like mRNA levels, studied in the same way, were different at these three developmental stages, with the highest and lowest levels found in fast growing newborn larvae and non-developing muscle larvae, respectively. Thus the two genes appear to be differently expressed, with thymidylate synthase mRNA level being constant, independent of the occurrence of proliferation (adults with female worms filled with proliferating embryos versus newborns and non-proliferating

newborns and muscle larvae) or development (quickly growing newborns versus non-growing and non-developing muscle larvae).

The steady-state level of thymidylate synthase mRNA, very low in quiescent (G_0) mammalian cell, is known to increase 10–20-fold as such cells progress to the S phase (Jen et al. 1985; Ayusawa et al. 1986; Griboudo et al. 2002) and decrease again with differentiation (Horie et al. 1992). When compared in a variety of cells and tissues, the amount of thymidylate synthase mRNA varies, reflecting differences in cell proliferation rates (Lee et al. 1999). The foregoing, persistent expression of thymidylate synthase mRNA at a constant level in all *T. spiralis* developmental forms studied, including developmentally arrested muscle larvae, points to an unusual pattern of cell cycle regulation and is in agreement with our previous hypothesis assuming muscle larvae cells to be arrested in the cell cycle. In contrast, low histone H3-like expression in muscle larvae, relative to newborn larvae and adults, could be expected, in view of previously described downregulation of *Caenorhabditis elegans* histone gene family in developmentally arrested dauer larvae relative to actively growing stages (Dalley and Golomb 1992). Interestingly, a gene homologous to histone H3 has been found to be heat-induced in *T. spiralis* muscle larvae (Mak et al. 2001; Niak et al. 2001).

Furthermore, the presence of high levels of thymidylate synthase protein in different developmental forms has been confirmed by immunofluorescent localization and confocal microscopy. Considering the cell cycle arrest hypothesis, of particular interest are the results concerning the developmentally arrested infective-stage larvae, studied not only following isolation, but also in an intact larva-nurse cell complex, localizing high levels of the enzyme mainly in cells of female and male gonad primordium, and in the vicinity of the stichocyte nuclei.

All enzyme activities studied were present at high levels throughout the life cycle of *C. elegans* (Table 1). This includes thymidine kinase, which could not be detected in muscle larvae of *Trichinella spiralis* and *T. pseudospiralis* (Results), as well as in certain other parasitic nematodes (Farland and MacInnis 1978; So et al. 1992), but was detected in *Brugia pahangi* and *Dirofilaria immitis* (Jaffe et al. 1982). Expression of high activity of this enzyme in dauer larvae, in relation to other developmental stages, provides further support for the cell cycle arrest hypothesis, as thymidine kinase activity is known to be dramatically lower in G_0 than in cycling cells (Pardee 1989). Moreover, the three remaining enzyme activities studied were also high in developmentally arrested dauer larvae, in spite of the fact that the three enzymes, similar to thymidine kinase, are known to be associated with cell proliferation or cell cycling (Santi and Danenberg 1984; Hokari et al. 1987; Vilpo 1983; Békési et al. 2004; Kit 1976; Blakley 1984; Johnson et al. 1978; Pestalozzi et al. 1995) and no cell divisions occur in metabolically depressed dauer larvae (Euling and Ambros 1996; Riddle 1988; Wadsworth and Riddle 1988). It is noteworthy that dauer larva specific activities of thymidylate synthase and dUTPase are comparable to those (0.1 nmol/min/mg protein and 0.7 nmol/min/mg protein, respectively) found in regenerating rat liver extracts (Cieśła et al. 1995b; Gołos and Rode 1999).

The pattern of thymidylate synthase mRNA level in *C. elegans* development is rather poorly correlated with proliferation, as dauer larvae, lacking cell divisions

(Euling and Ambros 1996; Riddle 1988; Wadsworth and Riddle 1988), show considerable level of this mRNA (Fig. 1a and b). In accord with thymidylate synthase specific activity, the enzyme mRNA level persists in dauer larvae for at least 6 weeks (Table 1; cf. Fig. 1). Additionally, comparison of the enzyme activity, enzyme protein and mRNA levels in adult worms, and L1, L3 and dauer larvae, shows only the enzyme protein and mRNA patterns (Table 1, Fig. 1, Results) to be correlated. Interestingly, the enzyme activity pattern parallels the two former patterns only with L1, L3 and dauer larvae, with the enzyme activity level in adult forms being low, relative to both mRNA and enzyme protein levels. The latter points to some regulation of enzyme protein expression and activity during the development. Of note is that *T. spiralis* thymidylate synthase mRNA levels showed no differences between adults, newborns and muscle larvae, with the enzyme specific activities in adults and muscle larvae also being comparable (Table 1, Fig. 1).

Expression patterns of the three enzymes, thymidylate synthase, dihydrofolate reductase and dUTPase, indicate the unusual cell cycle regulation pattern to be shared by developmentally arrested larvae of *C. elegans* and the two *Trichinella* species (Table 1, Fig. 1). Thus, the cell cycle arrest hypothesis concerning *Trichinella* muscle larva is supported, as with *C. elegans* dauer larva, a life cycle stage corresponding to *T. spiralis* muscle larva (Bürglin et al. 1998), a state of global cell cycle arrest has been hypothesized also based on other observations (Euling and Ambros 1996; Hong et al. 1998). However, the apparent global cell cycle arrest in muscle larvae, reflected by high expression of the enzymes involved in thymidylate biosynthesis, does not seem to be specific for *Trichinella* parasites, as in view of the present results it may be characteristic also for developmentally arrested forms of other species of nematodes.

In accord with the foregoing, *T. spiralis* muscle larvae (*T. pseudospiralis* larvae were not tested) contained also high levels, comparable with those found in mouse leukemia L1210 cells, of DNA polymerase α , a key enzyme of the eukaryotic replication complex (Hübscher et al. 2002), and ribonucleotide reductase, a key regulatory enzyme responsible for the reduction of the four ribonucleotides to deoxyribonucleotides (Jordan and Reichard 1998), as the corresponding genes (both suggested to be expressed in nematodes (Boxem et al. 1999; Encalada et al. 2000) are also known to be regulated in association with cell proliferation (Wahl et al. 1988; Wong et al. 1988) and cell cycle (Wahl et al. 1988; Takemura et al. 2001; Engstrom et al. 1985; Björklund et al. 1990). It should be mentioned that genes coding DNA polymerase α and ribonucleotide reductase, similar to several other genes coding enzymes of DNA biosynthesis, including thymidylate synthase, dihydrofolate reductase and thymidine kinase, undergo in mammalian cells regulation by E2F-1 transcription factor (DeGregori et al. 1995; Helin 1998), its reversible binding to the retinoblastoma protein (Rb) playing a key role in the cell cycle G1/S transition (Wu et al. 1996; Cam and Dynlacht 2003; Helin 1998). An orthologous regulatory system was found to be functional in *C. elegans* (Ouellet and Roy 2007; Schertel and Conradt 2007).

Notably, the possibility of global cell cycle arrest in non-developing *T. spiralis* muscle larvae poses a question of the relation of this unusual cell cycle regulation to changes induced by the larvae in muscle cells. Those changes, involving cell cycle

re-entry and induction of DNA synthesis, followed by cell cycle arrest, believed to be G2/M arrest (Jasmer 1995) but recently suggested to be of senescence-associated G1- like type (Dąbrowska et al. 2008), point to muscle larvae as a source of cell cycle-specific signals. In this respect, of interest is immunolocalization of thymidylate synthase around stichocyte nuclei, as well as in extracellular region of stichocyte, in canalicular ducts and lumen of adult female esophagus, pointing to a possibility that the enzyme protein might be secreted with the excretory-secretory granules (cf. Nagano et al. 2009), known to be produced within stichosome and secreted (Despommier and Müller 1976), their contents detected in the cytosol and nuclei of infected muscle cells (Despommier et al. 1990; Lee et al. 1991) and found also to be secreted into epithelial cells (Capo et al. 1986). Although larval excretory-secretory proteins are implicated in the formation/maintenance of the nurse cell (Leung and Ko 1997), only a few have been identified (Robinson and Connolly 2005; Robinson et al. 2007) and the regulatory mechanisms responsible for the reorganisation of hosts skeletal muscle are currently unknown. A possibility of thymidylate synthase playing a non-catalytic role in *T. spiralis* and/or *C. elegans* appears attractive, as high levels of the enzyme shown by developmentally arrested forms both cics, muscle and dauer larvae, appear to be in contradiction to the enzyme's catalytic activity seeming to be irrelevant (in the apparent absence of DNA biosynthesis in those larvae, dTMP may well not to be synthesized, as both events appear to be coupled, at least in mammalian cell (cf. Rode et al. 1980). Moreover, possible non-catalytic functions of the enzyme protein in translational regulation (Liu et al. 2002) and neoplastic transformation (Rahman et al. 2004) of mammalian cells have been suggested. Another interesting aspect of a hypothetical regulatory (in addition to the catalytic) function of thymidylate synthase protein in *T. spiralis* muscle larva development would be its potential chemotherapeutic exploitation.

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References

- Ayusawa D, Shimizu K, Koyama H, Kaneda S, Takeishi K, Seno T (1986) Cell-cycle-directed regulation of thymidylate synthase messenger RNA in human diploid fibroblasts stimulated to proliferate. *J Mol Biol* 190:559–567
- Békési A, Zagyva I, Hunyadi-Gulyás E, Pongrácz V, Kovári J, Nagy ÁO, Erdei A, Medzihradský KF, Vértessy BG (2004) Developmental regulation of dUTPase in *Drosophila melanogaster*. *J Biol Chem* 279:22362–22370
- Bürglin TR, Labos E, Blaxter ML (1998) *Caenorhabditis elegans* as a model for parasitic nematodes. *Int J Parasitol* 28:395–411
- Björklund S, Skog S, Tribukait B, Thelander L (1990) S-phase-specific expression of mammalian ribonucleotide reductase R1 and R2 subunit mRNAs. *Biochemistry* 29:5452–5458
- Blakley RL (1984) Dihydrofolate reductase. In: Blakley RL, Benkovic SJ (eds) *Folates and Pterines*, vol 1. Wiley, New York, pp 191–253
- Bollum FJ (1959) Mammalian enzymes of desoxyribonucleic acid synthesis. *Ann N Y Acad Sci* 81:792–793

- Boxem M, Srinivasan DG, van den Heuvel S (1999) The *Caenorhabditis elegans* gene *ncc-1* encodes a cdc2-related kinase required for M phase in meiotic and mitotic cell divisions, but not for S phase. *Development* 126:2227–2239
- Brenner S (1974) The genetics of *Caenorhabditis elegans*. *Genetics* 77:71–94
- Cabaj W (1986) The response of NIH mice to *Trichinella pseudospiralis*. *Acta Parasitol Pol* 31:77–85
- Cam H, Dynlacht BD (2003) Emerging roles for E2F: G1/S transition and DNA replication. *Cancer Cell* 3:311–316
- Capo V, Silberstein D, Despommier DD (1986) Immunocytolocalization of two protection-inducing antigens of *Trichinella spiralis* during its enteral phase in immune and non-immune mice. *J Parasitol* 72:931–938
- Carpenter NJ (1973) Thymidylate synthetase in mutants of *Drosophila melanogaster*. *Genetics* 75:113–122
- Carreras CW, Santi DV (1995) The catalytic mechanism and structure of thymidylate synthase. *Annu Rev Biochem* 64:721–762
- Cieśla J, Weiner KXB, Weiner RS, Reston JT, Maley GF, Maley F (1995a) Isolation and expression of rat thymidylate synthase cDNA: phylogenetic comparison with human and mouse thymidylate synthases. *Biochim Biophys Acta* 1261:233–242
- Cieśla J, Gołos B, Dzik JM, Pawełczak K, Kempny M, Makowski M, Bretner M, Kulikowski T, Machnicka B, Rzeszutarska B, Rode W (1995b) Thymidylate synthases from *Hymenolepis diminuta* and regenerating rat liver: purification, properties, and inhibition by substrate and cofactor analogues. *Biochim Biophys Acta* 1249:127–136
- Dalley BK, Golomb M (1992) Gene expression in the *Caenorhabditis elegans* dauer larva: developmental regulation of Hsp90 and other genes. *Dev Biol* 151:80–90
- Dąbrowska M, Zieliński Z, Wranicz M, Michalski R, Pawełczak K, Rode W (1996) *Trichinella spiralis* thymidylate synthase: developmental pattern, isolation, molecular properties and inhibition by substrate and cofactor analogues. *Biochem Biophys Res Commun* 228:440–445
- Dąbrowska M, Jagielska E, Cieśla J, Płucienniczak A, Kwiatowski J, Wranicz M, Boireau P, Rode W (2004) *Trichinella spiralis* thymidylate synthase: cDNA cloning and sequencing, and developmental pattern of mRNA expression. *Parasitology* 128:209–221
- Dąbrowska M, Skoneczny M, Zieliński Z, Rode W (2008) Nurse cell of *Trichinella spp.* as a model of long-term cell cycle arrest. *Cell Cycle* 7:2167–2178
- DeGregori J, Kowalik T, Nevins JR (1995) Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G₁/S-regulatory genes. *Mol Cell Biol* 15:4215–4224
- Despommier DD (1993) *Trichinella spiralis* and the concept of niche. *J Parasitol* 79:472–482
- Despommier DD (1998) How does *Trichinella spiralis* make itself at home? *Parasitol Today* 14:318–323
- Despommier DD, Müller M (1976) The stichosome and its secretion granules in the mature muscle larva of *Trichinella spiralis*. *J Parasitol* 62:775–785
- Despommier DD, Gold AM, Buck SW, Capo V, Silberstein D (1990) *Trichinella spiralis* secreted antigen of the infective L1 larva localizes to the cytoplasm and nucleoplasm of infected host cells. *Exp Parasitol* 71:27–38
- Encalada SE, Martin PR, Phillips JB, Lyczak R, Hamill DR, Swan KA, Bowerman B (2000) DNA replication defects delay cell division and disrupt cell polarity in early *Caenorhabditis elegans* embryos. *Dev Biol* 228:225–238
- Engstrom Y, Eriksson S, Jildevik I, Skog S, Thelander L, Tribukait B (1985) Cell cycle-dependent expression of mammalian ribonucleotide reductase. Differential regulation of the two subunits. *J Biol Chem* 260:9114–9116
- Euling S, Ambros V (1996) Reversal of cell fate determination in *Caenorhabditis elegans* vulval development. *Development* 122:2507–2515
- Farland WH, MacInnis AJ (1978) *In vitro* thymidine kinase activity: present in *Hymenolepis diminuta* (Cestoda) and *Moniliformis dubius* (Acanthocephala) but apparently lacking in *Ascaris lumbricoides* (Nematoda). *J Parasitol* 64:564–565

- Friedkin M (1973) Thymidylate synthetase. *Adv Enzymol* 38:235–292
- Garkavi BL (1972) Species of *Trichinella* isolated from wild animals. *Veterinariya* 10:90–91
- Georgopapadakou NH, Walsh TJ (1996) Antifungal agents: chemotherapeutic targets and immunologic strategies. *Antimicrob Agents Chemother* 40:279–291
- Gółoś B, Rode W (1999) An isotopic assay of dUTPase activity based on coupling with thymidylate synthase. *Acta Biochim Pol* 46:837–840
- Gould SE (1970) Clinical pathology: diagnostic laboratory procedures. In: Gould SE (ed) *Trichinosis in man and animals*. Charles C. Thomas, Springfield, pp 190–221
- Gribaudo G, Riera L, Rudge TL, Caposio P, Johnson LF, Landolfo S (2002) Human cytomegalovirus infection induces cellular thymidylate synthase gene expression in quiescent fibroblasts. *J Gen Virol* 83:2983–2993
- Heidelberger C, Danenberg PV, Moran RG (1983) Fluorinated pyrimidines and their nucleosides. *Adv Enzymol* 54:57–119
- Helin K (1998) Regulation of cell proliferation by the E2F transcription factors. *Curr Opin Genet Dev* 8:28–35
- Hokari S, Hasegawa M, Sakagishi Y, Kikuchi G (1987) Deoxyuridine triphosphate nucleotidohydrolase activity and its correlation with multiplication of erythroid cells in rat spleen. *Biochem Int* 14:851–857
- Hong Y, Roy R, Ambros V (1998) Developmental regulation of a cyclin-dependent kinase inhibitor controls postembryonic cell cycle progression in *Caenorhabditis elegans*. *Development* 125:3585–3597
- Horie N, Nozawa R, Takeishi K (1992) Identification of cellular differentiation-dependent nuclear factors that bind to a human gene for thymidylate synthase. *Biochem Biophys Res Commun* 185:127–133
- Hübscher U, Maga G, Spadari S (2002) Eukaryotic DNA polymerases. *Annu Rev Biochem* 71:133–163
- Jaffe JJ, Comley JC, Chrin LR (1982) Thymidine kinase activity and thymidine salvage in adult *Brugia pahangi* and *Dirofilaria immitis*. *Mol Biochem Parasitol* 5:361–370
- Johnson LF, Fuhrman CL, Wiedemann LM (1978) Regulation of dihydrofolate reductase gene expression in mouse fibroblasts during the transition from the resting to growing state. *J Cell Physiol* 97:397–406
- Kaiserman HB, Benbow RM (1987) Characterization of a stable major DNA polymerase alpha species devoid of DNA primase activity. *Nucleic Acids Res* 15:10249–10265
- Kaufman BT (1974) Methotrexate-agarose in the purification of dihydrofolate reductase. *Methods Enzymol* 34:272–281
- Kit S (1976) DNA synthesis and cancer. *Mol Cell Biochem* 11:161–182
- Lee DL, Ko RC, Yi XY, Yeung MH (1991) *Trichinella spiralis*: antigenic epitopes from the stichocytes detected in the hypertrophic nuclei and cytoplasm of the parasitized muscle fibre (nurse cell) of the host. *Parasitology* 102:117–123
- Lewis JA, Fleming JT (1995) Basic culture methods. *Methods Cell Biol* 48:3–29
- Liu J, Schmitz JC, Lin X, Tai N, Yan W, Farrell M, Bailly M, Chen T, Chu E (2002) Thymidylate synthase as a translational regulator of cellular gene expression. *Biochim Biophys Acta* 1587:174–182
- Jasmer DP (1995) *Trichinella spiralis*: subversion of differentiated mammalian skeletal muscle cells. *Parasitol Today* 11:185–188
- Jenh C-H, Geyer PK, Johnson LF (1985) Control of thymidylate synthase mRNA content and gene transcription in an overproducing mouse line. *Mol Cell Biol* 5:2527–2532
- Jong AY, Yu K, Zhou B, Frgala T, Reynolds CP, Yen Y (1998) A simple and sensitive ribonucleotide reductase assay. *J Biomed Sci* 5:62–68
- Jongwutiwes S, Chantachum N, Kraivichian P, Siriwasatien P, Putaporntip C, Tamburrini A, La Rosa G, Sreesunparsirikul C, Yingyoud P, Pozio E (1998) First outbreak of human trichinellosis caused by *Trichinella pseudospiralis*. *Clin Infect Dis* 26:111–115
- Jordan A, Reichard P (1998) Ribonucleotide reductases. *Annu Rev Biochem* 67:71–98

- Knudsen KL, Hansen MB, Henriksen LR, Andersen BK, Lihme A (1992) Sulfone-aromatic ligands for thiophilic adsorption chromatography: purification of human and mouse immunoglobulins. *Anal Biochem* 201:170–177
- Ko RC, Fan L, Lee DL, Compton H (1994) Changes in host muscles induced by excretory/secretory products of larval *Trichinella spiralis* and *Trichinella pseudospiralis*. *Parasitology* 108:195–205
- Kuratli S, Lindh JG, Gottstein B, Smith DF, Connolly B (1999) *Trichinella* spp differential expression of two genes in the muscle larva of encapsulating and nonencapsulating species. *Exp Parasitol* 93:153–159
- Lee Y, Shen G, Johnson LF (1999) Complex transcriptional initiation pattern of the thymidylate synthase promoter in mouse tissues. *Archiv Biochem Biophys* 372:389–392
- Leung RK, Ko RC (1997) In vitro effects of *Trichinella spiralis* on muscle cells. *J Helminthol* 71:113–118
- Mak CH, Sun KW, Ko RC (2001) Identification of some heat-induced genes of *Trichinella spiralis*. *Parasitology* 123:293–300
- Mathews CK, Scrimgeour KG, Huennekens FM (1963) Dihydrofolate Reductase. *Methods Enzymol* 6:364–368
- Miller DM, Shakes DC (1995) Immunofluorescence microscopy. *Methods Cell Biol* 48:365–394
- Nagano I, Wu Z, Takahashi Y (2009) Functional genes and proteins of *Trichinella* spp. *Parasitol Res* 104:197–207
- Niak CH, Su KW, Ko RC (2001) Identification of some heat-induced genes of *Trichinella spiralis*. *Parasitology* 123:293–300
- Ouellet J, Roy R (2007) The *lin-35/Rb* and RNAi pathways cooperate to regulate a key cell cycle transition in *C. elegans*. *BMC Dev Biol* 7:38
- Pardee AB (1989) G_1 events and regulation of cell proliferation. *Science* 246:603–608
- Pestalozzi BC, McGinn CJ, Kinsella TJ, Drake JC, Glennon MC, Allegra CJ, Johnston PG (1995) Increased thymidylate synthase protein levels are principally associated with proliferation but not cell cycle phase in asynchronous human cancer cells. *Br J Cancer* 71:1151–1157
- Rahman L, Voeller D, Rahman M, Lipkowitz S, Allegra C, Barrett JC, Kaye FJ, Zajac-Kaye M (2004) Thymidylate synthase as an oncogene: A novel role for an essential DNA synthesis enzyme. *Cancer Cell* 5:341–351
- Rathod PK (1997) Antimalarial agents directed at thymidylate synthase. *J Pharm Pharmacol* 49(Suppl 2):704–711
- Riddle DL (1988) The dauer larva. In: Wood WB (ed) *The nematode Caenorhabditis elegans*. Cold Spring Harbor Laboratory Press, New York, pp 393–412
- Riddle DL, Albert PS (1997) Genetic and environmental regulation of dauer larva development. In: Riddle DL, Blumenthal T, Meyer BJ, Priess JR (eds) *C. elegans* II. Cold Spring Harbor Laboratory Press, New York, pp 739–768
- Robinson MW, Connolly B (2005) Proteomic analysis of the excretory–secretory proteins of the *Trichinella spiralis* L1 larva, a nematode parasite of skeletal muscle. *Proteomics* 5:4525–4532
- Robinson MW, Greig R, Beattie KA, Lamont DJ, Connolly B (2007) Comparative analysis of the excretory–secretory proteome of the muscle larva of *Trichinella pseudospiralis* and *Trichinella spiralis*. *Int J Parasitol* 37:139–148
- Rode W, Scanlon KJ, Moroson BA, Bertino JR (1980) Regulation of thymidylate synthetase in mouse leukemia cells (L1210). *J Biol Chem* 255:1305–1311
- Rode W, Szymanowska H (1976) Developmental pattern of thymidylate synthetase activity in silkworm: *Bombyx mori* L. *Insect Biochem* 6:333–337
- Rode W, Zieliński Z, Dzik JM, Kulikowski T, Bretner M, Kierdaszuk B, Cieśla J, Shugar D (1990) Mechanism of inhibition of mammalian tumor and other thymidylate synthases by N^4 -hydroxy-dCMP, N^4 -hydroxy-5-fluoro-dCMP, and related analogues. *Biochemistry* 29:10835–10842
- Rosowsky A (1992) Development of new antifolate analogs as anticancer agents. *Am J Pharm Edu* 56:453–463
- Sagata N (1996) Meiotic mataphase arrest in animal oocytes: its mechanisms and biological significance. *Trends Cell Biol* 6:22–28

- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, New York, pp 1.25–1.31
- Santi DV, Danenberg PV (1984) Folates in pyrimidine biosynthesis. In: Blakley RL, Benkovic SJ (eds) Folates and pterines, vol 1. Wiley, New York, pp 345–398
- Schertel C, Conradt B (2007) *C. elegans* orthologs of components of the RB tumor suppressor complex have distinct pro-apoptotic functions. *Development* 134:3691–3701
- Schweitzer BI, Dicker AP, Bertino JR (1990) Dihydrofolate reductase as a chemotherapeutic target. *FASEB J* 4:2441–2452
- So NNC, Wong PCL, Ko RC (1992) Precursors of pyrimidine nucleotide biosynthesis for gravid *Angiostrongylus cantonensis* (Nematoda: Metastrongyloidea). *Int J Parasitol* 22:427–433
- Spector T (1978) Refinement of the coomassie blue method of protein quantitation. *Anal Biochem* 86:142–146
- Stewart GL (1983) Biochemistry. In: Campbell WC (ed) *Trichinella and Trichinosis*. Plenum Press, New York, pp 153–172
- Stewart GL, Read CP (1973) Deoxyribonucleic acid metabolism in mouse trichinosis. *J Parasitology* 59:264–267
- Takada N, Tada T (1988) Collection of newborn larvae of *Trichinella spiralis* in vitro. *Jap J Parasitol* 37:251–253
- Takemura Y, Jackman AL (1997) Folate-based thymidylate synthase inhibitors in cancer chemotherapy. *Anti-Cancer Drugs* 8:3–16
- Takemura M, Yamamoto T, Kitagawa M, Taya Y, Akiyama T, Asahara H, Linn S, Suzuki S, Tamai K, Yoshida S (2001) Stimulation of DNA polymerase α activity by Cdk2-phosphorylated Rb protein. *Biochem Biophys Res Commun* 282:984–990
- Tsukamoto I, Taniguchi Y, Miyoshi M, Kojo S (1991) Purification and characterization of thymidine kinase from regenerating rat liver. *Biochim Biophys Acta* 1079:348–352
- Vilpo JA (1983) Mitogen induction of deoxyuridine triphosphatase activity in human T and B lymphocytes. *Med Biol* 61:54–58
- Wadsworth WG, Riddle DL (1988) Acidic intracellular pH shift during *Caenorhabditis elegans* larval development. *Proc Natl Acad Sci U S A* 85:8435–8438
- Wahl AF, Geis AM, Spain BH, Wong SW, Korn D, Wang TS-F (1988) Gene expression of human DNA polymerase α during cell proliferation and the cell cycle. *Mol Cell Biol* 8:5016–5025
- Wińska P, Gołos B, Cieśla J, Zieliński Z, Frączyk T, Wałajtyś-Rode E, Rode W (2005) Developmental arrest in *C. elegans* dauer larvae leaves high expression of enzymes involved in thymidylate biosynthesis, similar to that found in *Trichinella* muscle larvae. *Parasitology* 131:247–254
- Wong SW, Wahl AF, Yuan P-M, Arai N, Pearson BE, Arai K-I, Korn D, Hunkapiller MW, Wang TS-F (1988) Human DNA polymerase α gene expression is cell proliferation dependent and its primary structure is similar to both prokaryotic and eukaryotic replicative DNA polymerases. *EMBO J* 7:37–47
- Wranicz MJ, Gustowska L, Gabryel P, Kucharska E, Cabaj W (1998) *Trichinella spiralis*: induction of the basophilic transformation of muscle cells by synchronous newborn larvae. *Parasitol Res* 84:403–407
- Wu CL, Classon M, Dyson N, Harlow E (1996) Expression of dominant-negative mutant DP-1 blocks cell cycle progression in G1. *Mol Cell Biol* 16:3698–3706
- Yasumasu I, Saitoh M, Fujimoto N, Kusunoki S (1979) Changes in activities of thymidylate synthetase and dihydrofolate reductase in sea urchin eggs after fertilization. *Dev Growth Differ* 21:237–243
- Young DW (1994) Tilden lecture: studies on thymidylate synthase and dihydrofolate reductase—two enzymes involved in the synthesis of thymidine. *Chem Soc Rev* 23:119–128
- Zierler MK, Marini NJ, Stowers DJ, Benbow RM (1985) Stockpiling of DNA polymerases during oogenesis and embryogenesis in the frog, *Xenopus laevis*. *J Biol Chem* 260:974–81

Immunodulation and Helminths: Towards New Strategies for Treatment of Immune-Mediated Diseases?

Fabrizio Bruschi, Lorena Chiumiento, and Gianfranco Del Prete

Abstract Parasitic helminths, and other persistent pathogens are able to produce molecules modulating the host immune response; hookworm for example produce the so-called neutrophil inhibitory factor (NIF), a protein which is the ligand of the integrin CD11b/CD18 present on the surface of neutrophil granulocytes, blocking the adherence of inflammatory cells to the endothelium. The cDNA for this protein derived from *Ancylostoma caninum* has been cloned and used in experimental models of neutrophil-mediated pathology, such as acute brain ischaemia, lung injury or experimental colitis and it was able to reduce tissue damage. For this reason, it has been postulated a possible therapeutic use of the protein in similar human conditions.

Other molecules derived from helminths have been shown to interfere with host immune cells: it is the case of taeniaestatin from *Taenia teniaeformis*, of the 45 kDa glycoprotein from *Trichinella spiralis* or of the antigen B from *Echinococcus granulosus*, just to give few examples.

The fact that most intestinal nematodes elicit a Th2 response in chronic infection has suggested the possibility to use experimental infections with such parasites for the treatment of Th1-mediated diseases, such as the inflammatory bowel diseases. Patients affected with Crohn's disease or ulcerative colitis were treated with the ova of a porcine whipworm *Trichuris suis*, which resulted in clinical amelioration of both diseases.

The persistent Th2 activation occurring in nematode infection might represent an interesting model to evaluate the efficacy of factors able to activate Th1 responses: it is the case of the neutrophil activating protein from *Helicobacter pylori*, a

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toll-like receptor 2 agonist which has been shown to down-regulate eosinophilia and IgE synthesis in an experimental trichinellosis model. These results would suggest the possible use of this protein in the treatment of Th2-mediated disorders, such as allergic diseases.

Keywords Immunomodulation • parasitic helminths • Th1 adjuvants • hygiene hypothesis

Introduction

Helminths colonize over a third of the human population, most of them living in developing countries (Weinstock et al. 2004) (Table 1). These worms are long-lived multicellular organisms, which have evolved various mechanisms to evade the host immune response and to limit parasite-induced pathology (Maizels et al. 2004; Belkaid et al. 2006). Helminths comprise two taxa phylogenetically very distant after the divergence occurred 600 million or more years ago (Knoll and Carroll 1999): the roundworm nematodes and the flatworm platyhelminths in which are grouped trematodes (such as *Schistosoma* spp.) and cestodes (*Taenia* spp., *Echinococcus* spp.). Although this wide divergence, the host immune response to these parasites is quite stereotyped (Maizels et al. 1993) with a strong polarization into a T-helper (Th) type-2 response (Abbas et al. 1996). Immune responses to

Table 1 Major parasitic helminths of humans

	Disease	Numbers (in millions) current estimates
Nemathelminthes		
<i>Ancylostoma duodenale</i>	Anaemia, malnutrition	1,300
<i>Necator americanus</i>		
<i>Ascaris lumbricoides</i>	Gastrointestinal complications	1,500
<i>Trichuris trichiura</i>	Gastrointestinal complications	1,000
<i>Trichinella spiralis</i>		?
<i>Wuchereria bancrofti</i>	Lymphatic filariasis, elephantiasis	100
<i>Brugia malayi</i>		
<i>Onchocerca volvulus</i>	Onchocerciasis (river blindness)	18
Plathelminthes		
Trematodes (flukes)		
<i>Schistosoma mansoni</i>	Schistosomiasis	200
Cestodes (tapeworms)		
<i>Taenia saginata</i>	Taeniosis	?
<i>Taenia solium</i>	Cysticercosis, taeniosis	?
<i>Echinococcus</i> spp.	Hydatidosis, alveolar echinococcosis	?

helminths are intriguing not only from the perspectives of understanding protective immunity and immunopathology, but also because a major branch of the mammalian immune system, the Th2 response, seems to have evolved specifically to deal with this class of pathogens. Th2 immunity involves the rapid activation and engagement of cells of both the innate (eosinophils and basophils) and adaptive (CD4⁺ T cells that commit to the Th2 pathway) immune systems (Voehringer et al. 2004). Th2 responses can prevent the survival of infecting parasites, expel adult parasites from the gut (Finkelman et al. 2004; Voehringer et al. 2006), allow host survival in a setting where the immune response cannot clear the parasites (Herbert et al. 2004), and/or mediate pathological fibrotic responses (Fort et al. 2001). A set of cytokines made by cells of the innate immunity promotes the development of defined CD4⁺ T cell lineages that in productive immune responses are appropriate to the particular situation. It is now clear that in addition to IL-4, IL-13, IL-5, IL-9, and IL-10, Th2 cells can make IL-25 and IL-31, and that these two cytokines can have crucial roles in promoting and/or regulating Th2 responses (Wynn et al. 2004; Dillon et al. 2004). Mice infected with *Brugia malayi* were found to preferentially expand the proportion of CD25⁺Foxp3⁺ cells within the CD4⁺ T cells with a shift of the balance of the host immune system towards the expansion and activation of regulatory T cells (Treg), in a manner dependent on live parasites but independent of a concomitant Th2 response (McSorley et al. 2008).

Recent advances by molecular and immunological helminthologists have indicated that helminths are a rich source of interesting molecules that could lead to innovation for many aspects of biomedicine. Parasites can produce different molecules which interfere with the host immune system at different levels (regulatory and effector responses). Some of them have been well characterised and purified or, in some cases, the corresponding cDNA was cloned.

This review will focus on immunomodulation by infections with nematodes or molecules of nematode origin; however also in other helminthic infections molecules have been identified with the ability to interfere with host immune system (van Riet et al. 2007). See also Table 2.

Nematodes

The major host–parasite interaction in parasitic nematodes is played by different molecules which are released by the parasite in the host tissues. These molecules are called excretory–secretory (ES) products since they are excreted/secreted by particular organelles, typical of nematodes. Among them, several have a potential immunomodulatory effect, for example chemotactic inhibiting factors, such as the 45 kDa glycoprotein purified from *Trichinella spiralis* muscle larvae (Bruschi et al. 2000), the platelet-activating factor hydrolase from *Nippostrongylus brasiliensis* (Blackburn and Selkirk 1992) and the eotaxin-degrading protease derived from hookworm (Culley et al. 2000), the glutathione peroxidase and superoxide dismutase from filarial nematodes (Selkirk et al. 1998), the phosphorylcholine (PC)-

Table 2 Helminth^a products modulating host immune response

Parasite	Molecule	Molecular weight and biochemical characters	Effects	Reference
<i>Echinococcus granulosus</i>	Antigen B	12 kDa protein present in the cystic fluid	Elastase and neutrophil chemotaxis inhibition	Shepherd 1987; Riganò et al. 2001
	EgTeg	12 kDa protein present on the tegument of protoscolex and germinal membrane	Influence maturation of DC toward a Th2 profile Neutrophil chemotaxis inhibition	Riganò et al. 2007 Ortona et al. 2005
<i>Fasciola hepatica</i>	Cathepsin L proteinases (CL1 and CL2), the major components of F. hepatica excretion/secretion products (ES)	Their 15 N-terminal residues were found to be identical to those of earlier described cathepsin L-like enzymes, isolated from the same source, reported as CL1 and CL2. Radioimmunoassay experiments have shown that these CL1- (25 kDa) and CL2-like (26 kDa) cysteine proteinases mediated kinin release from high molecular weight kininogen (HMWK)	Elicit a preferential type 1 response based on IgG2a antibodies	Bentancor et al. 2002
	Cathepsin L proteinase (FheCL), a major component of ES products	The predicted open reading frame encodes a putative protein of 326 aminoacids in length which has homology to members of the thiol cathepsin family of cysteine proteinases	FheCL was capable of suppressing IFN- gamma production during Bordetella pertussis infection. FheCL suppresses sheep T cell proliferation and reduces surface CD4 expression on both human and ovine T cells	O'Neill et al. 2001 Prowse et al. 2002
	Antioxidant thioredoxin peroxidase (TPx)	60-kDa enzyme	Induces the recruitment and alternative activation of macrophages towards a Th2 pattern	Maggioli et al. 2004
	Papain or cathepsin B-like proteolytic enzyme		The secreted enzymes cleave immunoglobulins (Ig) of mouse, rat, rabbit and sheep in vitro	Wilson et al. 1998

<i>Schistosoma mansoni</i>	Generic endoprotease with trypsin-like activity and a metalloaminopeptidase		Proteolytic cleavage of IgG after binding to the Fc receptor of <i>Schistosoma mansoni</i> schistosomula	Auriault et al. 1981
	Lacto- <i>N</i> -fucopentose III (LNFPIII)	C ₃₂ H ₅₅ NO ₂₅ ·H ₂ O polylectosamine sugar, is the predominant carbohydrate found in <i>S. mansoni</i> egg	Inducers of a Th2-like response that is associated with an increased Th2-type cytokine and IgE production, and eosinophilia	Okano et al. 2001
<i>Spirometra mansoni</i>	Cathepsin S-like protease		Proteolytic cleavage of IgG in Fab and Fc fragments	Kong et al. 1994
<i>Taenia crassiceps</i>	p66, an excretory/secretory product of the larva		p66 possesses activities that mimic some characteristics of murine IFN-γ.	Spolski et al. 2002
			p66 was shown as well to upregulate nitric oxide production in macrophages	
<i>Taenia solium</i>	A generic peptidase activity released into the fluid of <i>T. solium</i> metacystode-culture		It can decrease CD4+ expression in cocultures of human lymphocytes	Molinari et al. 2000
	Proteic extracts of <i>T. solium</i>		Immunoglobulin digestion	White et al. 1992
<i>Taenia taeniaeformis</i>	Taeniaestatin	19.5 kDa protein (serine protease inhibitor)	Neutrophil chemotaxis inhibition	Leid et al. 1987

^a Nematodes excluded

containing glycoprotein ES-62 purified from the filarial nematode *Acanthocheilonema viteae* (Harnett and Harnett 1993), inhibitors of antigen processing cells, so called cystatins from *B. malayi* (Manoury et al. 2001), and an adenosine-generating 5' nucleotidase from *T. spiralis* (Gounaris 2002). Homologues of transforming growth factor- β (TGF- β) have been found in the filarial nematodes *B. malayi* and *B. pahangi* (Gomez-Escobar et al. 1998). Orthologues of MIF have been found in *T. spiralis*, *Trichuris trichiura*, as well as in *B. pahangi* (Pennock et al. 1998), and an IFN- γ -like molecule has been suggested (Maizels and Yazdanbakhsh 2003).

Neutrophil Inhibiting Factor (NIF)

The purification and cloning of a ~41 kDa glycoprotein from canine hookworm, *Ancylostoma caninum* has been reported. This protein inhibits the adhesion of activated human neutrophils to vascular endothelial cells and the release of hydrogen peroxide from activated, adherent neutrophils. This molecule, designated neutrophil inhibitory factor (NIF), appears to bind with high affinity to the integrin CD11b/CD18 present on the neutrophil surface (Moyle et al. 1994). Once obtained in recombinant form, this protein was tested in different experimental models of neutrophil-mediated pathology, such as lung pathology (Barnard et al. 1995), transient focal cerebral ischaemia in the rat (Mackay et al. 1996), experimental colitis (Meenan et al. 1996), rat embolic model of stroke (Zhang et al. 2003). In all these studies NIF was proven to reduce tissue pathology.

Trichinella Products

gp45

A 45 kDa. glycoprotein (gp45) was purified by affinity chromatography from ES products of *T. spiralis* muscle larvae; this protein belongs to the TSL-1 family of glycoproteins which are characterised by the glycosilation with the β -arabinohexose (β -tyvelose), a glycan uniquely found in *Trichinella* spp. in β configuration (Wisniewski et al. 1993). Gp45 inhibits human neutrophil chemotaxis, as well as the oxidative metabolism of these cells, and also diminishes the up-regulation of CD11b molecules induced by formyl-methionyl-leucyl-phenylalanine (Bruschi et al. 2000). Gp45 corresponds to the protein obtained by cloning the cDNA of the 43 kDa gene which codes for a protein characterised by a sequence homology with DNase II and containing a helix-loop-helix (HLH) motif (Vassilatis et al. 1992). No information is available about which portion of the protein is responsible for modulating effects.

Migration–Inhibition Factor (MIF)

Orthologues (dopachrome tautomerase) of MIF acting on macrophages were described in *T. spiralis*, and in other nematodes (*B. pahangi*, *Trichuris muris*). MIF is an essential stimulator of T-cell-dependent immunity in mammals, consequently the parasite would use it to subvert the host immune response. Significant dopachrome tautomerase or phenyl-agarose-purifiable MIF-like protein was not detected in the soluble fraction of other nematodes (*Heligmosomoides polygyrus* and *N. brasiliensis*), of cestodes (*Hymenolepis diminuta*), or of trematodes (*S. mansoni*, *S. japonicum* and *S. haematobium*) (Pennock et al. 1998). cDNAs were obtained for MIF/L-dopachrome methyl ester tautomerase homologues from the nematodes *T. spiralis* and *T. trichiura*. The translated sequences showed 42% and 44% identity, respectively, with human or mouse MIF (Tan et al. 2001).

The gene of a homologue of MIF from *T. spiralis* and *T. pseudospiralis* has been expressed in *Escherichia coli* and better characterised, resulting again with a high homology (~40%) with mammalian counterparts. Normal macrophage accumulation around beads transplanted in mice subcutaneously was profoundly inhibited when the beads were pretreated with MIF recombinant protein. The production of this factor by *Trichinella* could explain the lack of macrophages around the encysted muscle larvae (Wu et al. 2003).

Filarial Product ES-62

ES-62 is a glycoprotein containing phosphorylcholine (PC) which was chromatographically purified from ES products of the filarial nematode *Acanthocheilonema viteae* (Harnett et al. 1989), with potent immunomodulatory activity. The activity of ES-62 appears to be due to the PC moiety, covalently bound via an N-linked glycan (Houston et al. 1997). Harnett and Harnett (1993) showed the ability of ES-62 to inhibit B-cell proliferation and to down-regulate protein kinase C, in mice. Furthermore, other effects were observed in vitro, such as energy induction in Jurkat T cells (Harnett et al. 1998), differential modulation of pathways essential in signalling cell proliferation (Deehan et al. 1998) and dendritic cell acquisition of a phenotype driving a Th2 response (Whelan et al. 2000). Also in vivo ES-62 was able to modify IgG subclass production, inducing high levels of IgG1 and poor IgG2a levels specific for non-PC epitopes (Houston et al. 2000).

Another effect of ES-62 was observed in macrophages which, after undergoing suppression of IL-12, IL-6 and TNF- α production, drive to an anti-inflammatory response (Goodridge et al. 2001).

In vivo administration of ES-62 amounts, similar to those encountered during natural infection, provokes B cell hyporesponsiveness in mice (Wilson et al. 2003a), but B1 cells producing IgM specific for non-peptide antigens, such as PC,

were activated (Wilson et al. 2003b). Interestingly, anti-PC IgM were detected in filariasis patients.

In the light of these multiple effects of ES-62, the use of this molecule in *in vivo* model of inflammatory diseases was tempted to verify a possible therapeutic effect. In a murine model of collagen-induced arthritis, this parasite-derived glycoprotein suppressed the pathology either when given during collagen priming or after the onset of the disease (McInnes et al. 2003). ES-62 was also tested in other experimental models of immune-mediated diseases. In a mouse model of allergic ovalbumin-induced airway hypersensitivity, ES-62 reduced peribronchial inflammation, mucosal hyperplasia, and prevented eosinophilia, as well as the release of Th2 cytokines, such as IL-4 (Melendez et al. 2007). ES-62 also reduced the immediate-type hypersensitivity to oxazolone in the skin, possibly due to the ability of this protein to inhibit mast cell degranulation and the Fc ϵ RI-induced release of allergy mediators, by selectively blocking key signal transduction events, including phospholipase D-coupled, sphingosine kinase-mediated calcium mobilization and nuclear factor- κ B activation (Melendez et al. 2007). In conclusion, all these results indicate ES-62 as a potential novel therapeutic tool for allergy (Harnett and Harnett 2008).

The hygiene hypothesis and further modifications: what derives from it.

Strachan (1989) proposed the so called hygiene hypothesis following an epidemiological study addressed to inquire into the reasons for the high increase of hay fever and eczema in early life of childhood in the post industrial era. Of several perinatal, social, and environmental factors evaluated the most striking associations with the allergic diseases were those for family size: hay fever and eczema in fact were less common in children from larger families, presumably exposed to a less hygienic habitat, than in children from smaller families (just one child). In western countries, over the past decades the decrease in family size, and improvement of life style have reduced the opportunity for cross infections. This may have resulted in a wider diffusion of clinically overt atopic diseases, such as hay fever.

The hygiene hypothesis included the exposure to a variety of microorganisms, virus and parasites, with which humans coexisted throughout much of the evolutionary history, as necessary for balanced and regulated immune system development, in particular during the prenatal period and early childhood, which are considered the critical intervals for the establishment of the Th1/Th2 balance.

According to this theory, viral and bacterial infections might prevent the induction of allergen specific Th2 cells because they establish Th1-biased immunity. Viral (except some respiratory viruses) and bacterial infections that induce strong Th1 responses tend to inhibit the development of bystander allergen-specific Th2 cells (Herz et al. 2000) through the production of IFN- γ , IL-12 and IL-18 at the site of naive T-cell priming.

Interestingly, in Guinea-Bissau urban community, the vaccination with BCG during infancy resulted protective against the subsequent risk of atopy (Aaby et al. 2000). Likewise, military students in Italy with a positive titer of anti-hepatitis-A antibodies showed fewer allergic manifestations as compared with hepatitis-A-negative subjects (Matricardi et al. 2000).

In contrast to viruses and bacteria, helminths are the most active Th2-cell inducers in both humans and experimental models (Finkelman et al. 1997; Lawrence 2003). Therefore, worm infections might promote atopy by inducing IL-4 expression, followed by an increased development of allergen-specific Th2 cells. Furthermore, eosinophilia and mastocytosis, which characterize worm infections, may directly increase type 1 hypersensitivity reactions. Helminth infection instead modifies the course of allergic disease (Wohleben et al. 2004).

Reports of an inverse correlation between helminth infection and allergy date back to the early 1970s (before the formulation of the hygiene hypothesis), and a series of epidemiological studies support a role for parasites in immune regulation. Chronic infection with either intestinal worms or schistosomes is associated with lower atopic reactivity in individuals living in parts of Africa and Central/South America where helminth infections are still prevalent. Increased levels of the immunosuppressive cytokine IL-10, produced in response to *S. haematobium* infection in Gabonese schoolchildren, correlate with decreased type-1 hypersensitivity (van den Biggelaar et al. 2000). Anthelmintic therapy results in a rise in allergic reactivity. In fact, *S. mansoni*-infected patients with asthma produce lower levels of Th2 cytokines than do helminth-free patients with asthma, and this modulation is likely played by IL-10. After helminth-infected patients with asthma received anthelmintic treatment, there was down-modulation of allergen-dependent IL-10 production in vitro by peripheral blood mononuclear cells from *S. mansoni*-infected patients (Araujo et al. 2004).

Understanding the insights, a lot of hypotheses have been proposed to explain the interplay between parasite and allergy (Maizels 2005).

Helminth-stimulated T regulatory (T-reg) cell populations can involve allergen specific T-reg to suppress the inappropriate immunopathological response to allergens (Maizels and Yazdanbakhsh 2003; Wilson and Maizels 2004). Alternatively, the parasite specific T-reg can provide an activation stimulus to pre-existing T reg in regulating the allergic immune response. In fact, in experimentally induced airway allergy to ovalbumin and house dust mite allergen of *Dermatophagoides pteronyssinus* the suppression of airway allergy was related to the transfer of mesenteric lymph node cells, containing high numbers of CD4⁺CD25⁺Foxp3⁺ T cells, higher TGF- β expression, and strong IL-10 responses in *H. polygyrus* infected animals (Wilson et al. 2005).

Also the “homeostatic model” is suitable for the reduced anti-allergic-response in the presence of parasite infection: when there are two distinct infections, the immune responsiveness capacity doesn’t fairly divide the effort, ameliorating the outcoming allergic diseases (Barthlott et al. 2003). To complicate further the immunological picture developed from different antigen stimuli, parasite might release in its host specific antagonists or receptor of chemokines, leukotrienes and prostaglandins, hampering T cells recruitment to the airways in asthma (Luster and Tager 2004).

Another hypothesis for the decreased rates of atopy observed among helminth-infected individuals is that parasite-induced polyclonal immunoglobulin E (IgE) compete with allergen-specific IgE for Fc ϵ RI binding on basophils and mast cells. Recently, however, this was not confirmed (Mitre et al. 2005).

Applying the protective role of parasite infection against inflammatory disorders, over the years a wide range of helminths and their derived products have been associated with the potential to modulate autoimmunity and allergic disorder, approaching the therapeutic window.

Here are some examples.

S. mansoni Multiple sclerosis (MS), is an autoimmune condition in which the immune system abnormally attacks the central nervous system (brain and spinal cord), leading to demyelination. MS has a prevalence that ranges between 2 and 150 per 100,000 individuals, depending on the country or specific population, but it is increasing in areas far from the equator (Kahana 2000). The animal model for multiple sclerosis (MS) is represented by the experimental autoimmune encephalomyelitis (EAE) mouse. In EAE mice immunized with *S. mansoni* ova, the severity of EAE is reduced (Sewell et al. 2003). Disease suppression is associated with immune deviation in the periphery and the CNS, demonstrated by decreased IFN- γ and increased IL-4 levels. One probable mechanism could be a simple Th2 polarization, supported by the finding that *S. mansoni* egg-induced-protection of EAE was dependent on STAT-6.

The NOD mouse strain is an excellent model of autoimmune disease and an important tool for dissecting tolerance mechanisms. The peculiarity of this mouse strain is that it develops spontaneous autoimmune diabetes, which shares many similarities to autoimmune or type 1a diabetes (T1D) in humans, including the presence of pancreatic islet-specific autoantibodies, and autoreactive CD4⁺ and CD8⁺ T cells.

Infection of NOD mice with *S. mansoni* inhibits the development of type 1 diabetes, both by injection of the Th2-inducing schistosome eggs (Cooke et al. 1999), or non living parasite extract (Zaccone et al. 2003). Crohn's disease results from dysregulated Th1-type mucosal inflammation of the digestive system, which may affect any part of the gastrointestinal tract from mouth to anus. Crohn's disease is rare in tropical countries but prevalent in developed countries with temperate climates, in which its incidence increased after 1940 (Elliott et al. 2003). In a chemically-induced (with trinitrobenzenesulphonic acid =TNBS) model of Crohn's disease, administration of 10,000 dead *S. mansoni* ova deviated the cytokine response of splenic and mesenteric lymph node cells, attenuating TNBS colitis and protecting mice from lethal inflammation. *Schistosome* egg exposure diminished IFN- γ and enhanced IL-4 production. The therapeutic potential of *S. mansoni* ES products was recently investigated in the model of TNBS-induced colitis in mice where they attenuated inflammation of the colon (Ruyssers et al. 2008).

T. spiralis Infection ameliorates experimental (dinitrobenzene sulfonic acid = DNBS)-induced colitis in mice, with a down-regulation of myeloperoxidase (MPO) activity in colonic tissue linked to an emerging Th2-type immune response characterized by high IL-4 and IL-13 production by spleen cells in *T. spiralis* infected mice (Khan et al. 2002).

T. spiralis infection in EAE of rats influences the severity of the disease (Gruden-Movsesijan et al. 2008).

In NOD mice infected by *T. spiralis* the outcoming diabetes is inhibited through the activation of a Th2 immune response pathway, in particular in the pancreatic islet-infiltrating lymphocytes (Saunders et al. 2007).

T. suis Live ova were used in a human trial against Crohn's disease. After 24 weeks of treatment Crohn's Disease Activity Index (CDAI) of enrolled patients showed a tendency towards a decrease to a better prognosis. Without adverse events, this new therapy may offer a unique, safe, and efficacious alternative for Crohn's disease management (Summers et al. 2005).

A. suum Induction of experimental sterile inflammation in animals is considered a valid model to study the inflammatory phase of arthritis. In zymosan-induced arthritis (ZYA) or collagen-induced arthritis (CIA) in animals, *A. suum* extract administration resulted in decreased arthritis symptoms. Cytokine levels assessed in joint exudates showed reduced levels of NO, IL-1 β , and IL-10 while synovial and femoral extremities histopathology and cartilage damage, assessed through the glycosaminoglycan (GAG) content, demonstrated an ameliorating synovitis arrangement (Rocha et al. 2008).

Also in the murine model of asthma, *A. suum* extract (Lima et al. 2002), especially the suppressive protein of *A. suum* (PAS-1) protein (Itami et al. 2005), has proven protective against hyper-responsiveness to allergens. Reduced lung inflammation and suppression of IL-5, IL-4 and of eotaxin contributes to the suppression of asthma-induced pathology.

Recently, it was shown, on the contrary, that mice infected with *Toxocara canis* and treated with ovalbumin undergo an exacerbation of allergic lung inflammation, eosinophilia, airway hyperresponsiveness, increased expression of IL-4 but not of IL-5 or IL-10 in the lungs (Pinelli et al. 2008).

H. polygyrus Although this gastrointestinal nematode does not enter the lung in its life cycle, it inhibits allergen-induced airway eosinophilia and bronchial hyper-reactivity in the murine model of asthma, by modulating IL-10 (Kitagaki et al. 2006). Interestingly, in experimentally induced colitis in IL-10-deficient mice, animals infected by *H. polygyrus* suppressed the persistent gut inflammation using an alternative pathway (Elliott et al. 2004).

Strongyloides stercoralis Similarly, this nematode suppresses pulmonary allergic responses to ovalbumin diminishing the airway eosinophil accumulation (Wang et al. 2001).

Necator americanus Infective larvae were inoculated percutaneously in patients affected by Crohn's disease ameliorating their pathology, similarly to what happened in the *T. suis* trial (Croese et al. 2006).

Filariasis We already discussed about the effects of ES-62 on experimental models of human diseases. The canine filarial nematode *Dirofilaria immitis*-derived antigen (DiAg) is a purified 15kDa protein able to induce antigen non-specific IgE in rats and mice, as well as polyclonal proliferation of B cells. A timely treatment of NOD mice at 6 weeks of life with DiAg completely prevented the insulin-dependent

diabetes onset, owing to the switching the immune response from a Th1 profile, spontaneous in the NOD mice, to a Th2 pattern, diverting the pathogenic accumulation of Th1 cells into Langerhans islets (Imai et al. 2001).

N. brasiliensis It is a gastrointestinal parasite of rodents with a similar life cycle and morphology to the human hookworms *N. americanus* and *Ancylostoma duodenale*. Infected animals by this nematode showed a decrease in allergen-induced airway eosinophilia and eotaxin levels before ovalbumin (OVA)-airway challenge; this doesn't occur in IL-10 deficient infected animals (Wohlleben et al. 2004).

In a LPS-induced inflammation model, *N. brasiliensis* ES antigens, obtained as culture supernatants of L3 parasite larvae, inhibit the infiltration of neutrophils as assessed by bronchoalveolar lavage, possibly through the release of anti-inflammatory molecules present in the ES products (Keir et al. 2004).

The Use of Th1 Adjuvants to Subvert the Th2 Response

Helminths are thought to have developed different strategies for survival in their human host. For example schistosomula can compromise complement function (Ouaissi et al. 1981) and degrade host immunoglobulins (Auriault et al. 1981). Helminths may shield themselves by molecular mimicry. Schistosomula can acquire surface molecules from the host, including blood group determinants and MHC molecules, or they can produce cytokine mimics (reviewed in Maizels et al. 1993). *S. mansoni* eggs were found to secrete a chemokine binding protein (smCKP) that blocks IL-8-induced neutrophil migration. Finally, as reported above, helminths can hamper the host adaptive immunity by down-regulation of T- and B-cell responses via the induction of regulatory T cells or the anti-inflammatory cytokines IL-10 and TGF- β in the chronic phase of infection (Maizels and Yazdanbakhsh 2003). The down-modulation of the immune response is not only directed to helminths, but also to non-related antigens. In helminth-endemic areas, the most prevalent and threatening diseases are malaria, tuberculosis, and HIV. An effective immune response against primary malaria infection requires a strong Th1 response. Therefore, helminth infections would decrease the development of protective immunity by inducing a Th2 response, and this has been confirmed in several studies (Nacher et al. 2002; Spiegel et al. 2003). As cerebral malaria has been associated with increased levels of proinflammatory cytokines, a helminth co-infection may help to suppress these cytokines by production of IL-10 and/or TGF- β which are up-regulated by helminths (Mahanty et al. 1996) and therefore decrease the chance of severe malaria.

Protection from tuberculosis is characterized by effective *Mycobacterium*-specific Th1 responses and it has been hypothesized that co-infections with helminths will prevent the necessary Th1 response by either disturbing the Th1/Th2 balance, or by driving the immune response towards a more anti-inflammatory status. Early observations showed that the incidence of lepromatous leprosy was twice as high in areas where onchocerciasis was endemic (Prost et al. 1979).

Dendritic cells (DC) and macrophages exposed to live microfilariae *in vitro* show reduced maturation after *M. tuberculosis* infection, indicating a compromised activation of the immune system by mycobacteria upon co-infection with helminths (Talaat et al. 2006), thereby possibly reducing the susceptibility of DC for infection by *M. tuberculosis*. Thus, *in vitro* studies indicate that helminths can suppress the immune response to mycobacterial infections. However, the *in vivo* relevance of *in vitro* findings has to be more intensively examined.

Helicobacter pylori, a Gram negative bacillus that infects more than 50% of the human population, is associated with various gastroduodenal diseases. *H. pylori* infection induces an immune response, which is not sufficient to either prevent or counteract bacterial colonization; rather it actually contributes to chronic gastric inflammation. Two of the major *H. pylori* virulence factors are the Vacuolating cytotoxin (VacA) and the Neutrophil Activating Protein (HP-NAP). VacA is able to interfere with antigen presentation and to inhibit T cell activation (Boncristiano et al. 2003). HP-NAP, an oligomeric protein of 150 kDa, initially identified as a promoter of production of oxygen radicals from neutrophils, is a Toll-like receptor (TLR)-2 agonist able to induce the expression of IL-12, and IL-23 by neutrophils and monocytes. Addition in culture of HP-NAP, as immune modulator, to allergen-induced T-cell lines resulted in a remarkable increase of IFN- γ -producing T cells and decrease of IL-4-secreting cells, thus shifting the cytokine profile of allergen-activated human T cells from a Th2 to a Th1 phenotype (D'Elios et al. 1997; Amedei et al. 2006). We have performed an *in vivo* study of the ability of HP-NAP to downmodulate the ongoing Th2 response induced in mice by *T. spiralis* infection. Infection with this parasite induces a stable Th2 activation after the very early Th1 response (Goyal et al. 1994). Groups of *T. spiralis*-infected BALB/c mice received intraperitoneal PBS/rat IgG2b (control animals) or 10 μ g HP-NAP with or without anti-TLR-2 antibody on days 10 and 28 after infection. Blood eosinophils, total and *T. spiralis*-specific IgE levels and cytokine levels were measured in the plasma up to day 42, when splenocytes were cultured for cytokine production. Control animals showed significant eosinophilia and increase of total and *T. spiralis*-specific IgE, IL-4, and IL-5 levels from days 10 to 14, whereas HP-NAP-treated animals showed less eosinophilia and total and excretory/secretory antigens of *T. spiralis*-specific IgE in the blood (Figs. 1 and 2). HP-NAP-treated animals also had higher IL-12 and IFN- γ levels in the plasma, and lower IL-4 and IL-5 levels. Interestingly, the addition of anti-TLR-2 antibody abrogated the anti-Th2/pro-Th1 activity of HP-NAP. In conclusion, *in vivo* delivery of HP-NAP enhances endogenous IL-12 and IFN- γ responses and efficiently targets both IL-5-induced eosinophilia and IL-4-mediated hyper-IgE responses induced by the parasitic infection (Del Prete et al. 2008). If the eosinophilia and the hyper-IgE response to *T. spiralis* antigens do actually represent a Th2-induced defence mechanism evolved against the worm, hampering the Th2 response by HP-NAP would expose the *T. spiralis*-infected host to a higher worm burden, and the higher number of larvae in skeletal muscle should be inversely correlated with the levels of eosinophilia and/or IgE response. An appropriate answer to this

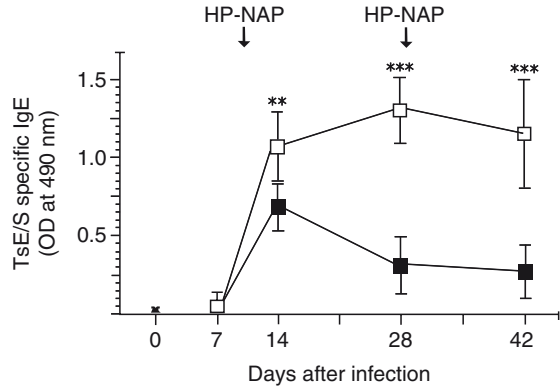


Fig. 1 Effect of HP-NAP treatment on plasma levels of total and TsE/S-specific IgE. In control animals (*open squares*) TsE/S-specific IgE levels were high up to day 42, whereas in HP-NAP-treated animals (*solid squares*) IgE levels progressively decreased after day 14. Results are presented as means $\pm$ SD in 2 groups of 45 animals. ** $P < .0005$ and *** $P < .0001$

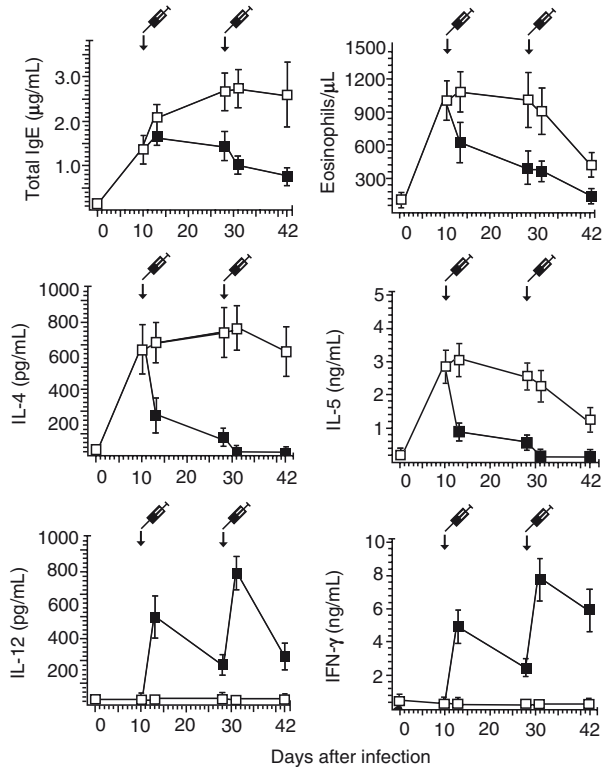


Fig. 2 Effect of HP-NAP treatment on blood Th2 and Th1 parameters. Eosinophils and total IgE, IL-4, IL-5, IFN- γ , and IL-12 levels were measured in the blood of control animals (*open squares*) and compared with values in animals treated on days 10 and 28 with HP-NAP (*solid squares*). Results are presented as means $\pm$ SD

question is required before HP-NAP-mediated Th2 suppression is proposed in a therapeutic strategy of trichinellosis.

As discussed above, the possibility of a correlation between allergies and helminth infections has been intensively studied, and results often show negative associations, suggesting that helminth infections might protect from allergic responses, although some studies find the opposite to be true. The intensity of infection might be the most important factor, as chronic helminth infections are increasingly associated with a regulatory response and protection from allergies, whereas acute, low level infections are thought to potentiate allergic reactions by increasing the Th2 response, without inducing regulation (Yazdanbakhsh et al. 2001; Cooper 2004). Interestingly, on the basis of large epidemiological studies, it has recently been reported a consistent negative association between *H. pylori* infection and the presence of allergic disorders, such as asthma and rhinitis (Blaser et al. 2008); however, the absence of a convincing molecular mechanism beside the epidemiological data raised criticism (Wjst 2008). Our recent studies on the anti-Th2 activity of HP-NAP may help in understanding such a complex issue and might offer an explanation for the decrement of allergy frequency in *H. pylori*-infected patients.

To assess whether HP-NAP might be beneficial for prevention and treatment of allergic bronchial asthma, HP-NAP was administered via intra-peritoneal (i.p.) or intra-nasal route using a mouse model of allergic asthma induced by inhaled ovalbumin (OVA). Groups of C57BL/6j, wild type or TLR-2^{-/-}, mice were treated with saline, or with OVA alone, or with OVA plus i.p. HP-NAP, or with OVA plus mucosal HP-NAP. In both systemic and mucosal protocols, mice were treated with OVA according to a standardized procedure consisting of a first phase of sensitization with OVA and a second phase of induction of the allergic response with aerosolized OVA on day 8, and finally exposed to the aerosolized antigen from day 15 to day 18 (Gonzalo et al. 1996; Vicentini et al. 2002). Control animals were injected with saline solution alone and then exposed to aerosolized saline (Fig. 3). In the systemic protocol, mice were treated with i.p. HP-NAP on day 1, whereas in the mucosal protocol mice received intranasal HP-NAP on days 7 and 8 (Codolo et al. 2008). After priming and repeated aerosol challenge with OVA, Th2 responses in the mouse lung were induced. Moreover, following OVA treatment, eosinophils were recruited and activated in bronchial airways, serum IgE levels increased (Table 3), and the elicited Th2 response correlated with the appearance of airway hyper-responsiveness. Both systemic and mucosal administration of HP-NAP strongly inhibited the development of airway eosinophilia and bronchial inflammation (Fig. 4). Likewise, HP-NAP treatment consistently inhibited the lung cytokine release, reducing the production of IL-4, IL-5, and GM-CSF in the bronchoalveolar lavage (Table 4). HP-NAP treatment also resulted in both significant reduction of total serum IgE and increase of IL-12 plasma levels. However, no suppression of lung eosinophilia and bronchial Th2 cytokines was observed in TLR-2 -knock-out mice following HP-NAP treatment, suggesting that interaction with TLR2 was required for HP-NAP to upregulate IL-12 production and to inhibit Th2 responses (Del Prete et al. 2008; Codolo et al. 2008).

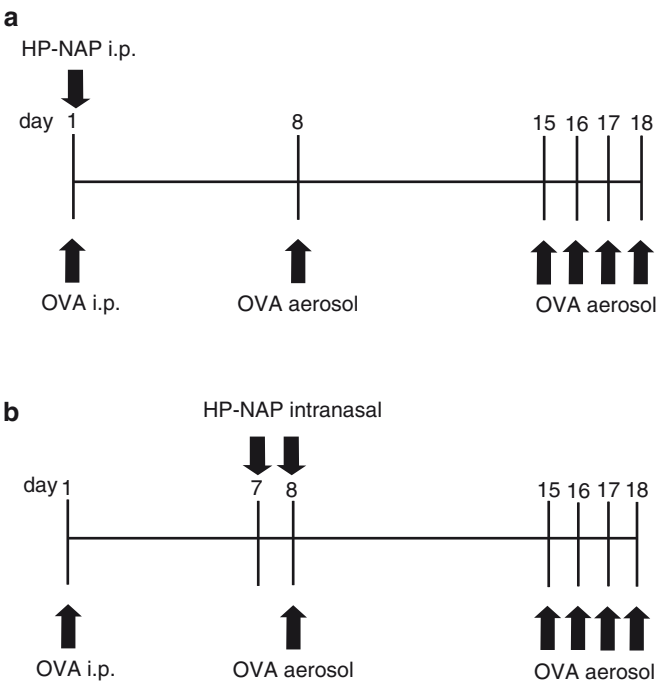


Fig. 3 Experimental protocol of the study. HP-NAP has been delivered systemically (i.p.) (**a**) or via mucosal route (**b**). Groups of C57BL/6j mice were treated with saline, with OVA alone, with OVA plus i.p. HP-NAP or with OVA plus mucosal HP-NAP. In both systemic and mucosal protocols, mice were treated with OVA according to a procedure consisting of a first phase of sensitization with OVA i.p. (100 µg/mouse) and a second phase of induction of the allergic response with aerosolized OVA (2% in PBS) for 5 min on day 8, and finally exposed to aerosolized antigen (1% in PBS) for 20 min daily on days 15–18. Control animals, designed as saline, were injected with PBS alone and then exposed to aerosolized PBS. In the systemic protocol (**a**) mice were treated with i.p. HP-NAP (10 µg/mouse) on day 1, whereas in the mucosal protocol (**b**) mice received intra-nasal HP-NAP (10 µg/mouse) on days 7 and 8

Table 3 HP-NAP delivery reduces serum IgE levels and eosinophil count in the blood of OVA sensitized mice

Treatment of mice	Blood WBC differential count/µl			Total serum IgE levels
	Lymphocytes	Neutrophils	Eosinophils	ng/ml (mean ± SE)
Saline	7,190 ± 700	1,298 ± 105	631 ± 79	44 ± 4
OVA alone	5,438 ± 528	1,333 ± 140	1,079 ± 131	561 ± 193
OVA + Sy HP-NAP	4,035 ± 350	842 ± 75	131 ± 27 ***	88 ± 35 **

On day 18, blood was taken from saline-, OVA- and OVA plus Systemic (Sy) HP-NAP-treated animals. Blood smears were stained to calculate the proportions and the mean (± SE) absolute counts of lymphocytes, neutrophils, and eosinophils. Levels of total serum IgE were assessed by a specific ELISA assay. ** $P < 0.01$, *** $P < 0.03$ OVA plus Sy HP-NAP versus OVA alone.

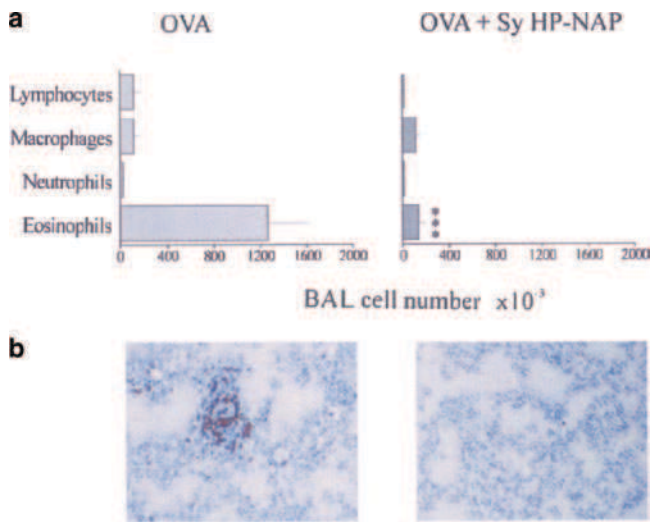


Fig. 4 Intrapерitoneal administration of HP-NAP inhibited the development of airway eosinophilia in OVA-sensitized animals. **(a)** On day 1, mice were sensitized with OVA alone or with OVA plus systemic HP-NAP (Sy HP-NAP), and then exposed to aerosolized OVA. Control animals were injected with PBS alone and then exposed to aerosolized PBS. Cytocentrifuge preparations from BAL of the three groups of animals on day 18 were stained to calculate the proportions of eosinophils, macrophages, neutrophils and lymphocytes. Absolute counts ($\pm$ SE) for each cell type were calculated from the number of total cells in the BAL. *** $P < 0.01$ versus OVA-treated mice. **(b)** Frozen sections of lungs from OVA-sensitized (*left*), and Sy HP-NAP-treated OVA-sensitized animals (*right*). Eosinophil infiltration in the lung is virtually absent in OVA-sensitized mice treated with Sy HP-NAP

Table 4 HP-NAP delivery reduces the Th2 cytokine release in the bronchoalveolar lavage of OVA sensitized mice

Treatment of mice	BAL cytokine levels (mean $\pm$ SE)		
	IL-4 (pg/ml)	IL-5 (pg/ml)	GM-CSF (pg/ml)
OVA alone	14.4 $\pm$ 5.0	16.1 $\pm$ 6.0	7.1 $\pm$ 2.9
OVA + Sy HP-NAP	4.0 $\pm$ 3.3*	2.9 $\pm$ 0.8*	1.6 $\pm$ 1.0*
OVA + Mu HP-NAP	0.7 $\pm$ 0.6**	2.8 $\pm$ 1.6*	0.7 $\pm$ 0.3**

On day 18, bronchoalveolar lavage samples were collected from OVA- and OVA plus Systemic (Sy) or mucosal (Mu) HP-NAP-treated OVA-sensitized mice. Cytokines were assayed in the supernatants. Mean values ($\pm$ SE) are reported. * $P < 0.05$, ** $P < 0.04$ OVA plus Sy or Mu HP-NAP versus OVA alone mice.

Conclusions

Even if it could seem bizarre nowadays, live parasites or their antigens would work for ameriolating immunological disorders, like allergies or autoimmune diseases. Helminth infection can control concomitant immune disease modulat-

ing the immune response. In animal models of type 1 diabetes, multiple sclerosis, inflammatory bowel disease, Crohn's disease, colitis and collagen-induced arthritis, the helminth infection cross-regulates the Th1-mediated disease shifting the response towards a Th2 profile, preventing or reducing the effects of an immoderate Th1 pattern.

In asthma and allergy, helminth alleviate the allergic Th-2 mediated pathology, too. The reverse is also true since bacterial proteins such as HP-NAP can shift Th2 response induced by helminths, such as *T. spiralis*, to a Th1-dominated condition.

To the point, a safe and controlled infection restore the immunological disease less harmful to the organism, following the strange strategy of the "minor injuries".

Therefore helminth and their derivatives could be an alternative therapeutical approach to a number of immune diseases, functioning like *in vivo* proactive medicaments.

References

- Aaby P, Shaheen SO, Heyes CB, Goudiaby A, Hall AJ, Shiell AW, Jensen H, Marchant A (2000) Early BCG vaccination and reduction in atopy in Guinea-Bissau. *Clin Exp Allergy* 30(5):644–650
- Abbas AK, Murphy KM, Sher A (1996) Functional diversity of helper T lymphocytes. *Nature* 383:787–793
- Amedei A, Cappon A, Codolo G, Cabrelle A, Polenghi A, Benagiano M, Tasca E, Azzurri A, D'Elis MM, Del Prete G, de Bernard M (2006) The neutrophil activating protein of *Helicobacter pylori* promotes T helper type 1 immune responses. *J Clin Invest* 116:1092–1101
- Araujo MI, Hoppe B, Medeiros M Jr, Alcântara L, Almeida MC, Schrieffer A, Oliveira RR, Kruschewsky R, Figueiredo JP, Cruz AA, Carvalho EM (2004) Impaired T helper 2 response to aeroallergen in helminth-infected patients with asthma. *J Infect Dis* 190(10):1797–1803
- Auriault C, Ouaisi MA, Torpier G, Eisen H, Capron A (1981) Proteolytic cleavage of IgG bound to the Fc receptor of *Schistosoma mansoni* schistosomula. *Parasite Immunol* 3(1):33–44
- Barnard JW, Biro MG, Lo SK, Ohno S, Carozza MA, Moyle M, Soule HR, Malik AB (1995) Neutrophil inhibitory factor prevents neutrophil-dependent lung injury. *J Immunol* 155:4876–4881
- Barthlott T, Kassiotis G, Stockinger B (2003) T cell regulation as a side effect of homeostasis and competition. *J Exp Med* 197(4):451–460
- Belkaid Y, Sun CM, Bouladoux N (2006) Parasites and immunoregulatory T cells. *Curr Opin Immunol* 18:406–412
- Bentancor A, Piacenza L, Carmona C (2002) Immunization with cathepsin L proteinases CL1 and CL2 secreted by *Fasciola hepatica* elicit a preferential type 1 response based on IgG2a antibodies in rats. *Helminthology* 76(3):199–205
- Blackburn CC, Selkirk ME (1992) Inactivation of platelet-activating factor by a putative acetylhydrolase from the gastrointestinal nematode parasite *Nippostrongylus brasiliensis*. *Immunology* 75:41–46
- Blaser MJ, Chen Y, Reibman J (2008) Does *Helicobacter pylori* protect against asthma and allergy? *Gut* 57:561–567
- Boncrisiano M, Paccani SR, Barone S, Olivieri C, Patrussi L, Ilver D, Amedei A, D'Elis MM, Telford JL, Baldari CT (2003) The *Helicobacter pylori* vacuolating toxin inhibits T cell activation by two independent mechanisms. *J Exp Med* 198:1887–1897
- Bruschi F, Carulli G, Azzarà A, Homan W, Minnucci S, Rizzuti-Gullaci A, Sbrana S, Angiolini C (2000) Inhibitory effects of human neutrophil functions by the 45 kD glycoprotein derived from the parasitic nematode *Trichinella spiralis*. *Int Arch Allergy Immunol* 122:58–65

- Codolo G, Mazzi P, Amedei A, Del Prete G, Berton G, D'Elios MM, de Bernard M (2008) The neutrophil activating protein of *Helicobacter pylori* down-modulates Th2 inflammation in OVA-induced allergic asthma. *Cell Microbiol* 10:2355–2363
- Cooke A, Tonks P, Jones FM, O'Shea H, Hutchings P, Fulford AJ, Dunne DW (1999) Infection with *Schistosoma mansoni* prevents insulin dependent diabetes mellitus in non-obese diabetic mice. *Parasite Immunol* 21(4):169–176
- Cooper PJ (2004) Intestinal worms and human allergy. *Parasite Immunol* 26:455–467
- Croese J, O'neil J, Masson J, Cooke S, Melrose W, Pritchard D, Speare R (2006) A proof of concept study establishing *Necator americanus* in Crohn's patients and reservoir donors. *Gut* 55(1):136–137
- Culley FJ, Brown A, Conroy DM, Sabroe I, Pritchard DI, Williams TJ (2000) Eotaxin is specifically cleaved by hookworm metalloproteases preventing its action *in vitro* and *in vivo*. *J Immunol* 165:6447–6453
- D'Elios MM, Manghetti M, De Carli M, Costa F, Baldari CT, Burrioni D, Telford JL, Romagnani S, Del Prete G (1997) T helper 1 effector cells specific for *Helicobacter pylori* in the gastric antrum of patients with peptic ulcer disease. *J Immunol* 158:962–967
- Deehan MR, Frame MJ, Parkhouse RM, Seatter SD, Reid SD, Harnett MM, Harnett W (1998) A phosphorylcholine-containing filarial nematode-secreted product disrupts B lymphocyte activation by targeting key proliferative signaling pathways. *J Immunol* 160:2692–2699
- Del Prete G, Chiumiento L, Amedei A, Piazza M, D'Elios MM, Codolo G, de Bernard M, Masetti M, Bruschi F (2008) Immunosuppression of TH2 responses in *Trichinella spiralis* infection by *Helicobacter pylori* neutrophil activating protein. *J Allergy Clin Immunol* 122:908–913
- Dillon SR, Sprecher C, Hammond A, Bilsborough J, Rosenfeld-Franklin M, Presnell SR, Haugen HS, Maurer M, Harder B, Johnston J, Bort S, Mudri S, Kuijper JL, Bukowski T, Shea P, Dong DL, Dasovich M, Grant FJ, Lockwood L, Levin SD, LeCiel C, Waggie K, Day H, Topouzis S, Kramer J, Kuestner R, Chen Z, Foster D, Parrish-Novak J, Gross JA (2004) Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. *Nat Immunol* 5:752–760
- Elliott DE, Li J, Blum A, Metwali A, Qadir K, Urban JF Jr, Weinstock JV (2003) Exposure to schistosome eggs protects mice from TNBS-induced colitis. *Am J Physiol Gastrointest Liver Physiol* 284(3):G385–G391
- Elliott DE, Setiawan T, Metwali A, Blum A, Urban JF Jr, Weinstock JV (2004) *Heligmosomoides polygyrus* inhibits established colitis in IL-10-deficient mice. *Eur J Immunol* 34(10):2690–2698
- Finkelman FD, Shea-Donohue T, Goldhill J, Sullivan CA, Morris SC, Madden KB, Gause WC, Urban JF Jr (1997) Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models. *Annu Rev Immunol* 15:505–533
- Finkelman FD, Shea-Donohue T, Morris SC, Gildea L, Strait R, Madden KB, Schopf L, Urban JF Jr (2004) Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunol Rev* 201:139–155
- Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, Menon S, Clifford T, Hunte B, Lesley R, Muchamuel T, Hurst SD, Zurawski G, Leach MW, Gorman DM, Rennick DM (2001) IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies *in vivo*. *Immunity* 15:985–995
- Gomez-Escobar N, Lewis E, Maizels RM (1998) A novel member of the transforming growth factor- β (TGF- β) superfamily from the filarial nematodes *Brugia malayi* and *Brugia pahangi*. *Exp Parasitol* 88:200–209
- Gonzalo JA, Lloyd CM, Kremer L, Finger E, Martinez AC, Siegelman MH, Cybulsky M, Gutierrez-Ramos JC (1996) Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J Clin Invest* 98:2332–2345
- Goodridge HS, Wilson EH, Harnett W, Campbell CC, Harnett MM, Liew FY (2001) Modulation of macrophage cytokine production by ES-62, a secreted product of the filarial nematode *Acanthocheilonema viteae*. *J Immunol* 167:940–945
- Gounaris K (2002) Nucleotidase cascades are catalyzed by secreted proteins of the parasitic nematode *Trichinella spiralis*. *Infect Immun* 70:4917–4924

- Goyal PK, Hermánek J, Wakelin D (1994) Lymphocyte proliferation and cytokine production in mice infected with different geographical isolates of *Trichinella spiralis*. *Parasite Immunol* 16(2):105–110
- Gruden-Movsesijan A, Ilic N, Mostarica-Stojkovic M, Stosic-Grujicic S, Milic M, Sofronic-Milosavljevic L (2008) *Trichinella spiralis*: modulation of experimental autoimmune encephalomyelitis in DA rats. *Exp Parasitol* 118:641–647
- Harnett W, Harnett MM (1993) Inhibition of murine B cell proliferation and down-regulation of protein kinase C levels by a phosphorylcholine-containing filarial excretory–secretory product. *J Immunol* 151:4829–4837
- Harnett W, Harnett MM (2008) Parasitic nematode modulation of allergic disease. *Curr Allergy Asthma Rep* 8:392–397
- Harnett W, Worms MJ, Kapil A, Grainger M, Parkhouse RME (1989) Origin, kinetics of circulation and fate in vivo of the major excretory-secretory product of *Acanthocheilonema viteae*. *Parasitology* 99:229–239
- Harnett MM, Deehan MR, Williams DM, Harnett W (1998) Induction of signalling anergy via the T-cell receptor in cultured Jurkat T cells by pre-exposure to a filarial nematode secreted product. *Parasite Immunol* 20:551–563
- Herbert DR, Hölscher C, Mohrs M, Arendse B, Schwegmann A, Radwanska M, Leeto M, Kirsch R, Hall P, Mossmann H, Claussen B, Förster I, Brombacher F (2004) Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. *Immunity* 20:623–635
- Herz U, Lacy P, Renz H, Erb K (2000) The influence of infections on the development and severity of allergic disorders. *Curr Opin Immunol* 12:632–640
- Houston KM, Cushley W, Harnett W (1997) Studies on the site and mechanism of attachment of phosphorylcholine to a filarial nematode secreted glycoprotein. *J Biol Chem* 272:1527–1533
- Houston KM, Wilson EH, Eyres L, Brombacher F, Harnett MM, Alexander J, Harnett W (2000) Presence of phosphorylcholine on a filarial nematode protein influences immunoglobulin G subclass response to the molecule by an interleukin-10-dependent mechanism. *Infect Immun* 68:5466–5468
- Imai S, Tezuka H, Fujita K (2001) A factor of inducing IgE from a filarial parasite prevents insulin-dependent diabetes mellitus in nonobese diabetic mice. *Biochem Biophys Res Commun* 286(5):1051–1058
- Itami DM, Oshiro TM, Araujo CA, Perini A, Martins MA, Macedo MS, Macedo-Soares MF (2005) Modulation of murine experimental asthma by *Ascaris suum* components. *Clin Exp Allergy* 35(7):873–879
- Kahana E (2000) Epidemiologic studies of multiple sclerosis: a review. *Biomed Pharmacother* 54(2):100–102
- Keir PA, Brown DM, Clouter-Baker A, Marcus YM, Proudfoot L (2004) Inhibition of neutrophil recruitment by ES of *Nippostrongylus brasiliensis*. *Parasite Immunol* 26(3):137–139
- Khan WI, Blennerhasset PA, Varghese AK, Chowdhury SK, Omsted P, Deng Y, Collins SM (2002) Intestinal nematode infection ameliorates experimental colitis in mice. *Infect Immun* 70(11):5931–5937
- Kitagaki K, Businga TR, Racila D, Elliott DE, Weinstock JV, Kline JN (2006) Intestinal helminths protect in a murine model of asthma. *J Immunol* 177(3):1628–1635
- Knoll AH, Carroll SB (1999) Early animal evolution: emerging views from comparative biology and geology. *Science* 284:2129–2137
- Kong Y, Chung YB, Cho SY, Kang SY (1994) Cleavage of immunoglobulin G by excretory-secretory cathepsin S-like protease of *Spirometra mansonii* plerocercoid. *Parasitology* 109:611–621
- Lawrence CE (2003) Is there a common mechanism of gastrointestinal nematode expulsion? *Parasite Immunol* 25:271–281
- Loid RW, Grant RF, Suquet CM (1987) Inhibition of equine neutrophil chemotaxis and chemokinesis by a *Taenia taeniaeformis* proteinase inhibitor, taeniaestatin. *J Immunol* 9:195–204

- Lima C, Perini A, Garcia ML, Martins MA, Teixeira MM, Macedo MS (2002) Eosinophilic inflammation and airway hyper-responsiveness are profoundly inhibited by a helminth (*Ascaris suum*) extract in a murine model of asthma. *Clin Exp Allergy* 32(11):1659–1666
- Luster AD, Tager AM (2004) T-cell trafficking in asthma: lipid mediators grease the way. *Nat Rev Immunol* 4(9):711–724
- Mackay KB, Bailey SJ, King PD, Patel S, Hamilton TC, Campbell CA (1996) Neuroprotective effect of recombinant neutrophil inhibitory factor in transient focal cerebral ischaemia in the rat. *Neurodegeneration* 5:319–323
- Maggioli G, Piacenza L, Carambola B, Carmona C (2004) Purification, characterization, and immunolocalization of a thioredoxin reductase from adult *Fasciola hepatica*. *J Parasitol* 90:205–211
- Mahanty S, Mollis SN, Ravichandran M, Abrams JS, Kumaraswami V, Jayaraman K, Ottesen EA, Nutman TB (1996) High levels of spontaneous and parasite antigen-driven interleukin-10 production are associated with antigen-specific hyporesponsiveness in human lymphatic filariasis. *J Infect Dis* 173:769–773
- Maizels RM (2005) Infections and allergy – helminths, hygiene and host immune regulation. *Curr Opin Immunol* 17(6):656–661
- Maizels RM, Yazdanbakhsh M (2003) Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nat Rev Immunol* 3:733–744
- Maizels RM, Bundy DAP, Selkirk ME, Smith DF, Anderson RM (1993) Immunological modulation and evasion by helminth parasites in human populations. *Nature* 365:797–805
- Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD, Allen JE (2004) Helminth parasites – masters of regulation. *Immunol Rev* 201:89–116
- Manoury B, Gregory WF, Maizels RM, Watts C (2001) *Bm*-CPI2 a cystatin homolog secreted by the filarial parasite *Brugia malayi* inhibits class II-MHC restricted antigen processing. *Curr Biol* 11:447–451
- Matricardi PM, Rosmini F, Riondino S, Fortini M, Ferrigno L, Rapicetta M, Bonini S (2000) Exposure to foodborne and orofecal microbes versus airborne viruses in relation to atopy and allergic asthma: epidemiological study. *BMJ* 320:412–417
- McInnes IB, Leung BP, Harnett M, Gracie JA, Liew FY, Harnett W (2003) A novel therapeutic approach targeting articular inflammation using the filarial nematode-derived phosphorylcholine-containing glycoprotein ES-62. *J Immunol* 171:2127–2133
- McSorley HJ, Harcus YM, Murray J, Taylor MD, Maizels RM (2008) Expansion of Foxp3+ regulatory T cells in mice infected with filarial parasite *Brugia malayi*. *J Immunol* 181:6456
- Meenan J, Hommes DW, Mevissen M, Dijkhuizen S, Soule H, Moyle M, Buller HR, Ten Kate FW, Tytgat GN, van Deventer SJ (1996) Attenuation of the inflammatory response in an animal colitis model by neutrophil inhibitory factor, a novel β 2-integrin antagonist. *Scand J Gastroenterol* 31:786–791
- Melendez AJ, Harnett MM, Pushparaj PN, Wong WS, Tay HK, McSharry CP, Harnett W (2007) Inhibition of Fc epsilon RI-mediated mast cell responses by ES-62, a product of parasitic filarial nematodes. *Nat Med* 13:1375–1381
- Mitre E, Norwood S, Nutman TB (2005) Saturation of immunoglobulin E (IgE) binding sites by polyclonal IgE does not explain the protective effect of helminth infections against atopy. *Infect Immun* 73(7):4106–4111
- Molinari JL, Mejia H, White AC Jr, Garrido E, Borgonio VM, Baig S, Tato P (2000) *Taenia solium*: a cysteine protease secreted by metacestodes depletes human CD4 lymphocytes in vitro. *Exp Parasitol* 94(3):133–142
- Moyle M, Foster DL, McGrath DE, Brown SM, Laroche Y, De Meutter J, Stanssens P, Bogowitz CA, Fried VA, Ely JA, Soule HR, Vlasuk GP (1994) A hookworm glycoprotein that Inhibits neutrophil is a ligand of the integrin CD11b/CD18. *J Biol Chem* 269:10008–10015
- Nacher M, Singhasivanon P, Yimsamran S, Manibunyong W, Thanyavanich N, Wuthisen R, Looareesuwan S (2002) Intestinal helminth infections are associated with increased incidence of *Plasmodium falciparum* malaria in Thailand. *J Parasitol* 88:55–58

- O'Neill SM, Mills KH, Dalton JP (2001) *Fasciola hepatica* cathepsin L cysteine proteinase suppresses *Bordetella pertussis*-specific interferon-gamma production in vivo. *Parasite Immunol* 23(10):541–547
- Okano M, Satoskar AR, Nishizaki K, Harn DA Jr (2001) Lacto-N-fucopentaose III found on *Schistosoma mansoni* egg antigens functions as adjuvant for proteins by inducing Th2-type response. *J Immunol* 167(1):442–450
- Ortona E, Margutti P, Delunardo F, Nobili V, Profumo E, Rigano R, Buttari B, Carulli G, Azzara A, Teggi A, Bruschi F, Siracusano A (2005) Screening of an *Echinococcus granulosus* cDNA library with IgG4 from patients with cystic echinococcosis identifies a new tegumental protein involved in the immune escape. *Clin Exp Immunol* 142: 528–538
- Ouaissi MA, Auriault C, Santoro F, Capron A (1981) Interaction between *Schistosoma mansoni* and the complement system: role of IgG Fc peptides in the activation of the classical pathway by schistosomula. *J Immunol* 127:1556–1559
- Pennock JL, Behnke JM, Bickle QD, Devaney E, Grecis RK, Isaac RE, Joshua GWP, Selkirk ME, Zhang Y, Meyer DJ (1998) Rapid purification and characterization of L-dopachrome-methyl ester tautomerase (macrophage-migration-inhibitory factor) from *Trichinella spiralis*, *Trichuris muris* and *Brugia pahangi*. *Biochem J* 335:495–498
- Pinelli E, Brandes S, Dormans J, Gremmer E, van Loveren H (2008) Infection with the roundworm *Toxocara canis* leads to exacerbation of experimental allergic airway inflammation. *Clin Exp Allergy* 38:649–658
- Prost A, Nebout M, Rougemont A (1979) Lepromatous leprosy and onchocerciasis. *Br Med J* 1:589–590
- Prowse RK, Chaplin P, Robinson HC, Spithill TW (2002) *Fasciola hepatica* cathepsin L suppresses sheep lymphocyte proliferation in vitro and modulates surface CD4 expression on human and ovine T cells. *Parasite Immunol* 24:57–66
- Riganò R, Profumo E, Bruschi F, Carulli G, Azzara A, Ioppolo S, Buttari B, Ortona E, Margutti P, Teggi A, Siracusano A (2001) Modulation of human immune response by *Echinococcus granulosus* antigen B and its possible role in evading host defenses. *Infect Immun* 69: 288–296
- Riganò R, Buttari B, Profumo E, Ortona E, Delunardo F, Margotti P, Mattei V, Teggi A, Sorice M, Siracusano A (2007) *Echinococcus granulosus* antigen B impairs human dendritic cell differentiation and polarizes immature dendritic cell maturation towards a Th2 cell response. *Infect Immun* 75:1667–1678
- Rocha FA, Leite AK, Pompeu MM, Cunha TM, Verri WA Jr, Soares FM, Castro RR, Cunha FQ (2008) Protective effect of an extract from *Ascaris suum* in experimental arthritis models. *Infect Immun* 76(6):2736–2745
- Ruyssers NE, De Winter BY, De Man JG, Loukas A, Herman AG, Pelckmans PA, Moreels TG (2008) Worms and the treatment of inflammatory bowel disease: are molecules the answer? *Clin Dev Immunol* doi:10.1155/2008:567314
- Saunders KA, Raine T, Cooke A, Lawrence CE (2007) Inhibition of autoimmune type 1 diabetes by gastrointestinal helminth infection. *Infect Immun* 75(1):397–407
- Selkirk ME, Smith VP, Thomas GR, Gounaris K (1998) Resistance of filarial nematode parasites to oxidative stress. *Int J Parasitol* 28:1315–1332
- Sewell D, Qing Z, Reinke E, Elliot D, Weinstock J, Sandor M, Fabry Z (2003) Immunomodulation of experimental autoimmune encephalomyelitis by helminth ova immunization. *Int Immunol* 15(1):59–69
- Shepherd JC, McManus DP (1987) Specific and cross-reactive antigens of *Echinococcus granulosus* cyst fluid. *Mol Biochem Parasitol* 25:143–154
- Spiegel A, Tall A, Raphenon G, Trape JF, Druilhe P (2003) Increased frequency of malaria attacks in subjects coinfecting by intestinal worms and *Plasmodium falciparum* malaria. *Trans R Soc Trop Med Hyg* 97:198–199
- Spolski RJ, Thomas PG, See EJ, Mooney KA, Kuhn RE (2002) Larval *Taenia crassiceps* secretes a protein with characteristics of murine interferon-gamma. *Parasitol Res* 88(5):431–438
- Strachan DP (1989) Hay fever, hygiene, and household size. *BMJ* 299:1259–1260

- Summers RW, Elliott DE, Urban JF Jr, Thompson R, Weinstock JV (2005) *Trichuris suis* therapy in Crohn's disease. *Gut* 54(1):87–90
- Talaat KR, Bonawitz RE, Domenech P, Nutman TB (2006) Preexposure to live *Brugia malayi* microfilariae alters the innate response of human dendritic cells to *Mycobacterium tuberculosis*. *J Infect Dis* 193:196–204
- Tan TH, Edgerton SA, Kumari R, McAlister MS, Roe SM, Nagl S, Pearl LH, Selkirk ME, Bianco AE, Totty NF, Engwerda C, Gray CA, Meyer DJ (2001) Macrophage migration inhibitory factor of the parasitic nematode *Trichinella spiralis*. *Biochem J* 357:373–383
- van den Biggelaar AH, van Ree R, Rodrigues LC, Lell B, Deelder AM, Kremsner PG, Yazdanbakhsh M (2000) Decreased atopy in children infected with *Schistosoma haematobium*: a role for parasite-induced interleukin-10. *Lancet* 356:1723–1727
- van Riet E, Hartgers FC, Yazdanbakhsh M (2007) Chronic helminth infections induce immunomodulation: consequences and mechanisms. *Immunobiology* 212(6):475–490
- Vassiliatis DK, Despommier D, Misek DE, Polvere RI, Gold AM, Van der Ploeg LHT (1992) Analysis of a 43-kDa Glycoprotein from the intracellular parasitic nematode *Trichinella spiralis*. *J Biol Chem* 267:18459–18465
- Vicentini L, Mazzi P, Cavegion E, Continolo S, Fumagalli L, Lapinet-Vera JA, Lowell CA, Berton G (2002) FcγR deficiency results in defective eosinophil recruitment to the lung during allergic airway inflammation. *J Immunol* 168:6446–6454
- Voehringer D, Shinkai K, Locksley RM (2004) Type 2 immunity reflects orchestrated recruitment of cells committed to IL-4 production. *Immunity* 20:267–277
- Voehringer D, Reese TA, Huang X, Shinkai K, Locksley RM (2006) Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system. *J Exp Med* 203:1435–1446
- Wang CC, Nolan TJ, Schad GA, Abraham D (2001) Infection of mice with the helminth *Strongyloides stercoralis* suppresses pulmonary allergic responses to ovalbumin. *Clin Exp Allergy* 31(3):495–503
- Weinstock JV, Summers R, Elliott DE (2004) Helminths and harmony. *Gut* 53(1):7–9
- Whelan M, Harnett MM, Houston KM, Patel V, Harnett W, Rigley KP (2000) A filarial nematode-secreted product signals dendritic cells to acquire a phenotype that drives development of Th2 cells. *J Immunol* 164:6453–6460
- White AC Jr, Molinari JL, Pillai AV, Rege AA (1992) Detection and preliminary characterization of *Taenia solium* metacystode proteases. *J Parasitol* 78(2):281–287
- Wilson MS, Maizels RM (2004) Regulation of allergy and autoimmunity in helminth infection. *Clin Rev Allergy Immunol* 26(1):35–50
- Wilson LR, Good RT, Panaccio M, Wijffels GL, Sandeman RM, Spithill TW (1998) *Fasciola hepatica*: characterization and cloning of the major cathepsin B protease secreted by newly excysted juvenile liver fluke. *Exp Parasitol* 88(2):85–94
- Wilson EH, Deehan MR, Katz E, Brown KS, Houston KM, O'Grady J, Harnett MM, Harnett W (2003a) Hyporesponsiveness of murine B lymphocytes exposed to the filarial nematode secreted product ES-62 in vivo. *Immunology* 109:238–245
- Wilson EH, Katz E, Goodridge HS, Harnett MM, Harnett W (2003b) In vivo activation of murine peritoneal B1 cells by the filarial nematode phosphorylcholine-containing glycoprotein ES-62. *Parasite Immunol* 25:463–466
- Wilson MS, Taylor MD, Balic A, Finney CA, Lamb JR, Maizels RM (2005) Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *J Exp Med* 202(9):1199–1212
- Wisniewski N, McNeil M, Greve RB, Wassom DL (1993) Characterization of novel fucosyl- and tyvelosyl-containing glycoconjugates from *Trichinella spiralis* muscle stage larvae. *Mol Biochem Parasitol* 61:25–36
- Wjst M (2008) Does *Helicobacter pylori* protect against asthma and allergy? *Gut* 57:1178–1179
- Wohlleben G, Trujillo C, Müller J, Ritze Y, Grunewald S, Tatsch U, Erb KJ (2004) Helminth infection modulates the development of allergen-induced airway inflammation. *Int Immunol* 16(4):585–596

- Wu Z, Boonmars T, Nagano I, Nakada T, Takahashi Y (2003) Molecular expression and characterization of a homologue of host cytokine macrophage migration inhibitory factor from *Trichinella* spp. *J Parasitol* 89:507–515
- Wynn TA, Thompson RW, Cheever AW, Mentink-Kane MM (2004) Immunopathogenesis of schistosomiasis. *Immunol Rev* 201:156–167
- Yazdanbakhsh M, van den Biggelaar A, Maizels RM (2001) Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease. *Trends Immunol* 22:372–377
- Zaccone P, Fehérvári Z, Jones FM, Sidobre S, Kronenberg M, Dunne DW, Cooke A (2003) *Schistosoma mansoni* antigens modulate the activity of the innate immune response and prevent onset of type 1 diabetes. *Eur J Immunol* 33(5):1439–1449
- Zhang L, Zhang ZG, Zhang RL, Lu M, Krams M, Chopp M (2003) Effects of a selective CD11b/CD18 antagonist and recombinant human tissue plasminogen activator treatment alone and in combination in a rat embolic model of stroke. *Stroke* 34:1790–1795

Parasites and Oncogenesis with a Special Reference to Gastro-Intestinal Neoplasia Induced by *Cryptosporidium parvum*

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Abstract In the last years a large number of infectious agents including virus, bacteria and parasites have been identified as direct causes or as risk factors to specific cancers around the world. Among the parasites linked to oncogenesis in humans there are strong associations. Particularly, *Schistosoma haematobium* is usually recognized as a cause of urinary bladder carcinoma. Some hepatic and colorectal cancers have been linked to infection by *S. japonicum* or *S. mansoni*. Moreover, a high proportion of cholangiocarcinoma in Far-East countries was imputable to Opisthorchiidae liver flukes. Among the parasitic Protists, the Apicomplexan *Theileria annulata* and *T. parva*, which are the agents of theileriosis in cows, induce often lethal lymphoproliferative process in these animals. The association between *Cryptosporidium* and digestive carcinomas has also been reported in a clinic study in Poland. More recently, the ability of *C. parvum* to induce neoplastic changes was established experimentally. This model revealed that *C. parvum* strains are able to induce gastrointestinal intraepithelial neoplasia in dexamethasone-treated SCID mice. Neoplastic lesions spread often to more than

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one digestive organ, and severity correlated with the inoculum size and the duration of the infection. Further studies are needed in order to characterize this process in mice, and to explore its occurrence in human cryptosporidiosis.

Keywords Parasites • cancer • oncogenesis • gastro-intestinal neoplasia • *Cryptosporidium parvum*

Infection and Cancer

The global burden of cancer associated with infectious agents approaches 20% of all malignancies (Ziegler and Buonaguro 2009). Best established as human oncogenic agents are Human Papillomavirus (HPV, cervical cancer), Epstein Barr Virus (EBV, B-cell lymphoproliferative diseases), Kaposi's sarcoma associated virus (KSHV, Kaposi's sarcoma), Human T Lymphocyte Virus 1 (HTLV1, T-cell leukemia), Hepatitis B virus, (HBV) and Hepatitis C Virus (HCV) (hepatocellular carcinoma) and *Helicobacter pylori* (gastric cancer) (Pagano et al. 2004). However, several parasites have been linked to oncogenesis in humans. Some of these associations are strong. Particularly, *Schistosoma haematobium* has been recognized as a definitive cause of urinary bladder cancer by the International Agency for Research in Cancer (IARC). Also, a high proportion of liver cholangiocarcinoma worldwide is usually attributable to Opisthorchiidae liver flukes (Parkin 2006).

Concerning parasite protozoa, the association, still speculative of some protozoa infections with cancer was commonly suggested on the basis of epidemiological data (Khurana et al. 2005) except for the cases of *Theileria parva* and *Theileria annulata*, pathogens of cattle (Dobbelaere et al. 2000; Dobbelaere and Rottenberg 2003; Lizundia et al. 2006). Regarding *Cryptosporidium* infection, the association of cryptosporidiosis and colonic adenocarcinoma was speculated in the case of a Spanish patient carrying both, who died rapidly after the onset of symptoms (Izquierdo et al. 1988). Likely more significant, an epidemiological study in Poland reported a high frequency (18%) of cryptosporidiosis in patients with colorectal cancer (Sulzyc-Bielicka et al. 2007). However, in these reports it was unclear if *Cryptosporidium* behaved as a carcinogenesis factor or simply as an opportunistic agent whose development was enhanced by host immunosuppression. Indeed, it was only recently that *Cryptosporidium parvum* could be related to carcinogenesis on a scientific basis in an experimental animal model (Certad et al. 2007; Certad et al. 2010).

How parasites can produce cancer? No data are available to answer this question except for *Theileria* infection, which is shortly evoked below. Infections in general can promote carcinogenesis by different mechanisms including: (1) oncogene transduction, with introduction of exogenous oncogenic genes; (2) activation of endogenous oncogenes, comprising those from endogenous retroviruses; (3) inactivation of constitutive suppressor genes, with enhanced susceptibility to exogenous

oncogenic agents. Furthermore, pathogens' indirect role is associated to cancer promotion through inflammation and angiogenesis (Ziegler and Buonaguro 2009).

However, it has been very difficult to identify pathogens as causative agents of cancers. Among all the possible factors, the latency periods between primary infection and cancer development usually long are one of the main reasons for this remarkable difficulty (zur Hausen 2009). In some cases, the epidemiology has provided clues for successful associations when there is a geographic coincidence of a specific infection with a particular type of cancer. For instance, hepatocellular carcinoma (HCC) and hepatitis B infection (zur Hausen 2009).

Trematodes

Schistosoma haematobium has been recognized as a definitive cause of urinary bladder cancer by the IARC. The relative risk for bladder cancer in *S. haematobium* infected patients is estimated between 1.8 and 23.5 (Parkin 2006). It has been calculated that 3% of bladder cancers around the world are attributable to *S. haematobium* (de Martel and Franceschi 2009). Several mechanisms have been suggested to explain the role of *S. haematobium* in this type of cancer. Eggs provoke granulomatous inflammation, ulceration and pseudopolypsis of the bladder and ureteral walls. Chronic lesions can then evolve into fibrosis, and carcinoma (de Martel and Franceschi 2009). In addition, chronic bacteriuria, which is often associated with *S. haematobium* infection, produces nitrosamines which are well known as bladder carcinogens (Hicks et al. 1982). Urinary stasis allows concentration of endogenous carcinogens leading to their absorption from urine and to the bladder epithelium (Bhagwandeem 1976).

The association between *Schistosoma mansoni* infection and HCC appears to be indirect. Patients with *S. mansoni* have higher incidence of HBV or HCV infection (two viruses associated with cancerogenesis process), compared to noninfected controls (Halim et al. 1999). The high exposure of schistosomiasis patients to HBV and HCV could be partly explained by transmission of these viruses during blood transfusion consecutive to haematemesis which is a relatively frequent complication of hepatosplenic schistosomiasis, via contaminated blood, syringes and needles (Darwish et al. 1993).

The role of *Schistosoma japonicum* in cancer occurrence is less clear, although this parasite has been associated with both liver and colorectal cancer. Some epidemiological and clinical studies in China and Japan support its role as one of the risk factors in HCC formation. Experimental studies have shown that liver cancer appears early in experimentally *S. japonicum* infected animals (Khurana et al. 2005).

Opisthorchiidae Digenetic trematodes are liver flukes found in the bile duct of domestic and wild animals but also in humans. *Opisthorchis viverrini*, *O. felinus* and *Clonorchis sinensis* are the most concerned species. They remain an important public health problem in many endemic areas in South-East Asia where they infect

at least 20 million of persons (Khurana et al. 2005). In addition to their association with hepatobiliary disease, they are considered as major etiological agents of hepatic cholangiocarcinoma (CCA) (Parkin 2006). After evaluating epidemiological studies, case series, and case control studies, *O. viverrini* was considered definitely carcinogenic by the IARC. Nowadays, hepatobiliary opisthorchiasis is the strongest link which could be occurred between a human malignancy and a Metazoan agent infection (Laha et al. 2007). However, the evidence relating infection with *O. felineus* and *C. sinensis* as human carcinogen is more limited (Parkin 2006). Nevertheless, liver cancer cases have also been reported in patients infected by *C. sinensis* in China, Hong Kong, Korea, Japan and also in North America among Asian immigrants (de Martel and Franceschi 2009).

The mechanisms of carcinogenesis due to liver fluke infection remain unknown, though are thought to be multifactorial. Chronic inflammation caused by the flukes has been suggested. The N-nitroso-compounds that may play a role are formed in the area of chronic infection around the bile ducts as the result of local generation of nitric oxide by inflammatory cells. NO is not only cytotoxic but may also lead to DNA damage, particularly in proliferating bile duct cells (Sripa et al. 2007). Furthermore, recent evidence suggests that *O. viverrini* secretes mitogenic proteins in host tissues where they might promote cell proliferation, mutagenesis and finally carcinogenesis (Laha et al. 2007; Sripa et al. 2007).

Protozoa

Some hypotheses have evoked the role of *Trichomonas vaginalis* and *Toxoplasma gondii* in several types of tumors (Khurana et al. 2005). Nevertheless, it has only been clearly established the transforming cancerogenic role of *Theilera* (Dobbelaere et al. 2000; Dobbelaere and Rottenberg 2003; Lizundia et al. 2006). Finally, the association between *Cryptosporidium* and digestive carcinomas has also been reported (Certad et al. 2007; Sulzyc-Bielicka et al. 2007; Certad et al. 2010).

Trichomonas vaginalis is a pathogenic protozoa sexually transmitted, found usually in the female lower genitourinary tract. Some histopathological and serological studies have reported the association between *T. vaginalis* infection and cervical neoplasia. However other studies have shown no association between the two. The prevalence of trichomoniasis could be more elevated in persons with high level of sexual activity. *T. vaginalis* is frequently coexisting with other infections like HPV or HIV potentially linked with malignancies. Thus, other risk factors for cancer could also be involved (Khurana et al. 2005). For this reasons, its role in the development of cancer is not clear.

The relationship between *Toxoplasma gondii* infection and neoplasia, including ocular tumours, gliomas, meningiomas, pituitary gland adenomas, leukemia and lymphomas has been reported (Khurana et al. 2005). In these studies is not clear if *Toxoplasma* has a role in tumorigenesis or if it is an opportunistic agent whose development is enhanced by host immunosuppression.

The Special Case of *Cryptosporidium*

Cryptosporidiosis represents a major public health problem. This infection, caused by protozoa of the genus *Cryptosporidium*, has been reported worldwide as a frequent cause of diarrhoea, and its prevalence varies according to different regions (Caccio and Pozio 2006). In developed countries, massive *Cryptosporidium* foodborne and waterborne outbreaks have been reported. In developing countries, *Cryptosporidium* affects mostly children under five (Xiao et al. 2004). Furthermore, cryptosporidiosis remains a clinically significant opportunistic infection in immunocompromised patients, causing potentially life-threatening diarrhoea, especially in those HIV-infected without access to highly active anti-retroviral therapy (HAART) (Pozio and Morales 2005). Additionally, these parasites not only infect humans, but also cause morbidity in farm animals, leading to economic losses (Sunnotel et al. 2006). Most *Cryptosporidium* species infect the epithelium of the gut but in severe infections, dissemination can occur to extra-intestinal sites (Lopez-Velez et al. 1995). Infection of the intestinal cells can result in blunting of the intestinal villi, crypt hyperplasia and inflammation. Epithelial cell apoptosis due to this parasite has also been described (Chen et al. 2001; Mele et al. 2004).

Interestingly, *Cryptosporidium* infection has been related to carcinogenesis. The association of cryptosporidiosis and colonic adenocarcinoma was speculated in the case of a Spanish patient carrying both, who died rapidly after the onset of symptoms (Izquierdo et al. 1988). More recently, an epidemiological study in Poland reported a high frequency of cryptosporidiosis in patients with colorectal cancer (Sulzyc-Bielicka et al. 2007). However, in these reports it was unclear if *Cryptosporidium* sp. behaved as a carcinogenesis factor or simply as an opportunistic agent whose development was enhanced by host immunosuppression.

Additionally, previous studies have associated cryptosporidiosis with the development of tumour lesions in vertebrates. One report described the association between *Cryptosporidium* sp. and aural-pharyngeal polyps in iguanas (Uhl et al. 2001). Cystic hyperplasia of the colonic mucosa was also described in nude mice (Heine et al. 1984). None of these studies has described the presence of pre-malignant lesions associated to cryptosporidiosis.

More recently, the ability of *C. parvum* to induce neoplastic changes have been established experimentally in infected severe combined immunodeficiency (SCID) mice treated with dexamethasone (Certad et al. 2007; Certad et al. 2010). Herein, adenomas with low or high-grade intraepithelial neoplasia associated with numerous *C. parvum* life stages were observed in different areas of the digestive tract including stomach, duodenum and ileocaecal region. The use of Ki-67 supported the neoplastic nature of the described *Cryptosporidium*-induced epithelial transformation, and showed that potential neoplastic alterations begin before histopathological lesions can be detected with standard stains. A highly significant correlation was found between intensity of cryptosporidiosis and severity of neoplastic lesions in dexamethasone-treated or untreated mice (Certad et al. 2010).

No data about the mechanism of *C. parvum*-induced neoplasia are available. Interestingly, it is well known that *Theileria parva*, another apicomplexan parasite, is responsible for a lymphoproliferative disorder of cattle (Dobbelaere et al. 2000). This organism infects and transforms bovine lymphocytes resulting in tumours with metastatic/invasive potential by a mechanism associated to inhibition of apoptosis (Dobbelaere et al. 2000). Inhibition of apoptosis has also been reported in other apicomplexan protozoa including *Cryptosporidium* (Heussler et al. 2001) which is able to activate NF- κ B pathway, preventing the induction of cell death early after infection. Apoptosis prevention probably benefits the parasite by stabilizing the host cell long enough to permit the completion of the life cycle (Heussler et al. 2001). Indeed, resistance to apoptosis could be an essential step in the progression to malignancy (Lowe and Lin 2000). Therefore, persistent infection with *C. parvum* could be a risk for gastro-intestinal neoplasia as an adverse effect due to the inhibition of intestinal cell apoptosis.

Concluding Remarks

In last years, important evidence has been found related to infection agents linked with cancer. Particularly, parasites with oncogenic potential have been shown to be highly prevalent within the population. Although the cancerogenic role of ubiquitous pathogens mentioned above, and the molecular mechanisms involved in the progression of chronic infections to cancers are still badly known, at least some of the malignancies can be preventable with early diagnosis, adequate treatment and prevention of infections, leading to a substantive public health benefit.

The particular interest, it is the recent discovery of the association between *C. parvum* infection and the development of gastrointestinal neoplasia. Because *C. parvum* is an opportunistic agent that causes significant morbidity and mortality in immunocompromised patients, it is possible that individuals infected with this parasite may have a higher risk of developing colorectal malignancies, especially when immunosuppression is more severe. A prospective observational cohort shows that the incidence of colorectal cancer was 2.3-folds higher in the HIV-infected population than in the general population (Patel et al. 2008). More studies have to be done in humans to evaluate cryptosporidiosis as a possible risk factor of gastrointestinal cancer.

References

- Bhagwande SB (1976) Schistosomiasis and carcinoma of the bladder in Zambia. *S Afr Med J* 50:1616–1620
- Caccio SM, Pozio E (2006) Advances in the epidemiology, diagnosis and treatment of cryptosporidiosis. *Expert Rev Anti Infect Ther* 4:429–443

- Certad G, Ngouanesavanh T, Guyot K, Gantois N, Chassat T, Mouray A, Fleurisse L, Pinon A, Cailliez JC, Dei-Cas E, Creusy C (2007) *Cryptosporidium parvum*, a potential cause of colic adenocarcinoma. *Infect Agent Cancer* 2:22
- Certad G, Creusy C, Ngouanesavanh T, Guyot K, Gantois N, Mouray A, Chassat T, Flament N, Fleurisse L, Pinon A, Delhaes L, Dei-Cas E (2010) Development of *Cryptosporidium parvum* induced gastro-intestinal neoplasia in SCID mice: Severity of lesions is correlated with infection intensity. *Am J Trop Med Hyg* 82:257–265
- Chen XM, Levine SA, Splinter PL, Tietz PS, Ganong AL, Jobin C, Gores GJ, Paya CV, LaRusso NF (2001) *Cryptosporidium parvum* activates nuclear factor kappaB in biliary epithelia preventing epithelial cell apoptosis. *Gastroenterology* 120:1774–1783
- Darwish MA, Raouf TA, Rushdy P, Constantine NT, Rao MR, Edelman R (1993) Risk factors associated with a high seroprevalence of hepatitis C virus infection in Egyptian blood donors. *Am J Trop Med Hyg* 49:440–447
- de Martel C, Franceschi S (2009) Infections and cancer: Established associations and new hypotheses. *Crit Rev Oncol Hematol* 70:183–194
- Dobbelaere DA, Fernandez PC, Heussler VT (2000) *Theileria parva*: taking control of host cell proliferation and survival mechanisms. *Cell Microbiol* 2:91–99
- Dobbelaere DA, Rottenberg S (2003) *Theileria*-induced leukocyte transformation. *Curr Opin Microbiol* 6:377–382
- Halim AB, Garry RF, Dash S, Gerber MA (1999) Effect of schistosomiasis and hepatitis on liver disease. *Am J Trop Med Hyg* 60:915–920
- Heine J, Moon HW, Woodmansee DB (1984) Persistent *Cryptosporidium* infection in congenitally athymic (nude) mice. *Infect Immun* 43:856–859
- Heussler VT, Kuenzi P, Rottenberg S (2001) Inhibition of apoptosis by intracellular protozoan parasites. *Int J Parasitol* 31:1166–1176
- Hicks RM, Ismail MM, Walters CL, Beecham PT, Rabie MF, El Alamy MA (1982) Association of bacteriuria and urinary nitrosamine formation with *Schistosoma haematobium* infection in the Qalyub area of Egypt. *Trans R Soc Trop Med Hyg* 76:519–527
- Izquierdo J, Antunez I, Calderon MT, Perez Giraldo C, Munoz Sanz A (1988) Diarrhea caused by *Cryptosporidium* and colonic neoplasia. *Rev Clin Esp* 182:393–394
- Khurana S, Dubey ML, Malla N (2005) Association of parasitic infections and cancers. *Indian J Med Microbiol* 23:74–79
- Laha T, Pinlaor P, Mulvenna J, Sripa B, Sripa M, Smout MJ, Gasser RB, Brindley PJ, Loukas A (2007) Gene discovery for the carcinogenic human liver fluke, *Opisthorchis viverrini*. *BMC Genomics* 8:189
- Lizundia R, Chaussepied M, Huerre M, Werling D, Di Santo JP, Langsley G (2006) c-Jun NH2-terminal kinase/c-Jun signaling promotes survival and metastasis of B lymphocytes transformed by *Theileria*. *Cancer Res* 66:6105–6110
- Lopez-Velez R, Tarazona R, Garcia Camacho A, Gomez-Mampaso E, Guerrero A, Moreira V, Villanueva R (1995) Intestinal and extraintestinal cryptosporidiosis in AIDS patients. *Eur J Clin Microbiol Infect Dis* 14:677–681
- Lowe SW, Lin AW (2000) Apoptosis in cancer. *Carcinogenesis* 21:485–495
- Mele R, Gomez Morales MA, Tosini F, Pozio E (2004) *Cryptosporidium parvum* at different developmental stages modulates host cell apoptosis in vitro. *Infect Immun* 72:6061–6067
- Pagano J, Blaser M, Buendia M, Damania B, Khalili K, Raab-Traub N, Roizman B (2004) Infectious agents and cancer: criteria for a causal relation. *Semin Cancer Biol* 14:453–471
- Parkin DM (2006) The global health burden of infection-associated cancers in the year 2002. *Int J Cancer* 118:3030–3044
- Patel P, Hanson DL, Sullivan PS, Novak RM, Moorman AC, Tong TC, Holmberg SD, Brooks JT (2008) Incidence of types of cancer among HIV-infected persons compared with the general population in the United States, 1992–2003. *Ann Intern Med* 148:728–736
- Pozio E, Morales MA (2005) The impact of HIV-protease inhibitors on opportunistic parasites. *Trends Parasitol* 21:58–63

- Sripa B, Kaewkes S, Sithithaworn P, Mairiang E, Laha T, Smout M, Pairojkul C, Bhudhisawasdi V, Tesana S, Thinkamrop B, Bethony JM, Loukas A, Brindley PJ (2007) Liver fluke induces cholangiocarcinoma. *PLoS Med* 4:e201
- Sulzyc-Bielicka V, Kuzna-Grygiel W, Kolodziejczyk L, Bielicki D, Kladny J, Stepień-Korzonek M, Telatynska-Smieszek B (2007) Cryptosporidiosis in patients with colorectal cancer. *J Parasitol* 93:722–724
- Sunnotel O, Lowery CJ, Moore JE, Dooley JS, Xiao L, Millar BC, Rooney PJ, Snelling WJ (2006) *Cryptosporidium*. *Lett Appl Microbiol* 43:7–16
- Uhl EW, Jacobson E, Bartick TE, Micinilio J, Schimdt R (2001) Aural-pharyngeal polyps associated with *Cryptosporidium* infection in three iguanas (*Iguana iguana*). *Vet Pathol* 38:239–242
- Xiao L, Fayer R, Ryan U, Upton SJ (2004) *Cryptosporidium* taxonomy: recent advances and implications for public health. *Clin Microbiol Rev* 17:72–97
- Ziegler J, Buonaguro F (2009) Infectious agents and human malignancies. *Front Biosci* 1:3455–3464
- zur Hausen H (2009) The search for infectious causes of human cancers: where and why. *Virology* 392:1–10

Smart Biosensors for Determination of Mycotoxines

Lyubov Yotova, Ivo Grabchev, Rossica Betcheva, and Dessislava Marinkova

Abstract The mycotoxins are biological pollutants. They are toxic metabolites produced by several fungi in foods and feeds. They are probably the best known and most intensively examined mycotoxins in the world. Contamination of foods with mycotoxines can be also object of bioterrorism.

Mycotoxins are non-volatile, relative low-weight molecules secondary metabolic products that may affect exposed persons in a variety of ways. These compounds are considered secondary metabolites because they are not necessary for fungal growth and are simply a product of the primary metabolic processes. The functions of mycotoxins have not been totally studied, but it is established that they play a key role in the antagonistic processes concerning microorganisms from the same environment. They are also believed to help parasitic fungi invade host tissues. The amount of toxins needed to produce adverse health effects varies widely among toxins, as well as to each person's immune system.

The aim of the present review is to present development of new biosensors for mycotoxines determination in foods, utilizing new polymeric membranes with immobilized enzymes and antibodies.

The following investigations concerning biosensors design are presented investigations concerning smart polymer characteristics, based on new synthesized polymers and dendrimers; simultaneously immobilization of mediators, fluorescent dyes and biochemical recognition molecules; amperometric and optical sensors constructions; investigations of the main parameters as a response time, linear range, reproducibility; mathematical models concerning optimization of biosensor measurements are described and compared with standard methods as HPLC and ELISA.

Keywords mycotoxines • bioterrorism • smart biosensors • electrochemical and optical sensors

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Introduction

The food safety policy of the Republic of Bulgaria is based on the functioning in the legal regulation. The basic law includes: *Low for the foods*, *National health Law*, *Veterinarian and medical activity Law*, *Plant safety law* *Feed safety law*. These laws state the legal food requirements, the producer, manufacturer's and merchant's obligations and the sequences of the complete food chain control actions (Valkov 2003).

Regulation No 31 of the Bulgarian Ministry of Health is functioning from 19 July 2004 and is dealing with the maximum allowable quantities of pollutants in the foods. The regulation defines the maximum allowable quantities of pollutants in the foods and the requirements for the sample preparation and analysis. The term "pollutants" includes any foreign substance that is released outside or is formed in or on the surface of different foods, during different procedures or the period of storage, and has harmful effect over the human organism.

The qualitative health-hygienic indexes of the food products are formed, according to the food specifications, manipulations, and the terms of storage. The health-hygienic regulations assessed by the manufacturer should correspond with those stated by the functioning legal regulations. The following indexes are included: organoleptical – appearance, color, odor, consistency etc.; physicochemical – dry weight, humidity, oiliness, quantity of salt, etc.; toxicological – quantity of heavy metals, pesticides, nitrates, nitrites, products and semi products of the production and storage process, additives; microbiological indexes, provisionally pathogenic microorganisms, mycotoxicological, mycological;

Foodborne pathogens may cause gastroenteritis fatal hemolytic uremic syndrome, abortion, meningitis, atherosclerosis and neurological malady. Centers for Disease Control and Prevention (CDC) estimates that there about 76,000,000 illnesses due to foodborne pathogens, 325,000 hospitalization and 5,000 deaths each year in the United States (Waldo 2003). Economic losses for foodborne diseases are estimated to be 6.9 billion dollars per year, and the losses are due to product recalls, medical intervention, loss of work hours, and death. In the recent years the threat of food bioterrorism unfolds a new perspective to the control and management of foodborne infections (Knoph et al. 2007a).

Regulations for aflatoxines and ochratoxin A (EC 466/2001), maximum level for *Fusarium toxins* in selected foodstuffs were recently set in European Commission Regulation 856/2005 which applied from 1 July 2006 (Rudolf and Alexandra 2007).

Contamination of food also incites product recalls bringing inevitable economic disasters to food manufacturers. In recent years, however, global political instability has also raised serious concerns about potential food bioterrorism. Detection tools are an essential component of food safety and control of food bioterrorism. Government and industrial agencies recognize the need for sensitive and low-cost detection tools. The validated official analytical methods based on chromatographic principles, rapid screening tests and several new techniques, for example use of biosensors.

Biosensors technology offers the best solutions because of its ultrasensitivity and potential for automation and online use; however, the specificity and cost must be addressed as we continue to develop sensors for specific applications.

The presence of harmful substances in foods is classified into the following groups: biological, chemical and physical.

Mycotoxines

Mycotoxines are biological pollutants. They are toxic metabolites produced by several fungi in foods and feeds. They are probably the best known and most intensively examined mycotoxins in the world.

Mycotoxins are non-volatile, relative low-weight molecules secondary metabolic products that may affect exposed persons in a variety of ways. These compounds are considered secondary metabolites because they are not necessary for fungal growth and are simply a product of the primary metabolic processes. The functions of mycotoxins have not been totally studied, but it is established that they play a key role in the antagonistic processes concerning microorganisms from the same environment. They are also believed to help parasitic fungi invade host tissues. The amount of toxins needed to produce adverse health effects varies widely among toxins, as well as to each person's immune system.

Some mycotoxins are carcinogenic, some are vasoactive, and some cause damage to the central nerve system. Often, a single mycotoxin can cause more than one kind of toxic effect. More than 240 fungi produce 100 toxic compounds, which cause lancinating and chronic diseases called mycotoxicoses.

The disappearing of several villages in Siberia after The Second World War is ascribed to mycotoxicose, caused by takes in of infected by mycotoxins grain bread. Mycotoxins in foods are thermo stable also.

Aflatoxines

The aflatoxin is a typical representative of the mycotoxins. The aflatoxins are toxic metabolites produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*.

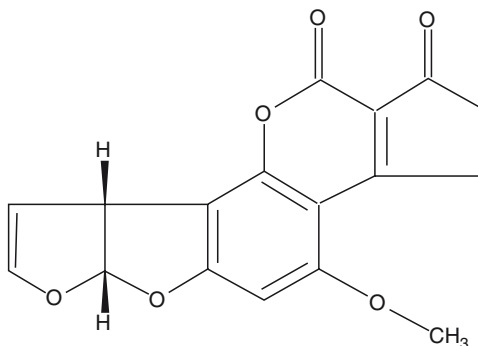
These genera are found everywhere, which explains the gross contamination of foods. The agricultural cultures grown in the tropical and subtropical climatic belts are more prone to aflatoxins contamination.

Asp. parasiticus is well adapted to the soil conditions and develops in peanuts synthesizing B1, B2, G1 and G2. Mycotoxins belonging to the B and G groups typically emit green fluorescent light when exposed to ultraviolet radiation.

Asp. Flavus develops on the leaves and blossoms of plants and prevails in maze, cottonseeds and walnuts. It synthesizes B1 and B2 aflatoxins (Santacroce et al. 2007).

The aflatoxins possess the following biological activity. They are the strongest carcinogenic substances in nature. They are also strongly toxic. They suppress the activity of the immune system. They possess an important biological specificity. They have a cumulative effect – manifested by evacuation through the liver, urine and faeces, a peak moment occurs, that is a carcinoma is formed in it, which is due to the accumulation of a certain quantity of aflatoxin. It has been proven that there is a connection between the virus causing hepatitis B and the occurrence of liver cancer when aflatoxins are taken in. These mycotoxins possess tetragenic effect (cause malformations) of the fetus when the mother has taken contaminated food that penetrates the fetus through the umbilical cord. Thus they cause irreversible deformations such as imbecility, defects in the heart valves, only one ear, no fingers or one hand shorter than the other (Vlahov and Ivanon 1996).

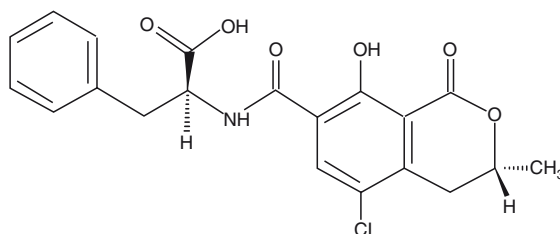
Of all the known mycotoxins aflatoxin B₁ (AFB₁) is the major and best studied one because it possesses exceptional toxicity and is widespread in market foods and foods in principle (such as peanuts, maize, cotton seeds).



The “threshold effect” (concentration above which foods become unusable) of aflatoxin B₁ is 20 µg L⁻¹ of the total content of aflatoxins. AFB₁ is formed when the temperature is 40–50°. Entering the organism through fodder it is metabolized to aflatoxin M₁ that is given off into the milk. The threshold of AFM₁ content in milk is 5 µg L⁻¹. For the maize used to feed milk cows this level is 20 µg L⁻¹, for calves used to produce meat and pigs it is 300 µg L⁻¹ and for breeding stock – 100 µg L⁻¹. The allowable quantities of aflatoxin B₁ in cotton seeds is 300 µg L⁻¹. An important condition for the toxic and carcinogenic effects of AFB₁ to be active is its conversion into a number of metabolites (Murphy et al. 2006).

Ochratoxin

The ochratoxins are mycotoxins produced by *Aspergillus ochraceus* and *Penicillium verrucosum*.



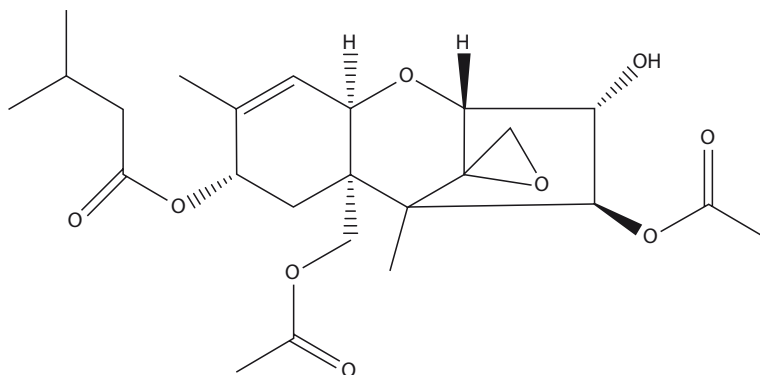
This mycotoxin occurs in a great number of foods with different physiological and environmental requirements. There are four ochratoxin homologs – A, B, C, D. Ochratoxin A is the most widely spread and toxic (Károva 1998).

This mycotoxin resides in contaminated wheat, maize, oats, cheese and meat products. Although ochratoxin can be found everywhere in the world the regions most threatened with contamination of some types of foods are Europe and Africa.

Ochratoxins belong to the mycotoxins group, affecting mainly the liver by causing acute and chronic damage to it. The international agency on cancer studies has classified ochratoxin A in group 2B as “potential carcinogen with people”. It is supposed that ochratoxin is one of the etiological factors, leading to the development of the severe liver disease known in this country as the Balkan endemic nephropathy (Maxwell et al. 2003).

Trichothecene

Trichothecenes are a group of over 150 structurally bound components synthesized from several fungus genera. The most important of them are the genus *Fusarium* or more exactly *Fusarium sporotrichoides* and *Fusarium graminearum*. They are the most often synthesized trichothecenes.



Low temperatures, high humidity and water content stimulate the synthesis of toxin. Examples of important trichothecenes are the toxin T-2, nivalenol, deoxynivalenol. Of these toxins T-2 is best studied as it is one of the first identified trichothecenes

in wheat crops although deoxinivalenol is the most often occurring mycotoxin in wheat and barley (Vlahov 2006).

The most toxic trichothecene, verrucarol Y, showed a lethal dose of 0.5 mg/kg in experiments with mice. In many vital systems T-2 appears to be the most toxic one followed by nivalenol and deoxynivalenol.

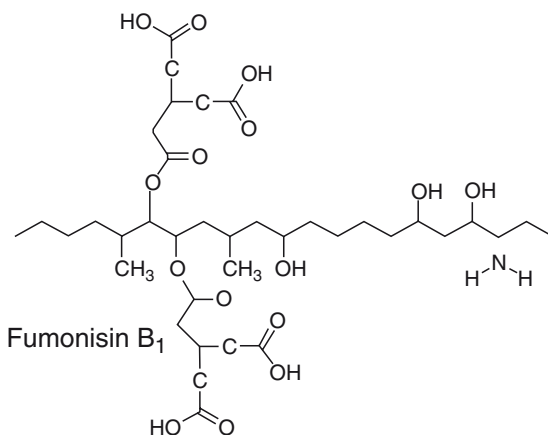
Trichothecenes possess a range of toxic effects. The acute trichothecene toxicity manifests as intestine disorders (vomiting, temperature, diarrhoea etc.). There are also haematological effects such as anemia and reduction of the number of leucocytes. These toxins suppress the synthesis of proteins in the organism.

Leukemia is the most frequent trichothecene mycotoxicosis in man. The effects of the trichothecenes appear soon after their taking in, causing burning sensation in the mouth, tongue, throat and intestine disorders.

The trichothecenes are powerful immune suppressing agents. The toxin significantly changes several immune indicators, mainly related to the immune response and the synthesis of protein (Murphy et al. 2006).

Fumosinins

These mycotoxins are produced by fungi pathogenic for maize like *Fusarium verticillioides* and *Fusarium proliferatum* and to a very small extent *Alternaria* in tomatoes. At least 15 varieties of fumosinins have been identified. They are readily dissolved in water and are distinguished from the other mycotoxins by their lack of aromatic structure or a unique chromophore, which does not allow easy analytic determination. Foods containing maize are the main hazard of contamination with fumosinins in the food industry (Vlahov and Ivanov 1996). Most of the foods on the market contain fumosinins to 500 ng g⁻¹ or less thanks to the control exercised on the maize and the other components. Fumosin B₁ is the most toxic representative of the group.

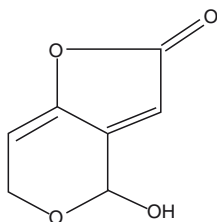


It is associated with the appearance of paralysis, restlessness, limping and inability to take water and food. These symptoms may appear a few hours after taking fumosinins. The changes in the liver are accompanied by changes in the central nervous system of horses and pigs. In pigs the smaller doses lead to a slow, progressive necrosis and the larger doses to acute pulmonary edema due to toxicity of the liver.

Besides these effects, FB_1 also has tumour activity. It is assumed that FB_1 is probably initiator of tumour in the liver. Since the wheat crops are an important food component in many parts of the world and because *Fusarium* is universal for them fumosinins are the cause of toxicosis in people as well as of other diseases (Maxwell et al. 2003).

Patulin

Another death causing mycotoxin is patulin, described by scientists, as “a substance for which there is no sufficient data on carcinogenity”.

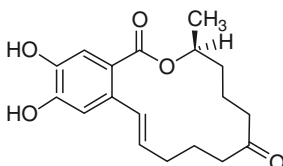


The fungus which produces it occurs in fermented apples and other decaying fruits, juices and nectars from fruit, strong drinks with alcohol content of up to 15°C and products from apples. It is produced by *Penicillium* (*Penicillium enpamsum*, *Penicillium islandicum*) and *Aspergillus* etc. (Murphy et al. 2006).

Zearalenon

Zearalenon is a macrolacton with a phenol ring bound to it.

Zearalenon is a natural contaminator of maize, wheat, oats, barley and hay. The synthesis of the toxin is enhanced by high humidity and low temperatures and is also often found together with trichothecenes. This mycotoxin is synthesized by several strains of *Fusarium* mainly by *Fusarium sporotrichoides* and *Fusarium graminearum* etc.



Zearalenone

Most prone to poisoning with ZEN are the pigs, cattle, poultry and laboratory animals. Poisoning occurs at quantities of $1 \mu\text{g L}^{-1}$, but it can also happen at $0.1 \mu\text{g L}^{-1}$. There is very little transmission of ZEN and its metabolites in milk. Zearalenon also causes changes in the breeding process. The toxin is mainly found in wheat crops with quantities varying from several nanograms/gram to several $1,000 \text{ ng g}^{-1}$ (Larinov and Pisareva 1997).

Conclusions Regarding Food Safety

Different degrees of the hazard of contamination with mycotoxins and hence the potentiality of occurrence of mycotoxicoses are most apparent in the under developed countries where the control is insufficient. In the light of the diseases of the immune system such as HIV infections and chronic protein undernourishment the additional weakening of the immune system due to mycotoxins deserves greater attention. It is little known about the effects of continuously taken small doses, especially when a combination of mycotoxins is taken. Besides, because of the varied mycotoxin contamination and the potential presence of high toxin content in some regions, it is difficult to take samples and carry out analyses regularly. For this reason it is imperative to develop low cost methods of assay employing simple tools and apparatus that will facilitate tracking the content of mycotoxins globally in order to care for people's health.

Thus, protection against food contamination with mycotoxins will be more effective for public health in the underdeveloped countries and deserves appropriate attention. Food industry should play a leading role in this respect as this will enhance the economic stability of the industry, will make foods and drinks safer, will intensify international trade and improve public health (Vlahov 2006).

Mycotoxine Analyse

Determination Methods with High-Performance Liquid Chromatography (HPLC)

Most methods for mycotoxins detection are based on HPLC using reversed phase columns followed by fluorescent detection. The methods used for purifying the sample are solid-phase extraction column (SPE) with normal phase or reversed phase absorbents. Recently, immune affinity columns have been used more often.

Immune Enzyme Method (ELISA)

Enzyme – linked immunosorbent analysis (ELISA) is a method known for decades. The technology is based on the ability of a specific antibody to distinguish the tri-dimensional structure of a given mycotoxin. The direct competitive

ELISA is usually used for the assay of mycotoxins. The commonly used ELISA needs equilibrium of the reaction antibody-antigen which requires an incubation period of approximately 1–2 h. Currently most ELISA tests for mycotoxins operate in the kinetic phase of linking antibody-antigen that reduces the incubation time to minutes. Although the reduction of the incubation time may lead to some loss of the sensitivity of the assay, the tests can produce accurate and reproducible results.

The antibodies are specific and sensitive. Their target compounds are mycotoxins, and not antigens, compounds with similar chemical groups that can react with the antibodies. This is the so-called matrix effect (Zheng and Richard 2006).

The ELISA tests are preferred because they require low volume of the sample and fewer clean-up procedures of the extracted sample compared to the conventional methods like TLC (thin layer chromatography) and HPLC (high-performance liquid chromatography). ELISA tests are rapid, simple, specific, sensitive and portable for use in the field for the detection of mycotoxins in foods and fodder (Zheng et al. 2005).

Membrane Based Assays

Flow-Through Immunoassay

The FTA for mycotoxins has been used since the late 1980s. It is based on the principle of the direct competitive ELISA. Anti-mycotoxin antibody is coated on the surface of a membrane.

The FTA is rapid, easy to use and suitable for testing mycotoxins. The interpretation of the results may be difficult when the mycotoxin concentration in the sample is close to the detection level (Paepens et al. 2004).

Lateral Flow Method

The technology of the immunochromatographic test, also called lateral flow test or strip test, has been used for many years. However, its application to food safety, especially in mycotoxin testing is quite recent. The typical immunochromatographic test strip consists of a sample pad; conjugate pad, membrane; absorbent pad and adhesive backing.

The control zone will always be visible regardless of the presence or absence of mycotoxin because the second antibody always binds to a second antibody gold particle complex thus showing the validity of the test. The benefits of the immunochromatographic test are that they are, very rapid, have long-term stability and are particularly suitable for testing for mycotoxins.

Fluorometric Assay

The fluorometric assay is an efficient quantitative method for mycotoxin analysis. To obtain accurate results it is very important to remove interferences before the

fluorometric measurement because other compounds in the extracted mycotoxin sample may have fluorescence that could alter the readings.

Available fluorometric assays use two sample clean-up methods.

- Immunoaffinity column clean-up (IAC)
- Solid phase extraction column clean-up (SPE)

to enhance the fluorescence signal, most mycotoxins are derivatized prior to the fluorometric measurement.

Fluorescent Polarization

Fluorescence polarization immunoassay (FP) is considered to be sufficiently reliable. This is a homogenous determination –the antibody-linked fractions and the free fractions of the antigens marked with fluorescein (Ag – F) can be distinguished in solutions without preliminary separation. The antigens marked with fluorescein are called fluorophores. FP is a competitive immunoassay in which a given quantity of marked antigens competes with the antigen from the sample for the binding sites of a limited number of antibodies.

The fluorescent polarization has two main differences from the ELISA method.

- The detection does not involve an enzyme reaction
- Separation of the bound and free compounds is not required

As a result, FP assays do not require a wash step and do not require waiting for an enzyme reaction for colour development.

The method is simple to use and applicable in practice. However, similarly to the ELISA methods the matrix effects can also exist in fluorescent polarization. Moreover, the FP is a highly throughput method. The samples have to be analysed in series and not as a batch (Tcvetkova and Danev 2000).

Radio Immunoassay (RIA)

Radio Immunoassay is an indirect immunological method in which radioactive isotopes of iodine – ^{131}I , ^{125}I or tritium ^3H are used as markers.

The method is based on competitive binding in which a certain quantity of antigens in the sample marked with an isotope competes with the antibodies for a limited number of specific binding sites. Both the isotope marked antigens and the non-marked ones are linked to the antibodies forming complexes of antigen-antibody. The concentration of the antigens in the sample is inversely proportional to the radioactivity of the complexes.

Benefits of the method:

The advantage of RIA is the utilization of ^{125}I for marking as it has a high gamma radiation providing for a sufficient sensitivity to detect antigens in pikomolar or lower concentration. The method is easy to automate, which allows large series to be analysed.

Shortcomings of the method:

The radioisotope marker has a short longevity (1–2 months) which often makes the RIA method inefficient and expensive because the reactive could not be used within the longevity period. The kits require special storage and waste disposal conditions. Because of the necessity to collect large series the processing of the samples and obtaining of the results happens some time after taking the biological material. Although the analytical system is fully automated the hazard of the operators being exposed should not be disregarded (Tcvetkova and Danev 2000).

Smart Biosensors for Mycotoxine Determination

After a brief section introducing smart biosensors, we proceed to describe biosensor systems that have been created and studied in our laboratory. They include optical, electrochemical, and piezoelectric-based systems designed to detect specific analyte molecules in solution.

Components of Biosensors

In terms of the simplest definition of its components, biosensors are typically thought to be comprised of a biological element (s) that is usually attached permanently to an underlying substrate. These are integrated with an appropriate signal transduction platform that provides a mechanism whereby the presence and usually the concentration of analyte being sensed by the biological element is converted into some type of quantitative electronic signal or output.

Immobilization Methods

Once the appropriate biological element has been identified and the platform chosen, a suitable surface attachment strategy must be devised. An effective attachment strategy should preserve the function and create a stable environment for the biological element, as well as facilitate and enhance coupling of the signal from the biological element to the platform to maximize the biosensors sensitivity to the analyte. There have been quite a large number and variety of physical and chemical attachment methods used in developing biosensors and we will not review them here. However, we will focus on one of our major thematic approaches in the Center for Intelligent Biomaterials (Knoph et al. 2007b), which has been to utilize polymeric thin films bound to the biosensors platform surface to immobilize biological elements. We have utilized both conducting and nonconducting polymers in various thin-film formats to carry out immobilization of different biological elements. As an example, some of these strategies have involved chemical synthesis of conducting polymers derivatized with biotin, which functions as a pendant biological

attachment site. General single-step reaction used widely to create derivatized conducting polythiophenes is presented, we outline the two-step synthesis of a representative biotinylated (B) polythiophene copolymer, B-PUMT, where the biotin is connected by an ester linkage to the pendant CH_2OH group on the thiophene polymer backbone (Marx et al. 1994). These biotin ligands recognize and bind each of the four subunits of the tetrameric proteins streptavidin and avidin with affinity constants approaching those of covalent bonds, around 10^{15} per M (Wilchek and Bayer 1989). These two proteins have been widely used to covalently derivatives biological elements can be immobilized upon the biotinylated polymer chain, following prior immobilization of the polymer upon the surface of the chosen platform (Knoph et al. 2007b).

During the last several years, optical biosensors gradually are involved in aflatoxins analyses. They are based on optical fibers or surface plasmon resonance.

Biosensors are devices, ideally small and portable, that allows the selective measurement of chemical and biochemical analytes. They consist of two components: the transducer and the chemical recognition element. Chemical recognition is accomplished by exploiting the natural selectivity of biochemical species such as enzymes, antibodies, chemoreceptors and nucleic acids. In the presence of the analyte, these agents, immobilized at the surface of the transducer, cause change in measurable property in the local environment. The transducer converts the biochemical signal in electronic signal. Transducers may measure electrochemical, optical, thermal or adsorption processes that change in the presence of analyte.

Polyamidoamine Dendrimers as Potential Substances for Obtaining Biosensors

The rapid detection of organic and biological compounds in the environment and in medicine is of a particular interest for identifying different biologically active substances of metabolic or industrial origin. Fluorescence as a signal for identifying the presence and quality of different compounds is widely used. For this purpose a number of appropriate sensors has been developed using fluorophores with different chemical structures. Among them polymer sensors are considered as quite promising. Synthesis of linear polymers has as a result obtaining of final products with heterogeneity that could influence the optical performance of the polymer sensor.

Poly (amidoamine) dendrimers (PAMAM) are new interesting class of star polymers. They have mono dispersive, well defined and developed three-dimensional structures comprising functional groups at high concentration. In its core they have amidic groups. Structurally modified PAMAM dendrimers with 1,8-naphthalimide derivatives are fluorescent dendrimers and they can be applied as effective and selective sensors for different metal cations and protons in organic solvents. This property can be transferred to other polymer matrixes (for example textiles).

In this study we present our first results about the possibility for the incorporation of bio-receptors into some new PAMAM dendrimers. Dendrimers under study contain peripherally bonded 1,8-naphthalimide derivatives (Figs. 1 and 2).

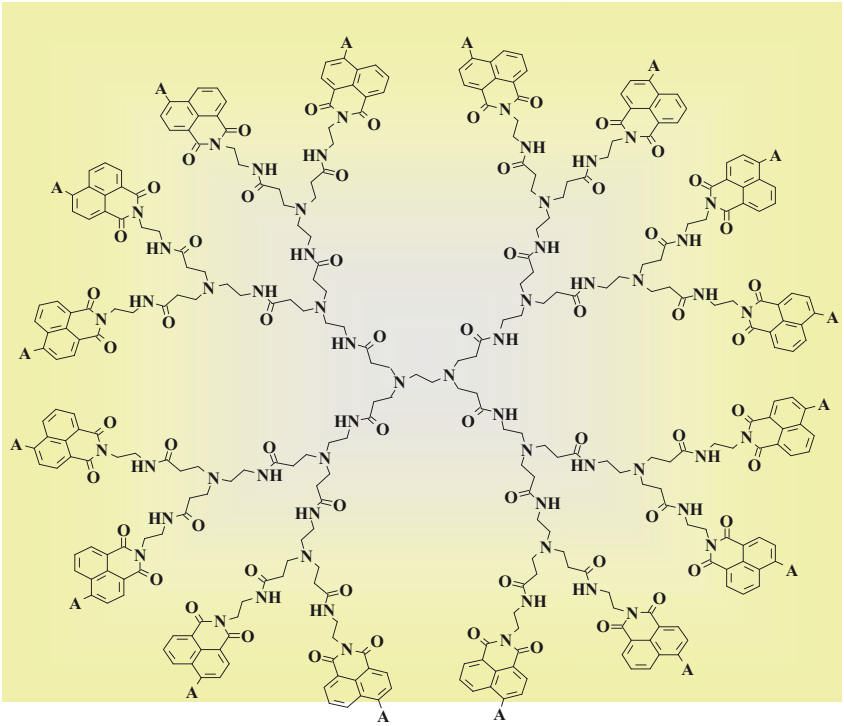


Fig. 1 Chemical structure of dendrimer



Fig. 2 Picture of dendrimer membrane with covalently immobilized fluorescent dye and enzyme

These dendrimers have been additionally modified with biologically active molecules, which can react with the available amidic functional groups. Result from the first photophysical and biochemical investigations of the new structurally modified dendrimers are discussed. The comparison between the bio-modified PAMAM dendrimers and some linear polymers is given (Grabchev et al. 2007a).

Photophysical and Biological Properties of Fluorescent PAMAM Dendrimer

Dendrimers are a relatively new category of star-shaped polymers. They are monodisperse, perfectly branched well-defined polymers, possessing a number of functional end groups that allow different modification of the dendrimers. A great deal of attention has been paid to this class of macromolecules owing to their new form of structure organization, which combines the properties of low, and high molecular weight compounds.

In this study we present the result on the preparation of fluorescence PAMAM dendrimer –acetyl cellulose membrane by spin coating method. Fluorescent PAMAM contained chemically bonded fluorescent dye. Glucose oxidase widely applied in clinical diagnostic was used as a model enzyme widely applied in order to check the possibility to use the membrane as a biosensor. The enzyme was covalently immobilized on the dendrimer membrane by preliminary oxidation of carbohydrate residues of the glucose oxidase with periodic acid. The chemical modification of PAMAM was supposed to occur in the dendrimer core where the enzyme can react with amidic functional groups. Thus the model enzyme and the immobilized dye form a stable conjugate with a high activity. Lipoxigenase, peroxidase and aflatoxine antibodies were covalently immobilized.

Membrane obtained was 40 m thick and several photophysical properties including the photostability were investigated. Fluorescence intensity was studied as well and the effect of Cu^{2+} and Co^{2+} cations in water solution was measured.

Based on preliminary photophysical and biological investigations a conclusion can be taken that the dendrimer membrane possesses promising properties for applying in biosensor and immunosensor constructions with fluorescent detection (Grabchev et al. 2007b) (Fig. 3).

Magnetic Particles

Magnetic nanoparticles modified with organic molecules have been widely used for biotechnological and biomedical applications because their properties can be magnetically controlled by applying an external magnetic field (Osaka et al. 2006a). They offer a high potential for numerous biomedical applications, such as cell separation (Safarik and Safarikova 2002), purification of nucleic acid (Berensmeier 2006), hyperthermia (Lao and Ramanujan 2004) and immunosensors (Sheng-Fu Wang and Tan Yu-Mei 2007).

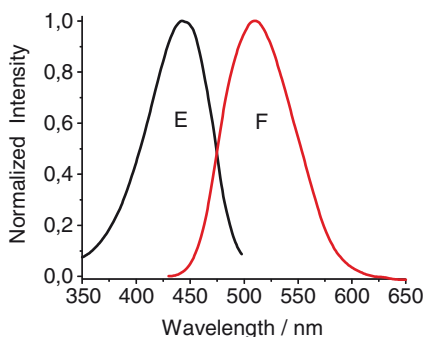


Fig. 3 Normalized excitation and fluorescence spectra of membrane

Magnetic nanoparticles as immobilization materials have the following advantages: (1) more specific surface area obtained for the binding of larger amounts of biomolecules; (2) lower mass transfer resistance; (3) selective separation of the immobilized biomolecules from a reaction mixture on application of a magnetic field. Among these materials, Fe_3O_4 magnetic nanoparticles are the most commonly studied. Fe_3O_4 magnetic nanoparticles have good biocompatibility, strong superparamagnetism, low toxicity, and an easy preparation process, and their use in biosensors has already shown attractive prospects (Sheng-Fu Wang and Tan Yu-Mei 2007).

Horseradish peroxidase (HRP) is an important peroxidase that contains heme, which is the protein active site with the resting state of the heme iron, Fe(III) , as prosthetic group. It can catalyze the H_2O_2 dependent one-electron oxidation of a great variety of substrates, and has been commonly employed to construct H_2O_2 biosensors (Hai-Li Zhang et al. 2008). Peroxidase is the most frequently used enzyme for the construction of immunosensors. Successful immobilization of horseradish peroxidase on modified magnetic particles and their employment in the amperometric biosensors are mentioned in (Hai-Li Zhang et al. 2008; Yu et al. 2006).

The objectives of this our study were to investigate the properties of immobilized horseradish peroxidase on magnetic particle coated with copolymer of acrylamide and acrylonitrile, used furdur in fibre optic smart biosensor constructions with simultaneously immobilized different antibodies for aflatoxines (Fig. 4).

Detection of Foodborne Pathogens Using Optical Biosensors

Biosensors use a combination of biological receptors and physical or chemical transducers, which represent a new and unique technology with great potential to meet the need for rapid detection of low levels of biomolecules (Baeumner 2003; Bhunia and Lathrop 2003; Ivnitcki et al. 1999; Vo-Dinh et al. 2001). Optical sensors measure a parameter of the reaction between a receptor and an analyte

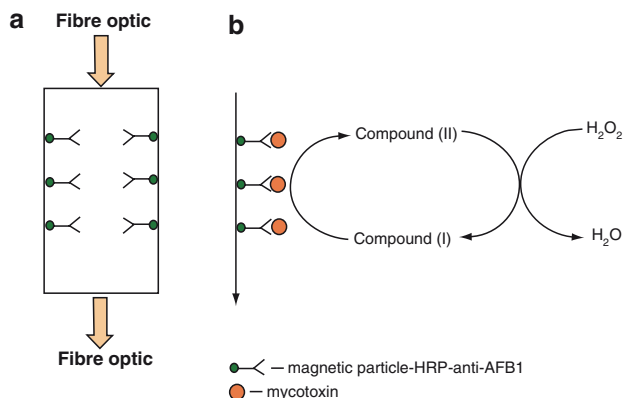


Fig. 4 The future immunosensor with fibre optic (a) and reaction with mycotoxin (b)

as a quantifiable optical signal. Optical biosensors offer the advantages of noninvasive, nondestructive, continuous, and simultaneous multianalyte detection (Taitt et al. 2005). Optical systems do not interfere with metabolism, and thus *in vivo* measurements are possible. In this chapter, some applications of the following optical sensing principles in foodborne pathogen detection are discussed: (1) SPR, (2) resonance mirror (RM), (3) fiber-optic biosensor, (4) array biosensor, (5) Raman spectroscopy, and (6) light-addressable potentiometric sensor (LAPS) (Knoph et al. 2007c).

Biosensors Based on Surface Plasmon Resonance (SPR)

The surface plasmon resonance is a measurement of the changes in the mass concentration that appear on the surface of the sensor and are due to the molecule binding. At specific wavelength and angle of incidence the free electrons in the thin metal film of the sensor surface will absorb the light and the intensity of the reflected light will decrease.

The angle of incidence in this process changes as mass concentration of the sensor surface changes. The variations in the mass concentration measured by the sensor are due to the binding and dissociation of interacting molecules. In this case those molecules are between the immobilised agent (the mycotoxin) and a specific antibody added to the sample.

There are immunochemical biosensors applied for mycotoxin assays. Gaag at all developed a method based on surface plasmon resonance for measuring of four different mycotoxins – aflatoxin B₁, zearalenone, ochratoxin A and fumartoxin B₁ (Paepens et al. 2004).

These systems utilize surface Plasmon resonance (SPR) and the Resonant Mirror, respectively and have proved especially convenient for the study of affinity

kinetic constants and screening of binding partners without the need for labeled molecules.

Surface Plasmon resonance occurs when plane – polarized light is internally reflected at an interface between media of different refractive indices, separated by thin metal film. Light energy is absorbed by the metal film but is at a maximum at a specific input angle. Typically it is possible to follow the binding of a macromolecular antigen to a surface immobilized antibody without need a label (Maragos 2002). Daly et al. (2000) gives an example for aflatoxine determination by a specific antibody too.

Sensor systems based on SPR now are commercially available (Nagata and Handa 2000).

Some of the latest investigations show that on the base of smart polymers with simultaneously immobilized biorecognition molecules as enzymes, antibodies, nucleic acids with fluorescent dyes can be constructed biosensors by means of SPR measurements.

In this method the fixed concentration of the mycotoxin specific antibodies are mixed with a sample containing some concentration of the mycotoxin. Then the mixture is passed over the sensor surface on which the mycotoxin is immobilised.

The surface plasmon resonance has a number of benefits.

- A very small volume of the sample is needed
- The metal chip can be reused
- The kinetics of the reaction antibody – antigen can be detected
- A number of analytes can be detected
- The method is easy to use

SPR was developed for several mycotoxins. Different mycotoxins have been found simultaneously within a period of 25 min. with a detection limit for aflatoxin B₁, zearalenon, ochratoxin A, fumonisin B₁ and deoxinivalenol respectively – 0.2, 0.01, 0.1, 50, 0.5 $\mu\text{g L}^{-1}$ (Zheng et al. 2005).

Fiber Optic Immunosensor

The Fiber optic immunosensors are the second major generation of biosensors. Many of the biosensors are a mini version of the classical spectrophotometry. The most frequently used is the fiber optic chain or membrane, covered with a biological element. The optical biosensors are only optical sensors where the element to be detected is of biological origin. This provides greater specificity for the analyte.

An evanescent wave is generated in the space between the optical fiber and the outside lower refractive index material. Fluorescent molecules in this region can absorb energy from the evanescent wave and fluoresce. A portion of the fluorescence will be coupled back into the fiber and can be detected. By immobilizing antibodies to the surface of an optical fiber, fluorescent interference from the bulk solution is almost completely eliminated. The signal generated in the assay corresponds

to the toxin concentration but varies depending on the assay format (Eaton and Groopman 1994).

Studies of the detection of fumonisin B1 by using a fiber-optic immunosensor showed that the sensor could detect fumonisin B1 in a quantitative range of 10–1,000 ng mL⁻¹ with a limit of detection of 10 ng mL⁻¹.

Benefits of the Method

- High specificity
- Ease of miniaturization
- Real-time monitoring
- Adaptability for remote sensing

However, the method may have limitations in sensitivity. Using immunoaffinity column clean up can enhance the sensitivity. Additionally, solvents may affect the accuracy of the method because they can change the refractive index of a medium (Zheng and Richard 2006).

Fibre Optic Oxygen Enzyme Biosensor

The purpose of this study was to design biosensors on the basis of covalently immobilised lipoxygenase and oxygen electrodes.

The measurements performed with the two types of biosensors show that the linear range of this type of enzyme electrodes using natural oxygen mediator manifest a wide range of measurement. The immobilised enzyme showed high operative stability, which makes the measurements easily reproducible. Both electrodes have very good correlation coefficients and a small standard deviation.

The performed investigations prove that they can be used for measuring real food (Yotova and Marinkova 2008).

Lipoxygenase is iron that contains dioxygenase and is found in plants and mammals, also produced by some microorganisms. It has been established that the lipoxygenase from soybeans can catalyse the oxidation of a wide range of endogenous chemicals (Kulkarni 1996).

For the first time bioactivation of aflatoxin B1 from soybeans lipoxygenase was demonstrated by Liu and Massey (Liu and Massey 1992). Datta and Kulkarni have also investigated it, but in the presence of linoleic acid which is the natural substrate of the enzyme (Datta and Kulkarni 1994).

Obtaining Polymer Membrane

Powder copolymer of acrylonitrile and acrylamide (200 mg) was diluted in 5 mL of dimethylformamide and the mixture was poured in petri and kept there until

dimethylformamide evaporated completely. The obtained membrane was activated with 50 mL phosphate buffer pH = 7 and 12.5 mL formaldehyde for 4 h at $t = 45^{\circ}\text{C}$ in a closed container being constantly stirred. The activated membrane was carefully washed with distilled water until there was no formaldehyde.

Enzyme Immobilization Procedure

The immobilization was performed with 1% solution of microbial lipoxygenase [E.C 1.13.11], from *Pseudomonas* sp., produced by Biovet, Peshtera, Bulgaria stirring it constantly in a closed container at $t = 25^{\circ}\text{C}$ for 21 h in phosphate buffers with pH = 5, 6 and 8 and acetate buffer pH = 4. After the completion of the procedure, the carrier granules were washed many times in buchner funnel with distilled water and 1 M NaCl until there was no absorption at $\lambda = 280$ nm.

Measurements of the Kinetics of the Enzyme Reaction

The measurements were performed spectrophotometrically at $\lambda = 234$ nm using substrate solutions with different concentrations as well as by accounting for the O_2 consumption in the enzyme reaction.

The latter procedure was performed with an electrode for O_2 . An optical sensor was constructed on the immobilised enzyme based on spectrophotometer with optical fibres and probe for oxygen measurement AvaSoft – Oxy, AvaSpec US B1 (Avantes – the Netherlands).

Using substrate solutions in the range 0.4–3.5 μM the dependency of the speed of the reaction on the concentration of the substrate (linoleic acid) was tracked.

The microbial lipoxygenase was purified using ultrafiltration. In this purification the specific activity of the enzyme preparation increased three times compared to the commercial preparation 2.214 E mg^{-1} (U mg^{-1}) and 860 E mg^{-1} , respectively.

The microbial lipoxygenase was immobilised on polymer membrane, which was done according to the procedure in the methodological part. Examination of the obtained conjugate for protein contents was performed spectrophotometrically in the way it was done for the enzyme immobilised on polymer granules. The relative activity of the immobilised enzyme is 65% and the protein contents 5.4 mg protein/g dry carrier.

In the Table 1 the main characteristics of free and immobilised at pH = 5, 6 microbial lipoxygenase are shown.

The stability of the microbial lipoxygenase immobilised on polymer carrier was determined by performing measurements of the activity with the same carrier with covalently bound enzyme for a period of 20 days (Fig. 5). As can be seen from the figure, the enzyme preserved its activity almost 100% for the whole period of investigation.

The biosensors were constructed with optical sensor for oxygen based on optical fibre Avaspec –Oxy (Avantes, Holland). The membrane with immobilised enzyme was attached to the electrodes with a dialysis membrane and a pipette tip.

Table 1 Catalytic properties of lipoxygenase from *Penicillium* sp

Enzyme	Amount of bound enzyme, mg g ⁻¹ dry carrier	Residual activity (%)	pH optimum	t (°C) Optimum	K _m [M]
Free	–	–	8.3	35	6 × 10 ⁻⁶
Immobilized on granulated carrier	2.9	94	7.5	35	1 × 10 ⁻⁶
Immobilized on membrane	5.4	65	7.5	35	–

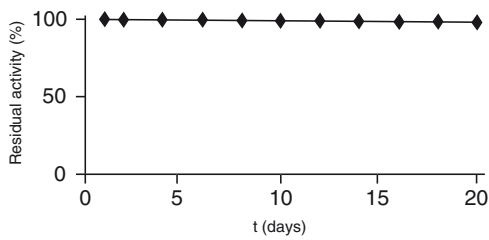


Fig. 5 Stability of microbial lipoxygenase immobilized on granulated polymer carrier in time

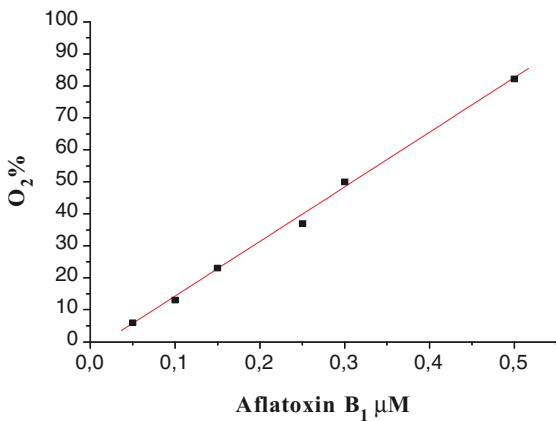


Fig. 6 Standard curve for determination of Aflatoxin B1 using biosensor AvaSpec – Oxy

In a measurement cell 20 mL of 0.1 Tris-buffer with pH 7.2 the respective sensor was incubated for 3 min with aflatoxin B1 in concentration range of 0.0 5–0.5 μM previously diluted in dimethyl sulphoxide. After 3 min of incubation at 37°C 0.1 mL linoleic acid was added to the samples so that the final concentration of the sample was 4 μM. Based on the measurements, standard curves were constructed for the optical enzyme electrode (Fig. 6).

Table 2 Statistical processing of standard curves

Electrode	Correlation coefficient	Standard deviation	Kohren criteria
AvaSpec – Oxy	0.09978	0.02014	0.1200

The obtained standard curves were statistically processed and are shown in Table 2. above.

The performed investigations prove that they can be used for measuring real food.

Application of Magnetic Particles in the Design of Biosensors

Nano particles are an attractive target due to their unique characteristics. Especially the magnetic nano particals are of interest not only because of their properties, but also because of their potential application in many fields as density particles, magnetic fluids and biomedical material for highly sensitive immune analyses (Osaka et al. 2006b).

An immunological method has been developed based on AFB₁ antibody-functionalised composite magnetic nano particles based on Fe₃O₄/SiO₂. For this purpose anti-AFB₁ is immobilized covalently on the surface of the nucleus. Te selectivity, reproducibility and stability of the immunological system are within the allowable error. Compared to the conventional ELISA the proposed immunosystem is simple and rapid (Li Wang and Xian-Xue Gan 2009).

Magnetic particles coated with copolymer of acrylamide and acrylonitrile has been prepared. Those particles were used as a matrix for a covalent binding of peroxidase. The periodic-oxidized enzyme was bound to the matrix by immobilization procedure at 4°C for 18 h. The immobilized enzyme showed relative activity of 86%. The following results were obtained for pH and optimum temperature of the immobilized enzyme – 7.0°C and 30°C, respectively. The analysis of the kinetic parameters of the immobilized enzyme showed values of $V_{\max} - 0.0517 \text{ M} \times 10^{-6} \text{ s}^{-1}$ and $K_m - 2.3 \times 10^{-4} \text{ M}$ (Yotova et al. 2008).

The copolymerization of acrylonitrile and acrylamide occurs by a free radical mechanism with an initial stage of initiation resulting in free radicals. FeSO₄, NaS₂O₅ and (NH₄)₂S₂O₈ are used as initiators. The following reagents were added to obtain copolymer containing 20% acrylamide: H₂O-174 mL, acrylonitrile-12 g, acrylamide-3 g, NaS₂O₅ – 0.125 g, FeSO₄ – 0.125 g, (NH₄)₂S₂O₈ – 0.125 g. Copolymerization takes 2 h at room temperature under constant stirring. The copolymer is filtered and profusely flushed with water in buchner funnel, and finally with methanol. It is dried in a vacuum oven at 50°C. The dried polymer is dissolved in dimethylformamide and then using a syringe it is poured drop by drop in a solution of ethanol and water (1:4) and surfactant. The spherical particles thus formed have an average diameter of ~1 mm. The water contents of the carrier thus formed were determined to be – 80%. The spherical particles with magnetic properties were obtained by producing a 10% solution of copolymer containing 5% magnetite.

Horseradish peroxidase (HRP, EC 1.11.17) was obtained from Fluka. The oxidation of carbohydrate moieties of enzyme with periodic acid (0.04 mM in 0.05 mM acetate buffer, pH 5.0) was performed according to Zaborsky and Ogletree (1974). The unreacted periodic acid was removed with 0.025-ethylene glycol. The oxidized enzymes were dialyzed against 50 mM phosphate buffer with pH 6.0 for 18 h.

The immobilization of HRP was performed in the following manner: 20 mL of oxidized dialytic solution of peroxidase was added to 1.0 g of absolutely dry particles. The process was implemented by constant stirring with a magnetic stirrer for 18 h at $t = 4^{\circ}\text{C}$, in dark.

The copolymer obtained according to the methodological part has a spherical shape with magnetic properties and average diameter of 0.6–0.8 mm (Fig. 7).

The magnetic particles have a high magnetic stability, which is a necessary condition to obtain the desired shape and size relevant to the requirements of the experiment. The mechanical robustness of the particles was preserved throughout the process.

The magnetic particles coated with copolymer were used as a matrix for covalent binding of the peroxidase.

The results from the investigations on the activity of the free and immobilized peroxidase at pH = 6 are shown in Table 3.

It can be seen from the table that the enzyme preserves its high relative activity.

The pH optima of free and immobilized peroxides were performed following the methodological part and are given in Fig. 8.



Fig. 7 Picture of received magnetic particle

Table 3 Investigation on the activity of the free and immobilized enzyme

Enzyme	Specific activity	Relative activity %	Amount of bound protein mg g^{-1} dry carrier
	U mg^{-1}		
Soluble enzyme	210	–	–
Immobilized enzyme	181	86	0.613

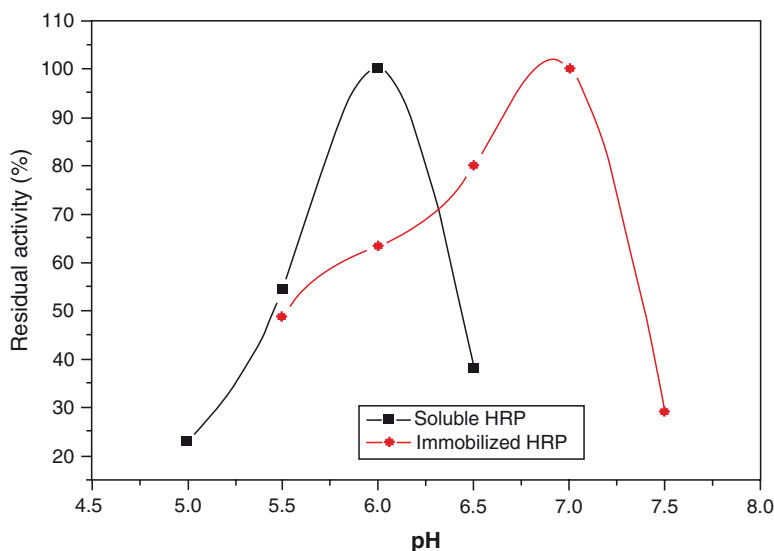


Fig. 8 Effect of pH on the activity of soluble and immobilized peroxidase

When examining the effects related to the pH optimum the effect of the matrices on the enzyme should be considered. In this case the pH optimum increases to 7.0. This change occurs due to the irregular distribution of the protons caused by the positive or negative loads of the employed carrier as well as the presence of diffusion limitations. In this case it can be assumed that the magnetic particles affect the enzyme molecules.

The experiments on the temperature optimum also showed a shift of the optimum of the immobilized enzyme at 30°C (Fig. 9).

The immobilization of enzymes to different carriers usually leads to a change in their kinetic behaviour characterized by different constants, the most important being the Michaelis constant (K_m).

Table 4 shows a comparison between the kinetic parameters of soluble and immobilized peroxidase and on magnetic particles.

In an investigation similar to ours, the following values for K_m of free and immobilized peroxidase, respectively 5.5 and 3.6 mM were obtained in immobilization on chitosan. The results from this investigation show that the immobilized enzyme has a greater affinity to the substrate compared to the free enzyme (Mohamed et al. 2008).

Magnetic particles coated with copolymer of acrylamide and acrylonitriles were successfully obtained. The peroxidase immobilized on these particles preserves its high relative activity. It was established that the catalytic parameters of the immobilized peroxidase change as follows: $t_{opt} - 30^\circ\text{C}$, $pH_{opt} - 7$ и $K_m - 2.3 \times 10^{-4}$.

Based on the results obtained from the covalent immobilization of peroxidase to magnetic particles coated with copolymer of acrylamide and acrylonitrile we can outline the steps in construction of a smart biosensor for mycotoxins.

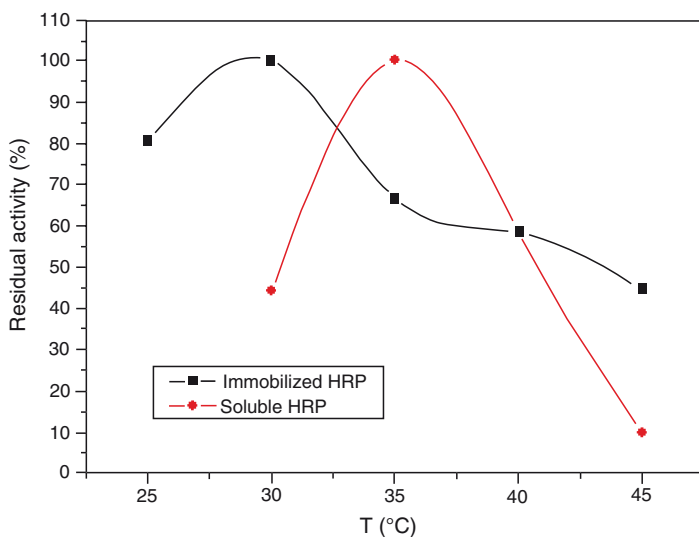


Fig. 9 Effect of temperature on soluble and immobilized peroxidase

Table 4 Kinetic parameters of soluble and immobilized peroxidase

Enzyme	Km [M]	Vm [$M \times 10^{-6} s^{-1}$]	R ²
Soluble enzyme	1.19×10^{-3}	—	—
Immobilized enzyme	2.3×10^{-4}	0.0517	0.99885

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References

- Baemner AJ (2003) Biosensors for environmental pollutants and food contaminants. *Anal Bioanal Chem* 377:434–445
- Berensmeier S (2006) Magnetic particles for the separation and purification of nucleic acids. *Appl Microbiol Biotechnol* 73:495–504
- Bhunia AK, Lathrop A (2003) Pathogen detection, food-borne. McGraw-Hill yearbook of science and technology. McGraw-Hill, New York, pp 320–323
- Daly S, Keating G, Dillon P (2000) Development of surface plasmon resonance-based immunoassay for aflatoxin B (1). *J Agric Food Chem* 48:5097–5104
- Datta K, Kulkarni A (1994) Oxidative metabolism of aflatoxin B₁ by lipoxygenase purified from human term placenta and intrauterine conceptual tissues. *Teratology* 50:311–317
- Eaton D, Groopman JD (1994) The toxicology of aflatoxins. Academic, New York, pp 383–426
- Grabchev I, Betcheva R, Yotova L (2007a) Polyamidoamine dendrimers as potential substances for obtaining biosensors. COST Action 868- Biotechnical fictionalization of renewable polymeric materials, Sitges, Spain, 16–17 April 2007

- Grabchev I, Betcheva R, Yotova L (2007b) Photophysical and biological properties of fluorescent PAMAM dendrimer. Biotechnical fictionalization of renewable polymeric materials, Graz, Austria, 12–14 September 2007
- Hai-Li Zhang, Guo-Song Lai, Han De-Yan, Yu Ai-Min (2008) An amperometric hydrogen peroxide biosensor based on horseradish peroxidase on an electrode modified with magnetic dextran microspheres. *Anal Bioanal Chem* 390:971–977
- Ivnitski D, Abdel-Hamid I, Atanasov P, Wilkins E (1999) Biosensors for detection of pathogenic bacteria. *Biosens Bioelectron* 14:599–624
- Karova E (1998) Microbiology. Academic Publishing house, Plovdiv
- Knoph GK, Bassi AS, Geng T, Bhunia A (2007a) Optical biosensors in foodborne pathogen detection, foodborne pathogens. In: Knopf GK, Bassi AS (eds) Smart biosensor technology. CRC Press/Taylor and Francis group, Boca Raton, FL, pp 506–507
- Knoph GK, Bassi AS, Marx KA (2007b) Toward understanding the intelligent properties of biological macromolecule-implications for their design into biosensors. In: Knopf GK, Bassi AS (eds) Smart biosensor technology. CRC Press/Taylor and Francis group, Boca Raton, FL 4–6
- Knoph GK, Bassi AS, Geng T, Bhunia A (2007c) Optical biosensors in foodborne pathogen detection, detection of foodborne pathogens using optical biosensors. In: Knopf GK, Bassi AS (eds) Smart biosensor technology. CRC Press/Taylor and Francis group, Boca Raton, FL 507
- Kulkarni AP (1996) Role of xenobiotic metabolism in developmental toxicity. In: Hood RD (ed) Handbook of developmental toxicology. CRC Press, Boca Raton, FL, pp 383–421
- Lao LL, Ramanujan RV (2004) Magnetic and hydrogel composite materials for hyperthermia applications. *J Mater Sci Mater Med* 15:1061–1064
- Larinov OG, Pisareva O Yu (1997) Structure of chemical compounds methods of analysis and process control. *Pharmaceutical* 31(7):49–53
- Li Wang, Xian-Xue Gan (2009) Biomolecule-functionalized magnetic nanoparticles for flow-through quartz crystal microbalance immunoassay of aflatoxin B1. *Bioprocess Biosyst Eng* 32:109–116
- Liu L, Massey T (1992) Bioactivation of aflatoxin B₁ by lipoxygenases, prostaglandin H synthase and cytochrome P450 monooxygenase in guinea-pig tissues. *Carcinogenesis* 13:533–539
- Maragos CM (2002) Novel assays and sensor platforms for the detection of aflatoxins. *Adv Exp, Med Biol* 504:84–93
- Marx KA, Samuelson LA, Kamath M, Lim JO, Sengupta S, Kaplan D, Kumar J, Tripathy SK (1994) Intelligent biomaterials based on Langmuir-Blodgett monolayer films. In: Birge BR (ed) Molecular and biomolecular electronics. Advanced in chemical series, Vol. 240. American Chemical Society Books, Washington, DC, pp 395–412
- Maxwell CK, Diaz-Lano LG, Smith TK (2003) Mycotoxins in pet food. *Agric Food Chem* 54:9623–9635
- Mohamed SA, Aly SA, Mohamed TM, Salah HA (2008) Immobilization of horseradish peroxidase on nonwoven polyester fabric coated with chitosan. *Appl Biochem Biotechnol* 144:169–179
- Murphy PA, Suzanne Hendich PHD, Landgren C, Cory M (2006) Food mycotoxins: an update. *Appl Biochem Biotechnol Hypotheses Food Sci* 71(5):51–55
- Nagata K, Handa H (2000) Real-time analysis of biomolecular interactions – applications of biacore, 1st edn. Springer, Tokyo
- Osaka T, Matsunaga T, Nakanishi T, Arakaki A, Niwa D, Iida H (2006a) Synthesis of magnetic nanoparticles and their application to bioassays. *Anal Bioanal Chem* 384:593–600
- Osaka T, Matsunaga T, Nakanishi T, Arakaki A, Niwa D (2006b) Synthesis of magnetic nanoparticles and their application to bioassays. *Anal Bioanal Chem* 384:593–600
- Paepens C, De Saeger S, Sibanda L, Barna-Vetro I, Van Hove F, Van Peteghem C. (2004) A flow-through enzyme immunoassay for the screening of fumonisins in maize. *Analytica Chimica Acta* 523:229–235
- Rudolf K, Alexandra M (2007) Mycotoxin analysis: state of the art and future trends. *Anal Bioanal Chem* 387:145–148
- Safarik I, Safarikova M (2002) Magnetic nanoparticles and biosciences. *Monatshefte für Chemie* 133:737–759

- Santacroce MP, Conversano MC, Casalano E, Lai O, Zizzadoro C, Centoducati G, Crescenzo G (2007) Aflatoxins in aquatic species: metabolism, toxicity. *Rev Fish Biol Fisheries* 18:99–130
- Sheng-Fu Wang, Tan Yu-Mei (2007) A novel amperometric immunosensor based on Fe_3O_4 magnetic nanoparticles/chitosan composite film for determination of ferritin. *Anal Bioanal Chem* 387:703–708
- Taitt CR, Anderson GP, Ligler FS (2005) Evanescent wave fluorescence biosensors. *Biosens Bioelectron* 20:2470–2487
- Tcvetkova T, Danev S (2000) Analytical principles and procedures in the clinical laboratory. *Biomedical and Life Sciences* 9:15–20
- Valkov P (ed) (2003) Chemical food pollutants. Academic Publishing House, Plovdiv
- Vlahov S (ed) (2006) Microbiology. Academic Publishing House, Plovdiv
- Vlahov S, Ivanov A (eds) (1996) General microbiology. Academic Publishing House, Plovdiv
- Vo-Dinh T, Cullum BM, Stokes DL (2001) Nanosensors and biochips: frontier in biomolecular diagnostic. *Sens Actuators B Chem* 74:2–11
- Waldo GS (2003) Genetic screens and directed evolution for protein stability. *Curr Opinion Chem Biol* 7:33–38
- Wilchek M, Bayer EA (1989) Avidin-biotin technology ten years on: has it lived up to its expectations? *Trends Biochem Sci* 14:408–412
- Yotova L, Marinkova D (2008) Application of immobilized lipoxygenase in the biosensors design for aflatoxin B1. *BioPS'08* November 4–5, III.37–III.46
- Yotova L, Ivanov T, Mironova V, Chuchuranova V (2008) Investigation of the properties of immobilized horseradish peroxidase on magnetic particles. *BioPS'08*, November 4–5, III.29–III.36
- Yu D, Blankert B, Bodoki E, Bollo S, Vire J, Sandulescu R, Nomura A, Kauffman J (2006) Amperometric biosensor based on horseradish peroxidase-immobilized magnetic particles. *Sens Actuators B Chem* 113:749–754
- Zaborsky O, Ogletree R (1974) Immobilization of enzymes on porous silica supports. *J Biochem Biophys Res Commun* 61:210–216
- Zheng MZ, Richard JL (2006) A review of rapid methods for the analysis of mycotoxins. *Mycopathologia* 161:261–273
- Zheng Z, Humprey CW, King RS, Richard JL (2005) Validation of an ELISA test kit for the detection of total aflatoxins in grain and grain products. *Mycopathologia* 159:255–263