From Morphology to DNA-Chips and Proteomics: Developments in Analytical Methods to Ascertain Food Safety and Authenticity

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A major fear for anyone would be to serve one's family infected food that will cause illness. The media have greatly contributed to the public awareness on food safety by providing information about some disease-causing agents: prions in meat, viruses specially in shellfish, bacteria such as *E. coli* in hamburgers, *Salmonella* in shrimp and eggs, or *Listeria monocytogenes* in diary products, and parasitic worms such as trematodes and nematodes in fish. A second consumer's concern is fraud by mislabeling and substitution of expensive raw materials by cheaper ones. This type of fraud has become much easier to perform since most of the morphological characters necessary to recognize the species are removed in an increasing amount of retail food items. Although mislabeling may not seem as grave as infectious food, one may reasonably assume that the same actor who intentionally misrepresented a declaration of ingredients, could easily have given false information about product standards and control analyses, and thus offer a dangerous, infected or contaminated foodstuff. Environmental issues are often at the root of mislabeling and many products are mislabeled because they contain protected species that should not have been harvested in the first place.

Consumers have the right to know what they purchase and to be offered only safe food items. To ensure these two issues, *i.e.*, composition and safety, not so long ago, food products should have had to go through a series of very different analyses requiring quite different expertise and equipment. Nowadays, thanks to the development of molecular biological techniques and in particular the polymerase chain reaction, the same technical principles and equipment can be applied to answer questions about the ingredients used and the safety of foodstuffs. The principal requirement is knowledge of a DNA sequence characteristic for the target. This work will describe some of the applications of these modern techniques to ensure seafood safety and identity with an indication of the near-future trends.

People in northen Spain have a high regard for their traditional seafood which includes "merluza" (hake, *Merluccius merluccius*). The catches of this species have decreased in recent years, and its price increased. This has encouraged the introduction of other hake species into the market, including *Merluccius australis, M. capensis, M. gayi, M. hubbsi, M. polylepis* and *Macruronus novaezelandie*. This introduction, unfortunately, has often been fraudulent: purchasers were led to believe that they were buying the "real" merluza. The first impression when the fraud was discovered was that they were being fooled and given a product of a lower quality grading, which is not necessarily true. In the opinion of a fisherman who had tasted the "alternative" hake species, the organoleptic properties of the "new" were not inferior to the traditional one. Therefore, if these new species had been introduced with their own names and characteristics, customers would have got used to them as "new" or "alternative" products, and the prices of the different commodities would have adjusted themselves according to preferences and supply.

Similarly, some capsules containing fish oils, marketed in Norway as containing Norwegian salmon oil, were shown to contain oil from pelagic fish from the South American Pacific coast (Aursand *et al.*, 1999). The composition of the marine oils was not of inferior quality in the fraudulent product, but the right of the customers to correct information had been breached.

In both cases it was not only the purchaser who suffered damage, but also the primary producer. Fishermen and breeders in poorer regions could see their income increased if their products had been established as a specific market niche. The alternative, to introduce them *in* an existing niche - to use their raw materials as substi-

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tutes - automatically and unnecessarily diminishes their long term value and condemns them as second class products, an image which may be very difficult to change later. In many cases only the intermediary who knows what he purchases and mislabels, obtains a profit - albeit a short term one.

Fraud and mislabeling may also occur immediately after capture: this may be the case for different species whose edible parts have similar organoleptic characteristics, captured in the same location, when one is protected and the other is not. For example, only the fins from sharks are used for human consumption; the usual practice is to cut the fins immediately after capture and discard the rest of the animal. Since it is not possible to visually identify the species from the fins, it is very easy for the fishermen to declare that the fins belong to a non-endangered species. Similarly, there has recently been a debate about the extension of illegal mislabeling of demersal species in northeast Atlantic waters: each boat has a quota specifying how much of each species they are allowed to take. It has been claimed that for economical reasons - to spare fishing costs and effort - fish are mislabeled so that it appears as if the boat had captured its allocated quota of each species, regardless of what they really harvested.

The examples above illustrate how easy it is to incorrectly label products being brought to the market, and is intended to demonstrate the importance of correct labelling, since, for example, the absence of correct labels *per se* may constitute a health hazard to consumers intolerant or allergic to certain products. This suggest the need for independent expertise to authenticate the true content of food.

Consumers must also be protected against the presence of pathogens in their seafood (Figure 1). Fortunately, the same type of techniques used to detect the species used in the manufacture of seafood can be applied to identify pathogens, genes for antibiotic resistance and genes used in the manufacture of gene modified organisms.

The reminder of this introductory note sets the focus for this paper: Labelling, authentication, bacterial detection and seafood-borne illnesses.

There are, according US General Accounting Office (1996), four bacterial pathogens of major concern in food-borne diseases. Three of them were not even considered pathogenic species 20 years ago. The existence of prions, the infective proteins supposed to cause scrapie, mad-cow disease and a new variant of human Creutzfeld-Jacob disease, was discovered by S. B. Prusiner in 1982, but it was not widely accepted until the mid-90's.

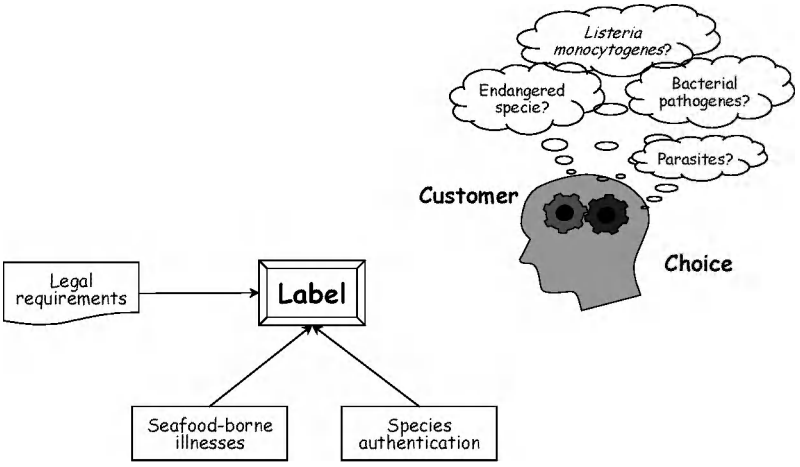

Figure 1 Consumers have to make decisions based on price, quality, expectations and information about the products

The discovery and characterisation of the pathogens and parasites has depended on their size and how different they are from each other. Bacteria, for example, were not observed until the development of the microscope by Antony van Leeuwenhoek in the 1680's. The existence of viruses, suspected since 1892 by Dimitry I. Ivanovski, could not be proven until the development of the electron microscope, in the 1940's, permitted the visualisation of individual viral particles. Nowadays, it is suspected that the real impact of viruses on food-borne infections has been greatly underestimated because the classical techniques for detection of viruses are complicated and expensive and many viruses cannot be cultured. Development of molecular techniques may make their detection more accessible and then their real effect on food safety may be mapped.

Parasites (primarily trematodes or flukes, but also nematodes) are a known source of seafood-borne diseases. Their study has been hampered because of their complicated life cycles, the fact that most of them do not cause severe disease or death in the short term, the difficulty of identifying the species and the life stage, and that they are endemic in countries with poor sanitary conditions. These are also usually poor countries where detection, treatment and eradication of this type of disease comes second to other priorities. Malaria is for example endemic in many of these countries. This has discouraged investment in the development of techniques for the specific detection and identification of these trematodes. The increase in international trade, travel, and new fashionable food habits, such as sushi and sashimi consumption in richer western countries where cooks have little experience in handling such delicate products, will undoubtedly force the development of more reliable screening techniques for the elimination of these organisms from the food chain. In addition, due to the depletion of the traditional fisheries (FAO, 2000b) and the need to cover the nutritional needs of increasing population, more and more of the seafood consumed comes from aquacultured species. It has been estimated that 85% of farmed fish comes from developing Asian countries, where parasite infections are endemic (Table 1).

Table 1 *Production of acquacultured species. About 63% of all aquacultured species and about 90% of all acquacultured fish species come from freshwater. Source: Brown, 2000; Howgate, 2000*

	Producer countries	% of the total fish farmed in 2000
Developing	China	68
	India	6.5
	Total produced by developing countries	85
Industrial	Japan	2.6
	United States	1.5
	Norway	1.3

It now seems generally recognised that it is impossible to fully guarantee the safety of food, but it is possible to reduce the likelihood of accidents happening by implementing HACCP-plans, good manufacturing (GMP) and good handling (GHP) practices. The first element of a HACCP-system is to identify the potential hazards and assess the risk (likelihood) of occurrence. Hazard is any biological, chemical or physical property that may cause a food to be unsafe for consumption, and it must be of such nature that its elimination or reduction to acceptable levels is essential for the production of safe food. Once the hazards are identified, one has to assess the *severity* - seriousness of the consequences if the hazard occurs in the product - and the *risk* - probability of the hazard occurring - of each hazard (NACMCF, 1992). Evidently then, design and implementation of HACCP-plans require a basic understanding of the hazards: the physiology, ecology, life cycle and impact on human health of pathogens. This information is also essential in order to estimate the risk imposed by the hazard. For example, if there is competitive flora or inhibitory substances in a product, the risk brought about by a particular hazard is diminished. It also requires the development of fast methods for targeting hazard microorganisms (FAO, 1999). Fortunately, with the advent of molecular techniques, in particular the polymerase chain reaction

(PCR[2]), and the increased accessibility of sequencing, there have been considerable advances during the last few years in the techniques to detect and identify unwanted organisms. This will help to reduce the impact of seafood-borne diseases in the population, to identify sources of contamination in natural reservoirs, processing environments and human carriers, and proceed accordingly to their elimination or treatment.

Nowadays, consumers purchase increasing amounts of products in which the morphological characteristics necessary for species identification are removed. In addition, alternative species are being introduced due to overexploitation of the traditional stocks and increased international trade. To protect consumer rights and endangered species, most countries have passed laws about correct labelling of retail products, which includes declaration of the species used in their manufacture and their origin (Anon, 2000; Commission of the European Communities, 2000). To ensure product identity, databases containing species-specific protein and DNA profiles and sequences are being developed in many countries.

Other elements that must generally be absent in seafood include: genes for antibiotic resistance, novel genes with undesirable properties in "traditional" genomes[3] and gene modified organisms. Antibiotic resistant strains of bacteria have increased in the recent years. This proliferation is attributed to the abuse of prophylactic and medical treatment with antibiotics by humans and in animal breeding.

This paper is intended to give a general view of the development in the techniques for detection and typing of pathogens in seafood, as well as the advantages and problems of the more modern molecular techniques. Secondly, it will address the methods available for authentication of seafood. As already mentioned, the same type of techniques used to identify pathogens can be applied to detect the species used in the manufacture of seafood, genes for antibiotic resistance and genes used in the manufacture of gene modified organisms.

Finally, mention is made of some of the most recent techniques in the field: DNA-chips and proteomics. The combination of computer manufacturing technology and molecular biology has been used to design DNA-chips or DNA microarrays (small chips or membranes to which probes complementary to the target to be detected have been attached). In this way it is possible to screen for several thousand targets in one analysis. Proteomics is devoted to the study of all the proteins - activities, regulation and interactions - in a cell or an organism, and is becoming increasingly important because it permits the assessment of stimuli of interest on many (theoretically thousands) proteins simultaneously. It allows the design of drugs for specific targets, the examination of host-infective agent interactions and, in an analogous manner, the interplay between pathogens and food matrices. With this type of information, the food industry could design food-grade antimicrobial products in the same way that the pharmaceutical industry designs specific drugs and vaccines.

It must also be borne in mind that the perceived risks do not always correspond to the real ones. Two examples of this are mad-cows disease *versus* trematode infections and GMO in foods *versus* dietary supplements. All European consumers are well aware of CJD a consequence of which is a decrease in the consumption of meat in most countries. Although the real cause of the new CJD disease is not fully known, the number of cases of CJD are counted by the hundreds, and the costs of eradication run to thousands of million euros. Trematode infections, on the other hand, are seldom heard of, there are no reliable test to detect these parasites and many consumers are not even aware of the fact fish may carry worms. Trematode infections affect tens of millions of persons and one of their consequences is the often fatal liver cancer (WHO, 1995). Similarly, there is not one single study showing any danger due to the consumption of foods containing GMO (Kryspin-Sørensen, 2000), while there is abundant documentation proving side-effects of dietary supplements. These include blood-clotting abnormalities, increased blood pressure, allergic reactions, cardiac arrythmia, exacerbation of autoimmune diseases, kidney and liver failures and death (Miller & van Doren, 2000). Nevertheless, politicians,

legislators and many researchers call for the implementation of the precautionary-principle, and claim labelling and moratoria (Bioteknologinemnda, 2000) on the introduction of GMO foods. On the other hand there does not seem to be much social concern - and indeed consumers use huge amounts of money in this market - about the many dietary supplements that continue to be sold over the counter in a completely chaotic and unregulated market (see Miller & van Doren, 2000).

Labels, trademarks and brands

Most products carry labels, trademarks or brands - be it on a fanciful printed wrapping or only as a small, simple handwritten sign in the shop-window. For many years now, governments in most countries regulate what sellers may or may not; shall and shall not, say or claim in their communication with customers. Hence, in addition to labels and signs, many packaged products will also carry compulsory information. We will deal with this in some detail below, but first some comments about brand labels, collective trademarks, "certificates of origin", and "other" quality labels.

Brands usually belong to a company (or consortium) who set their own standards for the brand. Companies that have established a reputation among their customers, and who intend to keep it, have also established certain standards - often more stringent than those demanded by the legislation - and deliver a certain level of quality that customers get used to expect (Kapferer, 1997). Companies use the brand name to distinguish themselves from other companies delivering similar products, and a major goal would be that customers use the brand name as the reference name or cue for their decision to buy.

Collective trademarks exist where the same mark is used by several independent producers that agree on common rules for producing and/or marketing the product. According to Menard & Valceschini (1999), collective trade-brands can be divided into:

1. "Simple" collective trademarks, in which there is no need of co-ownership, but there are rules established by the holders of rights that all users of the trademark (who often pay for the use of the name) must implement; and
2. "Certified" trademarks, which are those collectively owned, often by a group of producers, that contractually agreed on a set of requirements with a legal status, so that they can be enforced by a third party

Most collective trademarks are of the second type and generally lead to the definition of a "label" that ensures that a product has a set of specific characteristics. The characteristics may be agreed upon by the private partners alone or, as is more common in Europe, among the authorities, producers and consumers. Then a certifying organisation controls and approves producers according to how they follow the standards. The certifying organisation must be a formal institution, public, private, or mixed, but always external to the parties dealing with the transaction. It is very important that the certifying organisation be reliable and trustworthy, because failure to comply to the standards by just one member may damage the reputation of the trademark and of all the other companies sharing the standards. Approval is never definitive and may be withdrawn. One example is the "Label Rouge" developed in the French agricultural sector, which guarantees an objective level of high quality, based on specific production and manufacturing rules, some of which may refer to a regional tradition (Menard & Valceschini, 1999).

If a product contains a "certificate of origin" label, it means that it has obtained an official certificate of its origin and how it was produced (Rioja wines, Roquefort cheese). Other similar products originating from different regions are not granted such certificate. Recently, in the Basque Country, the sectors involved in the sale of fresh fish have agreed on the improvement of the labelling system proposed by the departments of Agriculture and Fisheries and Consumers and Tourism in order to discriminate fish that come on shore at Basque harbours. The objective is to create a "label" based on the

name of the fishing town that can be used as a quality symbol by the buyers.[4)]

Other quality labels may, or may not, be backed up by certain specifications about the product. For example, some products carry an "organic" label, meaning that they have been produced according to certain practices. It does not necessarily say anything about whether the product itself meets the standards for "organic", for example, about its content of pesticides. Other "organic" labels can mean that the product itself has fulfilled the requirements of an organic certifying body. Other labels may have no information at all about the product: in Norway, many products carry the sign of the association of allergic persons. A television program showed that most customers assumed that this meant that the product had been tested and declared "safe" for allergic people. In reality the label only means that the firms allowed to use the label had made a financial contribution to the association.

The expressed need for labels among French consumers of oysters differed depending on their knowledge about the products: older - who are big consumers of oysters - and people in the coastal cities, rely basically on their knowledge, praise the products of the region and do not wish labels or quality marks. Inhabitants of bigger cities pay higher prices for a certain appellation, "Marenes Oléron", although they did not know the specific characteristics of the product. The trend among distributors paralleled that of consumers: 85% of the inland distributors, but only 36% of the coastal ones, would rely on a certified quality approach (Erwan & Paquotte, 1998). On the other hand, to those Norwegian consumers included in the study of Norberg (2001), who lacked knowledge of certification marks, such labels did not have much value. However, they all showed great faith in the food control authorities and were not troubled about the safety of the products. This may be because Norway is a small and rather closed food market, where consumers have access mainly to national products. Therefore, for these consumers, the fact that the products were on the shelves was probably sufficient guarantee of their approval - and therefore certification of a minimum level of quality - by the health authorities.

Norberg's results basically agree with those of Wessells *et al.* (1999) about consumer preferences for "ecolabelled" seafood. An important ecolabelling initiative, with support from important major supermarket chains is underway in many countries: the companies wishing to sell products from accredited fisheries will be able to purchase the right to ecolabel their products to inform the consumer that the fish was harvested from a sustainable source. Wessells *et al.,* (1999) found that the consumer's acceptance of the ecolabel depended on the gender (women declaring being more prone to buy ecolabelled products), price of the commodity, frequency of seafood consumption, the budget allocated to seafood and, primarily, trust of the consumers in the organism granting the label and knowledge about the status of the fisheries.

In summary, labels and quality certificates are only correctly understood and used by consumers if they are linked to a basic understanding of what they are and what they mean. It is also very important to keep in mind that to achieve a "name", *i.e.,* a good and solid reputation, may be a long task, requiring a significant investment in marketing and information. On the other hand, only one scandal - a food poisoning or mislabeling incident, badly handled - may be enough to ruin a hard earned reputation.

As mentioned in the introduction to this paragraph, product labelling also has some legal requirements - in the next paragraph we will briefly discuss the requirements in the European Community and in USA.

Legal requirements to labelling in EU and USA

One major difference between the philosophies behind the labelling of food products between the EU and the USA is reflected by the recent debate on the labelling of food containing ingredients from gene modified organisms (GMOs) (see Ethics in Food and Agriculture Series. No.2 , 2000). In the USA the labels are required to provide meaningful information and to warn and instruct the consumer. Misleading or unnecessary information is considered to conflict with the

right of consumers to be able to choose wisely, and to lessen the effectiveness of essential information of the label. Therefore, if GMOs are equivalent to their traditional counterparts in terms of nutrition, composition or safety, labelling is considered unnecessary and perhaps misleading. In the EU, on the other hand, labelling is intended to ensure the consumers' right to know any fact they may deem important to exercise the right to choose. Therefore, labelling of foods manufactured from GMOs is obligatory under the EC Regulation number 1139/98 of the 26th of May 1998, from the European Council of Ministers and Agriculture of the EU. This regulation went into effect in the whole EU on September 3rd, 1998. A later regulation (no. 49/2000) has set up a tolerance level of 1% for each single ingredient to account for the problem of adventitious contamination, provided that the operator has taken appropriate steps to avoid the use of GMOs as a source.

Legislation in the European Union

Authentication of fishery products in the European Community is based in three core premises as indicated in Directive 2000/13/EC of the European Parliament (and of the Council of 20 March 2000 on the approximation of the laws of the Member States):

- The prime consideration for any rules on the labelling of foodstuffs should be the need to inform and protect the consumer (Article number 6)
- Detailed labelling, in particular giving the exact nature and characteristics of the product which enables the consumer to make his choice in full knowledge of the facts, is the most appropriate since it creates fewest obstacles to free trade (Article number 8)
- The rules on labelling should also prohibit the use of information that would mislead the purchaser or attribute medicinal properties to foodstuffs. To be effective, this prohibition should also apply to the presentation and advertising of foodstuffs. (Article number 14)

Further, it is specified in the second article that labelling must not be misleading to the purchaser, among other properties, as to: *"the characteristics of the foodstuff and, in particular, to its nature, identity, properties, composition, quantity, durability, origin or provenance, method of manufacture or production"* (article number 2). Article number 3 follows this up by giving a list of compulsory particulars for the labelling of foodstuffs, including *"list of ingredients"* and *"particulars of the place of origin or provenance, where failure to give such particulars might mislead the consumer to a material degree as to the true origin or provenance of the foodstuff"*. However, it is specified that as an alternative to a list of ingredients, naming all species of fish where *"the fish constitutes an ingredient of another foodstuff and provided that the name and presentation of such foodstuff does not refer to a specific species for fish"*, the ingredient may be designated by the name of the category, *i.e.*, "fish", rather than by the specific name.

Legislation in the USA

In the USA, the Food and Drug Administration (FDA) is responsible for assuring that foods sold in the United States are safe, wholesome and properly labelled. The Federal laws governing food products under FDA's jurisdiction are: *The Federal Food, Drug, and Cosmetic Act* and *The Fair Packaging and Labeling Act.*

The Fair Packaging and Labeling Act, Title 15 - Commerce and Trade , Chapter 39 - Fair Packaging and Labeling Program '1451, Congressional Delegation of Policy states that: *"Informed consumers are essential to the fair and efficient functioning of a free market economy. Packages and their labels should enable consumers to obtain accurate information as to the quantity of the contents and should facilitate value comparisons. Therefore, it is hereby declared to be the policy of the Congress to assist consumers and manufacturers in reaching these goals in the marketing of consumer goods."*

The regulations specify that the commodity shall *"bear a label specifying the identity of the commodity and the name and place of business of the manufacturer, packer, or distributor"* (*ibid.*)

About labelling of food in packaged form, it is stated that the principal display panel shall: *"bear as one of its principal features a statement of the identity of the commodity".* Such statement of identity shall be in terms of:

1. The name now or hereafter specified in or required by any applicable Federal law or regulation; or, in the absence thereof,
2. The common or usual name of the food; or, in the absence thereof,
3. An appropriately descriptive term, or when the nature of the food is obvious, a fanciful name commonly used by the public for such food.

In addition, nutritional information must be provided on the label for almost all packaged food products intended for retail sale. The regulations specify which nutrition information that must be on the label and the format in which it is to be presented. Regulations also prescribe conditions under which nutrient content claims or health claims may be made on the label or in labelling for a food product.

It is estimated (Kurtzweil, 2000) that the new food label will cost FDA-regulated food processors between US$ 1.4 and 2.3 billion over the next 20 years. However, the benefits to public health - measured in monetary terms - are estimated to well exceed the costs. Potential benefits include decreased rates of coronary heart disease, cancer, osteoporosis, obesity, high blood pressure, and allergic reactions to food *(ibid.).*

Table 2 Classification of viruses associated to food-borne human diseases (Lees, 2000 and Svensson, 2000)

Name	*Genome*	*Detection*	*Novel methods*	*Problems*	*Relationship to seafood consumption*
Caliciviruses	Positive sense single stranded RNA	Difficult to work with, cannot be cultured by conventionalvirological techniques, great progress by using molecular techniques. NLV and Sapporo virus cloned and sequenced (*Liu et al.*, 1995)	Molecular techniques (Berke *et al.*, 1997; Clarke & Lambden, 1997)	**NLV** are the major cause of epidemic gastrointestinal illnesses in families or community outbreaks. Infection occurs in all age groups and they are the major cause of gastroenteritis in adults (Kapikian, 1996; Kaplan *et al.*, 1982). **Sapporo like viruses** Genetically distinct from NLV. They may comprise several strains.	**NLV**: Frequently associated to seafood (shellfish and bivalve) co **Sapporo like viruses:** Not documented to have caused diseases following seafood consumption.
Hepatitis E virus		Cloned and sequenced (Bradley, 1995)		Symptoms similar to hepatitis A. Endemic in many developing countries, particularly in Asia. High mortality rate in pregnant women.	Outbreaks mostly linked to consumption of contaminated drinking water.
Astroviruses	Positive sense single stranded non-segmented RNA	Difficult to grow in culture. Recently specialised cell lines have been used to grow astroviruses directly from stool samples (Wilcocks *et al.*, 1990)		Symptoms similar to those of NLV: vomiting, diarrhoea, fever and abdominal pain, mainly in children and elderly. Usually full recovery and immunity after natural infection. Occur throughout the world, mainly in temperate climates and during the winter.	Their importance as causative agents of gastroenteritis following seafood consumption is unclear. Molecular techniques are expected to clarify this point.

Table 2 Classification of viruses associated to food-borne human diseases (Lees, 2000 and Svensson, 2000). **Cont.**

Name	*Genome*	*Detection*	*Novel methods*	*Problems*	*Relationship to seafood consumption*
Rotaviruses	11 separate strands of double-stranded RNA	Adapted to grow in cell cultures. Together with the large amounts of particles recuperated in stools has led to the development of diagnostic kits and assays (Yolken & Wilde, 1994)	Triplex reverse transcriptase PCR (Tsai *et al.*, 1994).	Group A rotaviruses have been frequently identified as the most common viral pathogen in diarrhoea requiring treatment or hospitalisation in children under 2. In developing countries they are considered to account for about 20% of all diarrhoeal-associated deaths in children under 5. Not considered to cause significant problems in adults. Group A rotavirus infections are detectable with antibodies in virtually all children by the age of 5	The infection is common and the viruses are shed in large numbers in stools (>10^{12} particles/g faeces) leading to detectable levels in sewage waters. Rotaviruses have been detected in bivalve shellfish grown in contaminated waters but they have not been linked to infectious disease, may be due to resistance to severe infection developed by active immunity acquired by repeated infection throughout life (Bishop, 1996)
Adenoviruses	Double stranded non-segmented DNA	Most are easily culturable except enteric adenoviruses serotypes 40 and 41	PCR (Girones *et al.*, 1995, Pina *et al.*, 1998) Nested PCR (Vantarakis & Papapetropoulou, 1998)	Associated with gastroenteritis (Wadell *et al.*, 1994). Reported to cause about 10% of infantile gastroenteritis (second only to rotaviruses). Less severe but longer than rotaviruses diseases. Shed into the gut and isolatable from faeces.	Detected in polluted sewage effluents, seawater and shellfish (Girones *et al.*, 1995, Vantarakis & Papapetropoulou, 1998) but no seafood related outbreaks have been reported.
Enteroviruses (fam. Picornaviridae)	Positive sense single stranded non-segmented RNA		Nested PCR (Vantarakis & Papapetropoulou, 1998), triplex reverse transcriptase PCR	All groups may become infected. Often mild or innaparent. In some cases the virus can spread to organs other than the intestinal tract and cause serious or fatal diseases (f.e. poliomyelitis, acute myocarditis, aseptic meningitis, haemorrhagic conjunctivitis, congenital infection of neonates and other non-specific febrile illnesses. Concern that they may cause or contribute to chronic diseases such as diabetes miellitus.	Isolated from sewage effluents and bivalve shellfish. Bivalve shellfish have not been linked to transmission of enterovirus diseases.
Hepatitis A virus	Like enteroviruses	Laboratory adapted hepatitis A virus strains can be grown in culture, but the wild type virus is more fastidious.	By PCR, it has been shown in stools (Yotsuyanagi *et al.*, 1996), sewage effluents, polluted waters (Tsai *et al.*, 1994) and bivalve shellfish	Diseases progresses from fever, headache, nausea and malaise to vomiting, diarrhoea, abdominal pain and jaundice. Hepatitis A is self-limiting and rarely causes death, although patients may be incapacitated for several months. Complete recovery with long-term immunity from reinfection. Endemic in developing countries with most children seropositive by the age of 6.	Bivalve shellfish frequently implicated in outbreaks.

Food safety: Seafood-borne illnesses

Each year in the USA millions of illnesses and thousands of deaths are traced to contaminated food (with an estimated cost from over US$ 5 to over 22 thousand million. Experts believe that the risk of food-borne diseases has increased during the last 20 years (United States General Accounting Office, 1996). Food-borne diseases can originate from consumption of viruses or bacterial pathogens, toxic substances and parasites. About half of the food-borne disease outbreaks remain unrecognised, mainly due to inadequate diagnostic methods and sampling (Svensson, 2000). The major food-borne infectious disease causing agents will be introduced under the headlines of:

- viruses
- pathogenic bacteria
- parasites

Viruses

Illnesses relating to the consumption of pathogenic viruses can cause polio, gastro-enteritis and hepatitis (see reviews by Lees, 2000; Svensson, 2000; and Table 2). Man is the only known reservoir for the major viruses causing food-borne diseases: calicivirus and hepatitis A virus (Svensson, 2000), and as far as it is known, all the viruses that cause food-borne illnesses are transmitted via the faecal-oral route. Shellfish borne gastro-enteritis was linked to viruses for the first time in the UK in 1976/77 when cooked cockles were epidemiologically linked to 33 incidents affecting almost 800 persons (Appleton & Pereira, 1977). A large outbreak involved over 2,000 persons in Australia (Murphy *et al.*, 1979), and other outbreaks have been registered in Japan, Canada, UK and the Scandinavian countries (Lees, 2000).

The high relevance of viruses in food-borne diseases was not suspected until the advent, in the 90's, of more sensitive molecular detection techniques. Caliciviruses, such as Norwalk virus, were then recognised as the major cause of seafood-associated gastro-enteritis: over 80% of the outbreaks of non-bacterial gastro-enteritis in US and Europe are currently attributed to caliciviruses (Svensson, 2000).

Bivalve molluscan shellfish have consistently been proven to be an effective vehicle for the transmission of viral diseases (Lees, 2000). The high risk comes from two facts: shellfish are filter feeders and many of them are consumed whole and raw or only lightly cooked. The risk is increased because many species are cultivated in near-coastal waters, where contamination with human sewage - which may contain high levels of viral particles - may easily occur. The largest known outbreak of hepatitis A occurred in Shanghai, China, in 1988, where almost 300,000 cases were traced back to the consumption of clams harvested from a sewage polluted area (Halliday *et al.*, 1991; Tang *et al.*, 1991).

In addition, shellfish harvested in contaminated areas may carry a mixture of viruses and patients may be simultaneously infected with more than one viral strain (Lees, 2000). This probably contributes to the very high attack rates registered (sometimes up to 100%) and explains why, in some outbreaks, patients may suffer hepatitis after an initial gastro-enteritis (Halliday *et al.*, 1991; Richards, 1985).

The need to re-evaluate the methodology used for the control of viral contamination is illustrated by the fact that in many outbreaks, the harvesting areas, treatment processes and products sold, complied with the current regulatory requirements established by the different countries involved (Fleet *et al.*, 2000; Lees, 2000).

Food-borne pathogenic bacteria

For convenience, Huss (1994) divided the pathogenic bacteria associated with seafood-borne diseases into two groups. The first contains bacteria naturally found in the aquatic environment, such as *Clostridium botulinum, Vibrio* spp, *Aeromonas hydrophila, Plesiomonas shigelloides* and *Listeria monocytogenes*. The more psychrotrophic *L. monocytogenes* and C. *botulinum* are more frequently found in Arctic and colder climates, while the more mesophilic *Vibrio* spp are more frequently found in warmer envi-

ronments. The second group consists of mesophilic bacteria that contaminate the aquatic environment from human or animal reservoirs such as *Salmonella* spp, *Escherichia coli*, *Shigella* spp or *Staphylococcus aureus*. The degree of processing of seafood has implications for the risks associated: botulism, listeriosis and cholera are more frequently associated with consumption of smoked, fermented, salted and pickled products, consumed without further processing (Huss, 1994; Jay, 1992).

The United States General Accounting Office (1996) targeted four bacteria as of major concern in food-borne diseases in the USA: *E. coli* O157:H7, *Salmonella enteriditis*, *Listeria monocytogenes* and *Campylobacter jejeuni*. It is worth noting that three of the four - *E. coli* O157:H7, *L. monocytogenes* and *C. jejeuni* - were not recognised as food-borne disease causing agents only 20 years ago.

Gilbert *et al.* (1999) reviewed the use and limitations of molecular methods for the diagnosis of infectious diseases. There is no universal method that permits identification and typing of all bacteria, as each species requires specific conditions for growth as well as antibodies, primers or probes for detection. We have chosen *Listeria monocytogenes* as an example to illustrate the methods of detection, identification and typing of bacteria.

Listeria monocytogenes

Listeria monocytogenes is an ubiquitous Gram-positive bacteria. There are seven species of *Listeria*: *L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii, L. grayi* and *L. murrayi*. Only *L. monocytogenes* is considered to be a pathogen of concern for humans (Ryser & Marth, 1991). *L. monocytogenes* is naturally found in soils, sewage, freshwater sediments and is frequently carried in the gastrointestinal tract of animals and humans (Farber & Peterkin, 1991). The existence of asymptomatic carriers is well known and it has been estimated that between 2-6% of the human population are positive at any given time (Rocourt, 1994). The importance of *L. monocytogenes* as a food-borne human pathogen has been recognised only since the 1980's (Farber & Peterkin, 1991; Gellin *et al.*, 1991). Between 85 to 95% of cases of listeriosis are food-borne (Buzby *et al*, 1996). Listeriosis can occur in healthy individuals, but the primary groups at risk are the immunocompromised, including pregnant women, the elderly, AIDS patients and those under medication to reduce their immune responses, such as cancer patients and organ transplant recipients. Listeriosis has a long incubation period, from one to several weeks (Ryser & Marth, 1991), making it very difficult to associate foodstuffs to sporadic cases of the diseases. The symptoms of the infection may vary from asymptomatic infection, to skin infections and flu-like symptoms, to miscarriage, stillbirth, sepsis, meningitis and death (Farber & Peterkin, 1991).

The death rate in the USA has been estimated to be between 23% (Tappero *et al.*, 1995) and 28% (Schwartz *et al.*, 1988), except for a Californian outbreak caused by Mexican-style cheese, where it was almost 34% (Jay, 1992). Between 1986 and 1988, the mortality rate in the UK was between 51% and 44% for perinatal and adult cases respectively (McLauchlin, 1987).

Although listeriosis has not often been associated with seafood consumption (Ryser & Marth, 1991), shellfish and raw fish are thought to have played a role in an outbreak in Auckland, New Zealand in 1980, (Lennon *et al.*, 1984). The outbreak lasted for 11 months, with 22 perinatal listeriosis cases registered, including 5 foetal and 1 liveborn deaths. Most of the isolates were serovar 1b and no cultures from foods were obtained.

L. monocytogenes has been isolated from retail frozen seafood in 9 of 12 countries in a study by Weagant *et al.* (1988) and from seafood in Taiwan (Wong *et al.*, 1990). Again, although the long incubation period of the disease makes it difficult to trace sporadic cases back to remaining food items, Loncarevik *et al.* (1998) in Sweden and Rørvik *et al.*, (1995, 2001) in Norway found that *L. monocytogenes* isolated from clinical cases and from some fish products were indistinguishable. Therefore fish products cannot be disregarded as source of illness. Curiously, and in spite of its ubiquitous nature, *L. monocytogenes* does not seem to be detectable in tropical seafood products (Ben

Embarek, 1994; Fuchs & Surendran, 1989; Muntiningish & Sunarya, 1998).

L. monocytogenes can contaminate estuarine environments though sewage, processing and agricultural effluents. The relatively low salt content in this environment does not affect its survival capacity. Up to 11% of unprocessed shrimps in the Gulf of Mexico were *L. monocytogenes* positive, as well as about 1.5% of retailed fresh water fish. The Food and Drug Administration found that 26% of frozen seafood products from various countries that were found to be positive were shrimp (raw and cooked), cooked crab meat and surimi.

Between 9% and 28% of the samples of ready-to-eat shrimps, crab and smoked fish analysed in several surveys contained *L. monocytogenes* (Dillon *et al.*, 1994; Farber & Peterkin, 1991; Rocourt & Bille, 1997). Harvey & Gilmour (1993) found that 18.3% of 513 food samples examined in a survey in Northern Ireland contained *L. monocytogenes.* While 229 of the 513 food items had received cooking presumed sufficient to eliminate *Listeria*, 12.2% of them were *L. monocytogenes* positive. In Norway, Rørvik & Yndestad (1991) examined 382 samples of retail food items (imported soft cheese, raw chicken, minced meat, fermented sausages, vacuum-packed processed meat products, smoked salmon, peeled shrimps, raw minced fish) and 78 carcass samples (sheep, pig, cattle) and found 16.3% of them to be positive. *L. monocytogenes* was most frequently isolated from raw chicken, sporadically from soft cheese, shrimps, processed meat products and smoked salmon, yet not from carcasses and fermented sausages. In southern Finland, 20% of 110 ready-to-eat vacuum packed fish products (hot- and cold-smoked and salted fish) were found to be *L. monocytogenes* positive in a survey carried out by Johansson *et al.* (1999). In a survey of Japanese retail foods, Inoue *et al.*, (2000) found between 12.2 and 37% of minced meats, 5.4% of smoked salmon samples and 3.3% of ready-to-eat seafood positive for *L. monocytogenes*, although they did not find the bacterium in any of 285 vegetable samples examined.

Often, different strains are isolated from raw materials and final products. Some studies show that *L. monocytogenes* can colonise the processing environment (utensils, brines, etc.) (Destro *et al.*, 1996; Giovannacci *et al.*, 1999; Lawrence & Gilmour, 1995; Rørvik *et al.*, 1995, 1997, 2001). Colonisation of the processing environment (FAO, 1999; Giovannacci *et al.*, 1999; Johansson *et al.*, 1999; Rørvik *et al.*, 1995, 1997) as well as job rotation among departments (Rørvik *et al.*, 1997) have been established as primary mechanisms for contamination of some products.

Detection of L. monocytogenes

There are two basic classical approaches to detection of the presence of any given micro-organism in a food product: direct plating from the food onto a selective agar medium and incubation of the product in a resuscitation and/or enrichment medium, followed by plating onto a selective agar medium.

Traditional detection methods for *L. monocytogenes* involved a cold enrichment procedure followed by plating (see Ben Embarek, 1994 for a review; Biester and Scharte, 1939). These methods may require months for detection of the bacterium. Newer methods use enriched selective media, shorter time (about 48 h) and higher temperature (30-37°C) (Loncarevic, 1998 and references therein). The selection of the most suitable medium for recovery of *L. monocytogenes* from food matrices depends on the food type, the strain infecting the product, the level of infection and the degree of injury to the bacterium (Loncarevic, 1998). The bacteria present in food matrices have usually been subjected to stressful conditions (cold storage, drying, freezing, smoking, heat shock, antibacterial substances etc.) that may have killed many of them and injured another fraction. A few may have survived the treatment but require optimal conditions for a period of time in order to start growing again. Therefore, it is convenient to incubate them in a resuscitation medium first, followed by a medium selective for the targeted micro-organism.

All virulent strains of *L. monocytogenes* produce a specific substance responsible for the β-haemolysis of erythrocytes and the destruction of phagocytic cells that engulf them. This substance has been called liste-

riolysin O (LLO) and is homologous with streptolysin O (SLO) and pneumolysin (PLO) (Mengaud *et al.*, 1987, 1988). Colonies of suspected *Listeria* spp are identified by their ability to produce β-haemolysis on blood agar and by biochemical tests (Rocourt *et al.*, 1983). The biochemical identification has been simplified by the introduction of a rapid test strip identification system that is claimed to reduce the time needed for conventional identification of suspected colonies to about 24 h (Bille *et al.*, 1992).

The lack of an optimal procedure is reflected by the fact that different official bodies have chosen different protocols (Loncarevic, 1998; Lovett, 1988; McClain & Lee, 1988; NMKL, 1990). It has also been shown that the enrichment and plating media may favour or suppress the growth of different strains of *L. monocytogenes* to different degrees Loncarevic *et al.* (1996, 1997).

Patel (1994) gives an account of the methods used in modern microbiology, all of which have been applied to the detection of a variety of micro-organisms, including *L. monocytogenes*. The introduction of membrane filtration techniques permitted the retention of bacteria in filters than can be placed directly on agar plates or soaked in nutrients which allow the growth of the micro-organisms on the filter.

Bacterial growth can also be measured by using automated electrical techniques. Metabolism and growth result in an increase in the number of charge-carrying molecules in the growth medium. These molecules are better carriers. Electrical techniques estimate bacterial growth by measuring changes in the conductivity of the medium. They have been applied to routine analysis following the development of suitably selective media for relevant bacterial species and of computer technology to record, store and present the data to the operator.

Bacteria can be selectively removed from a medium by using immunomagnetic beads which are magnetic particles coated with streptavidin and antibodies specific for the target. Since the particles are magnetic, they stick to the walls of the tube when in contact with a magnetic particle concentrator. The medium and non-targeted organisms can be pipetted away while the immunomagnetic particles, with the targeted pathogen, can be resuspended in a new medium or used directly for subsequent analyses such as PCR amplification.

Molecular techniques are mainly based on the use of PCR. New genes are being characterised and sequenced every day, so the number of sequences available for PCR detection of micro-organisms is continuously increasing. *L. monocytogenes* has been successfully detected by PCR targeting virulence or invasion genes, such as the listeriolysin O (*hly*) gene (Agersborg *et al.*, 1997; Furrer *et al.*, 1991; Golsteyn Thomas *et al.*, 1991; Niederhauser *et al.*, 1992) and the invasion-associated protein (*iap*) gene (Agersborg *et al.*, 1997; Jaton *et al.*, 1992). Other bacteria detected by PCR techniques are *Campylobacter coli, C. jejuni* (targeting flagellar protein gene and other *Campylobacter* spp; *Clostridium botulinum* (targeting the gene for the neurotoxin). *E. coli, Salmonella typhimurium* and *Shigella flexineri* were detected by specific PCR amplification of the *lamB* gene (which encodes the bacteriophage lambda receptor) and enterotoxigenic strains of *E. coli* by targeting the gene for the enterotoxin.

Since only a few µl[5)] are used for the amplification (typically 10-50 µl), and it is required the absence of inhibitors, it is necessary to design protocols both to increase the concentration of the target organism and to eliminate those inhibitors. In our own experience it is necessary to incubate the food samples for at least 48h to be able to detect low levels of *L. monocytogenes* (1-5 bacteria per 5 g product) and simultaneously avoid false positives due to the presence of high levels of non-viable bacteria. Incubations of only 24h can render false negative and false positive results respectively (Agersborg *et al.*, 1997). Unfortunately, the use of immunomagnetic beads, which could significantly reduce the time of sample preparation, did not show promising results (Skjerve *et al.*, 1990; Uyttendaele *et al.*, 2000) .

After PCR amplification, hybridisation of the amplicon with a probe recognising an internal sequence, has been shown by Xiaoming *et al.* (2000) to be effective, not only to confirm that the correct target had been amplified, but also to increase the sensitivity of detection. These authors successfully used this approach for the simultaneous

detection of *Salmonella typhimurium* and *L. monocytogenes* from pure bacterial mixtures and seeded milk.

Typing of L. monocytogenes

Strain identification is critical to determining the source or sources of infection - the index case, the product originating the outbreak, or the focus of contamination in a factory - as well as the routes of spreading of a given infection.

Serotyping is based on the ability of the bacteria to agglutinate antibodies anti-somatic and anti-flagellar antigens. The seven species of *Listeria* possess antigens that give rise to 17 serovars: the somatic O antigens give 15 serovars and there are 5 flagellar antigens H (Table 3). One of the problems with serotyping is that some of the 13 serovars of the pathogenic *L. monocytogenes* are shared by *L. innocua* and *L. seeligeri*. The other is that most isolates from food and clinical cases belong to serotypes 1 and 4, with serogroup 1/ 2 more frequently encountered in food isolates and serogroup 4 in clinical isolates (Farber and Peterkin, 1991). Thus, the low discrimination of serotyping makes it of limited value for epidemiological studies.

Multilocus enzyme electrophoresis (MEE) estimates the genomic relationship among isolates by determining the relative electrophoretic mobilities of a set of water soluble polymorphic bacterial enzymes, after starch electrophoresis and specific enzymatic staining techniques (Selander *et al.*, 1986). The result of the application of this technique to each bacterial isolate and enzyme, is called an *electromorph*. The combination of electromorphs for all the enzymes is called the electrophoretic type (ET). Typically, MEE typing requires the analysis of between 15 and 25 polymorphic enzymes (Jay, 1992). Differences in the electrophoretic mobility of allelic variants are dictated by differences in the amino acid sequences and therefore in the nucleotide sequence. Since some enzymes may be polymorphic among different strains of the same species, MEE can be used in epidemiological studies, giving additional information to that obtained by serotyping alone. All isolates can be typed by MEE and there is good correlation between MEE types and serotypes (Jay, 1992).

Table 3 Serovars of Listeria spp according to Seeliger and Jones (1986). ()[a] not always present

Listeria spp	*Serovars*	*O antigens*	*H antigens*
monocytogenes	1/2a	I II (III)[a]	AB
monocytogenes, seeligeri	1/2b	I II (III)	ABC
monocytogenes	1/2c	I II (III)	B D
monocytogenes	3a	II (III) IV	AB
monocytogenes	3b	(III) IV (XII XIII)	ABC
monocytogenes	3c	(III) IV (XII XIII)	B D
monocytogenes	4a	(III) (V) VII IX	ABC
monocytogenes, innocua	4ab	(III) V VI VII IX X	ABC
monocytogenes	4b	(III) V VI	ABC
monocytogenes, seeligeri	4c	(III) V VII	ABC
monocytogenes, seeligeri	4d	(III) (V) VI VIII	ABC
monocytogenes	4e	(III) V VI (VIII) (IX)	ABC
ivanovii	5	(III) (V) VI (VIII) X	ABC
monocytogenes	7?	(III) XII XIII	ABC
innocua, welshimeri	6a (4f)	(III) V (VI) (VII) (IX) XV	ABC
innocua, welshimeri, seeligeri	6b (4g)	(III) (V) (VI) (VII) IX X XI	ABC
grayi		(III) XII XIV	E
murrayi		(III) XII XIV	E

Phage-typing classifies bacterial isolates according to the phages that can lyse them. Phages are viruses that can infect bacteria and were first described in 1945 (Jay, 1992). They contain double stranded DNA and belong to two groups: Siphoviridae (phages with non-contractile tails) and Myoviridae (phages with contractile tails). Phage-typing is usually considered to show good reproducibility (Audurier *et al.*, 1984; Loessner & Busse, 1990; McLauchlin *et al.*, 1986) but no correlation seems to exist between lytic patterns and species and serovar types (see Loessner & Busse, 1990). One major disadvantage of this technique is that at present there are not enough phages to lyse all *L. monocytogenes* isolates (Audurier & Martin, 1989).

For typing by Restriction Enzyme Analysis (REA), the whole isolated bacterial chromosome is digested by restriction endonucleases. The resulting high molecular mass fragments (from a few kilo-bases to over 10 mega-bases) are resolved by agarose-gel electrophoresis or pulsed-field gel electrophoresis (PFGE), depending on their size (Southern and Elder, 1995). Since the restriction enzymes cut the double stranded bacterial DNA at specific sequences, the number and size of the resulting fragments will depend on the nucleotide sequence. The analysis will therefore reflect genomic differences among isolates. REA alone has been used to characterise strains of *L. monocytogenes* isolated from listeriosis outbreaks (Baloga & Harlander, 1991; Nocera *et al.*, 1993) and shown that isolates from major human epidemics exhibit unique restriction patterns (Wesley & Ashton, 1991). The combination of low-frequency cutting enzymes for REA with PFGE has made the patterns easier to interpret and has proved to be a highly discriminatory technique (Brosch *et al.*, 1991, 1994; Carrier *et al.*, 1991).

Ribotyping is based on the characterisation of isolates by identifying polymorphisms in the ribosomal RNA genes. The genes coding for the 16S and 23S rRNA contain some hypervariable regions suitable for genus and species identification, but there are also some highly conserved regions that permit the use of rRNA from *E. coli* as a universal probe. Moreover, since most bacteria contain multiple ribosomal operons, - operon is a genetic regulatory system found in bacteria and their viruses in which genes coding for functionally related proteins are clustered along the DNA - ribotyping provides patterns complex enough to be suitable for subtyping.

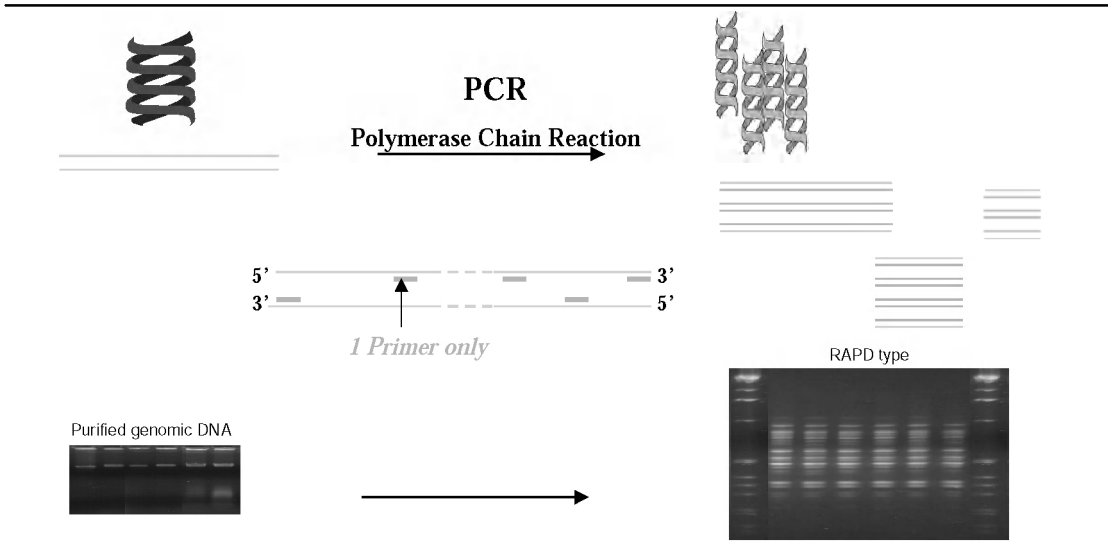

Figure 2 *Random Amplification of Polymorphic DNA (RAPD) is based on the non-stringent amplification of genomes by using arbitrary - usually short - primers. The number and length of the amplicons produced depends on the loci that are complementary to the sequence of the primer and therefore dependant on the primer-DNA combination. The amplicons produced are separated and visualised, usually by agarose gel electrophoresis, staining and ultraviolet light, and a more or less complex pattern -the RAPD-type - is produced*

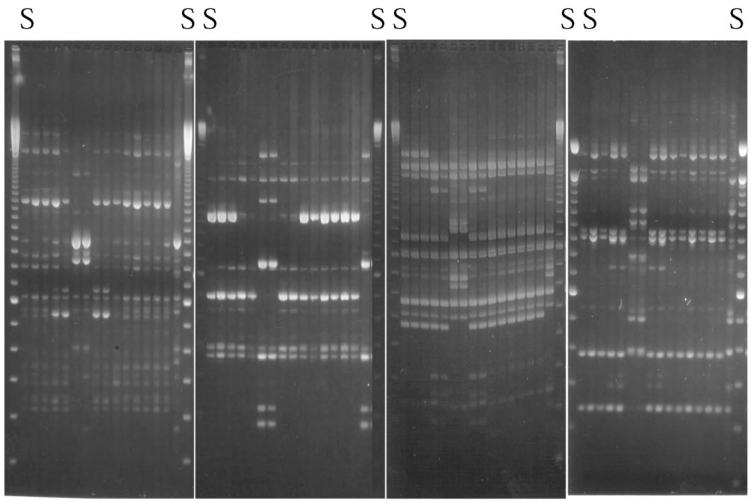

Figure 3 *Example of RAPD-typing of* L. monocytogenes *isolates. Each gel is the result of typing with a different primer. Each lane, in each gel, contains a different isolate. All gels contain the same isolates in the same order. S, lanes containing 100bp ladder molecular mass standards. The 3rd gel shows distortions that took place during the electrophoretic run*

Ribotyping has been applied to epidemiological investigations of *L. monocytogenes* and other pathogens, both in sporadic cases and in outbreaks (Baloga & Harlander, 1991; Graves *et al.*, 1991, 1994; Grimont and Grimont, 1986; Nørrung & Gerner-Smidt, 1993; Stull *et al.*, 1988). It is a reproducible typing method but it usually fails to discriminate between 4b serovars (Baloga & Harlander, 1991; Nocera *et al.*, 1993; Saunders *et al.*, 1989; Wesley *et al.*, 1990). These are most frequently associated with sporadic and epidemic listeriosis (Pinner *et al.*, 1992; Schuchat *et al.*, 1991a, 1991b).

Random Amplification of Polymorphic DNA (RAPD) (Welsh & McClelland, 1990; Williams *et al.*, 1990), is a PCR amplification, under non-stringent conditions, with arbitrary - usually short - primers. (Figure 2). RAPD analysis has been used to type isolates of *L. monocytogenes* (Lawrence & Gilmour, 1995; MacGowan *et al.*, 1993; Mazurier *et al.*, 1992; Mazurier & Wernars, 1992). By using the appropriate primers, RAPD is a very discriminating technique because it targets many different loci simultaneously. Moreover, its discriminatory power can easily be increased by using different primers, since the supply of arbitrary primers is practically unlimited (Figure 3), and all genomes can be typed. However, the results of RAPD do not always agree with other typing methods. Strains of different serotypes may occasionally share the same RAPD-type (Destro *et al.*, 1996; Mazurier & Wernars, 1992; and the authors' unpublished results), and the results of RAPD and phage typing have not always shown a correlation (Mazurier *et al.*, 1992).

The World Health Organization (WHO) sponsored an international collaborative study to evaluate methods for subtyping of *L. monocytogenes* (Bille & Rocourt, 1996). No single method was found to be optimal to type all isolates used in the exercise.

PCR-based detection of viruses and bacteria: some problems

In addition to obtaining suitable primers to amplify only the targeted sequences, some of the problems encountered in applying PCR to the detection and identification of pathogenic micro-organisms in seafood are common to the application of this technique to all food matrices. These include elimination of inhibitors, concentration of the target, and detection of non-viable forms of the targeted organism. Other problems are specific to the nature of the target: a difference to bacteria, viruses do not multiply in the

infected shellfish tissues. Since usually less than 100 µl of sample are analysed, the targeted particles have to have minimum concentration that permits its detection.

Design of suitable consensus primers has been more problematic for species whose strains show high degree of diversity, in the case of viruses, the NLV strains. The use of combinations of primers pairs to cover the spectrum of sequenced types has solved this problem and most of the known clinical NLV types can now be detected. For viruses that show little variation, such as hepatitis A, the application of PCR-detection has been straight-forward (Lees, 2000).

Crude shellfish extracts seem to contain PCR inhibitors. The problem of elimination of inhibitors can be diminished, and simultaneously the target concentration increased, by extracting only the digestive organ of the shellfish, where viruses accumulate, followed by the usual purification and nucleic acid concentration steps included in most sample preparation protocols for PCR amplification (Lees, 2000). As in the case of bacteria, most of these studies have been made on artificially contaminated samples and it seems that the detection of viruses in naturally contaminated samples is more difficult. One reason may be that naturally contaminated samples have lower levels of the target sequence. Increasing the sensitivity of the amplification by nested PCR has helped to overcome this problem (Dore *et al.,* 1998; Green *et al.,* 1998).

Target-recognition techniques can produce false positives due cross-reactions with similar sequences of usually related organisms. In addition, as long as the targeted fragment of nucleic acid is not destroyed, non-viable forms of the viruses will also be detected. The solution to the cross-reactions may be the design of more specific primers and increase the stringency of the reaction, or sequencing of the obtained amplicon. Avoidance of the detection of non-viable forms is more complicated. The genome of most of these viruses is made up of RNA, which is more readily degraded than DNA, and therefore it is quickly degraded by the digestive enzymes of the shellfish or the associated bacterial flora. Yet, some samples artificially seeded with feline calicivirus (used as a model for Norwalk group viruses) that had been heat-treated to inactivate the viruses and that failed to yield virus on culture, gave positive results by reverse transcriptase (RT)-PCR (Slomka & Appleton, 1998). We have obtained similar results in the PCR-based detection of *L. monocytogenes* when the level of targeted sequence was high and the samples analysed immediately after the heat-destruction of the organism. In any case, the detection of non-viable forms is a problem that needs to be addressed and some researchers have proposed to start with an initial round of culture in cells followed by PCR-detection or a combination of immunological and molecular techniques. The first approach will not be applicable to non-culturable viruses, it will significantly delay the analysis time, and the analysis will again be restricted to laboratories with facilities for virus culturing in cell lines. The second approach, a combination of immunological and molecular techniques, may present the advantage of simultaneously enriching the sample, eliminating contaminants and collecting only viable particles by immunological techniques, thus simplifying the sample preparation procedure for PCR detection. However, the goodness of the analysis will be highly dependant on the quality and reactivity of the antibodies used.

Parasites

Protozoa, Platylhelminthes and Nematoda can also cause food-borne diseases. Of special relevance to fish products are trematodes and some nematodes, whose taxonomical classification is shown in Table 4. Trematodes, or flukes, infect the liver, lungs or blood of mammals. *Paragonimiasis* is caused by species of the genus *Paragonimus. P. westermani* is predominant in Asia, but is also found in Africa and South and Central America, while *P. kellicotti* is more frequent in North and Central America. *P. westermani* is a lung fluke. *Clonorchiasis* is caused by *Clonorchis (Opisthorchis) sinensis,* the Chinese liver fluke that causes oriental biliary cirrhosis. *Opisthorchiasis* is caused by *Opisthorchis felineus* and *O. viverrini. Clonorchis* and *Opisthorchis* are very similar both morphologically and in their life cycles (Figures 4 and 5). The infections

are mostly contracted from the ingestion of raw or improperly cooked crabs or fish.

Table 4 Taxonomical classification of some parasites causing food-borne diseases

Phylum Platyhelminhes
Class Trematoda (flukes)
Subclass Digenea
Order Plagiorchiata
Family Troglotrematidae
Genus *Paragonimus*
Species *P. westermani* in Asia, *P. kellicotti* in Africa and South and Central America.
Order Opisthorchiata
Family Opisthorchiidae
Genus *Opisthorchis*
Species *O. felineus* in Kazakhstan, Russia and Ukraine
Species *O. viverrini* in Thailand and Laos
Genus *Clonorchis*
Species *C. sinensis* in China
Class Cestoidea
Subclass Eucestoda(tapeworms)
Order Pseudophyllidea
Family Diphyllobothriidae
Genus *Diphyllobothrium*
Species *D. latum* in USA, Scandinavia
Phylum Nematoda
Class Secernentea (Phasmidia)
Order Ascaridida
Genus *Ascaris*
Subfamily Anisakinae
Genus *Anisakis*
Genus *Pseudoterranova (Phocanema)*

Clonorchiasis is due to the ingestion of fish containing the metacercariae: the cyst wall dissolves in the intestine, the young flukes emerge and migrate through the body to the bile ducts of the liver (Figure 4). *C. sinensis* causes, among other complaints, cirrhosis and liver cancer. *Paragonimiasis* is acquired by ingesting crustaceans infected with metacercariae which hatch out of the shells, bore their way as young flukes through the walls of the duodenum and then move to the lungs, where they become enclosed in connective tissue cysts (Figure 5). Golden brown eggs may appear in sputum 2 to 3 months later. Both paragonimiasis and clonorchiasis are diagnosed by demonstrating the presence of eggs in sputum, stools or duodenal fluid. There are some ELISA tests available (for the infection, not for the parasite) but they give cross-reactions with infections caused by related species.

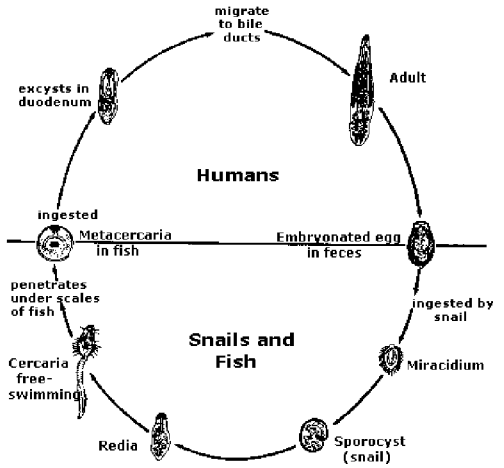

Figure 4 Life cycle of Clonorchis sinensis *(the Chinese or oriental liver fluke),* Opisthorchis viverrini *(South-east Asian liver fluke) and* O. felineus *(cat liver fluke). The source of the figure is* DPDx, the CDC web site for parasitology diagnosis. *http://www.dpd.cdc.gov*

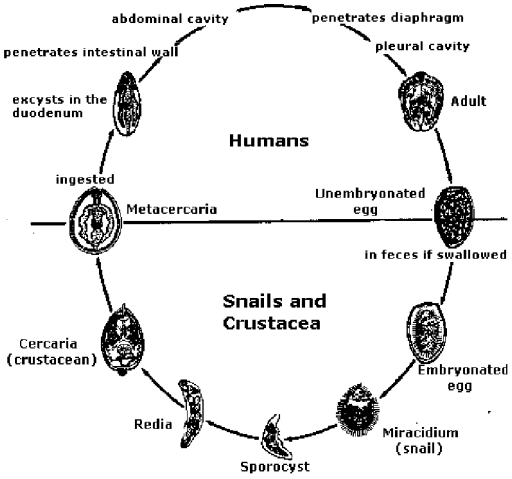

Figure 5 Life cycle of Paragonimus westermani, *the oriental lung fluke. The source of the figureis* DPDx, the CDC web site for parasitology diagnosis. *http://www.dpd.cdc.gov.*

Anisakiasis is caused by the ingestion of fish flesh infected with nematodes: either *Anisakis simplex* (herring- or whaleworm) or *Pseudoterranova (*formerly *Phocanema) decipiens* (cod- or sealworm). Both parasites have several intermediate hosts and generally more than one definitive host. Humans are not the final host for either of them, and infections occur upon ingestion of fish containing from the second to the fourth molting larva. The worms do not mature in humans and the disease comes from the activities of the juvenile worms: *A. simplex* is usually more harmful than *P. decipiens* because it can penetrate the mucosal lining while most *P. decipiens* are passed in faeces, coughed up or vomited after irritating the mucosa. The diagnosis of this condition is complicated by the absence of eggs from faeces. Larvae can be seen in the intestinal tract by endoscopy and the affected tissue can be removed surgically (Jay, 1992).

Impact of fish-borne trematode infections

Food-borne parasitic diseases affect millions of people world-wide. About 700 million people are considered to be at risk of contracting food-borne trematode infections (FBT) and 40 to 50 million are believed to be infected by one or more trematode parasites. (FAO, 2000a; WHO, 1995). Table 5 shows where the diseases are endemic, the estimated number of infected people, and the sources of infection.

Most of these infections occur in Asia as a direct result of the consumption of raw or improperly cooked fish and crustaceans containing the viable and infective stage of the parasite. People's eating habits are associated with cultural and social practices and, therefore, extremely difficult to change. Moreover, most FBT infections occur in areas where there is poverty, pollution and increasing population growth. The use of water containing human and animal faeces to fertilise plants and to feed fish, allows the life cycle of these parasites to be completed and perpetuates the infection.

As mentioned above, detection and identification of *Opisthorchis*, *Clonorchis* and *Paragonium* are usually based by the microscopical examination of clinical specimens by expert personnel to detect the eggs (Sukontason *et al.,* 1999). However, the eggs of *Opisthorchis* are practically identical to those of *Clonorchis*. Slemenda *et al.* (1988) and Kim (1998) used specific immunological methods to diagnose paragonimiasis and clonorchiasis respectively. These methods were only useful to detect infected patients and are obviously dependant on the quality on the antigen preparation. One could expect variable results depending on the quality of the batch used for the analysis.

Table 5 Location and estimates for some endemic fish-borne trematode infections (FBT) (WHO, 1995)

Disease	*Caused by*	*Endemic in*	*Estimated infected*	*Acquired from*
Clonorchiasis	*Clonorchis sinensis*	China The Republic of Korea Japan Hong Kong Viet Nam Russian Federation	7 million (more than half in China)	113 fish ssp, mainly Cyprinidae
Opisthorchiasis	*Opisthorchis felineus*	Kazakhstan Russia Ukraine	10 million 7 million in Thailand alone	Cyprinidae
	O. viverrini	Thailand Laos		
Paragonimiasis	*Paragonimus westermani* *Paragonimus* spp	At least 20 countries. In Asia endemic in regions of China, Republic of Korea Laos Philippines Thailand	22 million	Crabs and crayfish

Emigration and international trade have the potential to exacerbate the problem of FBT. Emigrants usually take their eating habits and preferences with them, and increased international trade and improvement in communications have allowed the transport of fresh fish and crabs from one part of the world to another in a matter of hours. For example, within the EU the consumption of improperly cooked freshwater fish has traditionally been rather limited. Nowadays, however, fresh fish from Asia, Africa and South America is more easily available. The increased access of the ethnic communities in the EU to their traditional food items as well as the fact that many of those habits have become fashionable (Chinese, sushi and sashimi restaurants) have increased the risk of FBT in Europe.

Another factor to keep in mind about the possible future impact of parasitic diseases in developed countries is the dominance of the production of aquacultured species by developing countries. Howgate (1998) estimated that about 63% of the total production and 90% of fish production comes from freshwater, and that the greatest cause for concern in this industry - regarding food safety - is the high incidence of trematode parasites in tropical and subtropical zones. According to Brown (2000), about 85% percent of the fish farmed in the year 2000 came from developing countries: almost 68% from China, followed by India with a 6.45%. Other developing countries with increasing aquaculture activies are Bangladesh, Indonesia, and Thailand. Among industrial countries, Japan (2.6%), the United States (1.5%), and Norway (1.3%) are the leaders. China produces fish primarily in ponds, lakes, reservoirs, and rice paddies. Some 5 million hectares of land are devoted exclusively to fish farming, much of it in carp polyculture. In addition, 1.7 million hectares of rice land is used to produce rice and fish together. China breeds four types of carp that feed at different levels of the food chain. Silver carp and bighead carp are filter feeders; the grass carp feeds largely on vegetation, while the common carp is a bottom feeder, living on detritus that settles to the bottom. Most of China's aquaculture is integrated with agriculture, enabling farmers to use agricultural wastes, such as pig manure, to fertilize ponds, thus stimulating the growth of plankton. This type of fish polyculture typically boosts the fish yield per hectare over that of monocultures by at least half, and it also dominates fish farming in the second major producer, India (Brown, 2000). This type of fish polyculture also demands the erradication of parasites from the land animals whose manure will be used to feed the fish in order to cut the life-cycle of the trematodes.

Confirming the 1995 report of the World Health Organization (WHO, 1995) we have not been able to find literature concerning fast, easy methods to detect the presence of trematodes in fish or crab samples. Similarly, there seems to be a lack of data about the survival of these parasites in foodstuffs submitted to different processing conditions and how the conditions that might inactivate or kill them would affect the organoleptic properties of the fish and therefore its acceptance by the consumers (WHO, 1995).

In summary, to eradicate FBT infections, there is a need to obtain information on the epidemiology of these diseases and determine to what extent aquaculture practices may enhance the problem; to map the resistance of metacercaria cysts in fish (crab) flesh to relevant processing conditions and to develop sustainable, effective management methods including rural sanitation and awareness of the problem. This demands the development of fast, uncomplicated methods for the detection and identification of the parasites in fish, snails, faeces, and environmental samples. Happily, the European Union has recently funded a research project to be carried out over 4 years, involving researchers in China, India, Thailand and Laos, three European institutions and FAO (FIIU). The participants come from several sectors including parasitology, medicine, biotechnology, food technology, fisheries and community health. The principal output of the research will be a diagnostic field test kit intended for use both in epidemiological research and assessing fish products.

Future work

With the increase in the field of genomics during the last few years, it would be rea-

sonable to expect that parasites will be targeted as organisms relevant to map. Large scale genome projects are well advanced for the six human parasites targeted by the World Health Organization: *Trypanosoma brucei, T. cruzi, Leishmania* spp, schistosomes, filariae and malaria parasites (Brindley, 2000). Unfortunately, none of the FBT previously mentioned were given priority and only some sequences corresponding *to P. westermanii* have been published (*ibid.*)

It is likely that the increased trade of potentially infected fish and crustaceans into more stringent markets (EU, USA, Japan), will force the control agencies to develop techniques for the fast and reliable identification and elimination of these parasites in food samples. The current techniques to identify these trematodes are fastidious and researchers involved in phylogenetic studies will require the development of straightforward techniques for their identification B including phenotypic and sequencing data. These techniques will have the potential to detect the targeted parasites in the tissues of snails, fish and crabs (see Andrews & Chilton, 2000). Just to illustrate the need for reliable data in parasite systematics and identification, Blair *et al.*, (1997) found, that the differences in the sequences of the cytochrome *c* oxidase subunit I (COI) of geographically separated populations of *P. westermani* were so large that they approached those seen between different species. At present, unfortunately, no such method seems to be available.

We will now turn to methods for species authentication, before listing some final comments and recommendations.

Methods for species authentication

Species identification is not possible by visual inspection when the morphological characteristics necessary for the identification, such as heads, fins, skin or bones, have been removed. Nevertheless, there are many other methods useful for species identification in these cases (see Table 6). Selection of the most appropriate method for a particular case will depend on the nature of the product (*eg.* whole pieces or comminuted, raw or heat-treated), and on the aim of the analysis: *i.e.* to assess whether it contains what is claimed on the label or to identify what it really contains.

Table 6 Common speciation methods used in fish and meat products their advantages and disadvantages

Type of technique	*Targeted molecules*		*Advantages*	*Disadvantages*
	Proteins	*DNA*		
Fingerprinting	SDS-PAGE IEF 2D-electrophoresis Peptide mapping	RFLP and probe hybridisation RAPD Using consensus sequences: SSCP RFLP	Always produce a result No "false positives: a "bad" fingerprint is unlike a "good" fingerprint of another species	Identification by comparison to standards Not useful: - For mixtures - If the target is degraded
Target recognition	Immunological techniques, with spp/stock specific antibodies: blot hybridisation ELISA	Using specific sequences: PCR Blot hybridisation	Smaller targets are less affected by processing Easier interpretation: optimally should produce +/- results	Need to be developed for each species/stock/strain Frequent cross reactivities with related species

Traditional species identification is usually based on the analysis of either the proteins or the DNA contained in the product (for a review, see Sotelo *et al.,* 1993). Both protein and DNA-based methods can be subdivided in two groups (Table 6): those that target many loci and produce a fingerprint-like pattern (SDS-PAGE (Piñeiro, *et al.,* 1999; Mackie *et al.,* 2000); IEF, (Mackie *et al.,* 2000; Rehbein *et al.,* 1999); RAPD (Lee & Chang, 1994; Martinez *et al.,* 2001; Martinez & Daniélsdóttir, 2000; Martinez & Malmheden Yman, 1999; Partis & Wells, 1996) and those that target one or a few loci (ELISA-like, immunological techniques, SSCP (Mackie *et al.,* 1999; Rehbein *et al.,* 1999a) or RFLP (Mackie *et al.,* 1999 ; Meyer *et al.,* 1995; Wolf *et al.,* 1999, 2000) of PCR amplified sequences.

DNA-based techniques have the advantage that one does not need a standard for each tissue, because all the cells in an individual have the same genomic DNA. This is not the case for proteins, since each tissue expresses the proteins necessary for its function, and in addition, the expressed proteins may change depending on the developmental stage of the individual. Therefore the protein pattern is species-, tissue-, and developmental stage-specific (Martinez *et al.,* 1991).

Which technique is best for product authentication depends mainly on how the sample has been treated. SDS-PAGE or RAPD are easy and relatively cheap to perform, and do not require any previous knowledge of the sample under study (Martinez & Malmheden Yman, 1999; Partis & Wells, 1996; Welsh & McClelland, 1990; Williams *et al.,* 1990). However, they do demand certain integrity of the proteins or of the genomic DNA. If the product has been subjected to severe processing conditions (*eg.* such as sterilisation) one has to rely on techniques that target small DNA fragments, because proteins (Martinez *et al.,* 2001; Sotelo *et al.,* 1993;) and DNA (Mackie *et al,* 1999; Martinez, 1997) are altered so much that they do not render recognisable patterns. Suitable techniques target one or a few loci, but require the use of consensus or degenerate primers, which should be optimised for each group of related species (Martinez & Daniélsdóttir, 2000). Also, once the target is amplified, its sequence has to be confirmed. This can be done by RFLP or SSCP analysis or by sequencing.

Species identification by PCR-RFLP usually requires the use of more than one restriction endonuclease, and which restriction endonucleases will give discriminant fragments depends on the species underanalysis (Quinteiro *et al.,* 1998; Wolf *et al.,* 1999, 2000). Authentication using PCR-SSCP is based on the size of only two bands (each of the two single strands of the DNA amplicon), therefore the results are dependant on the specificity of the primers. Accordingly, primers should be developed for each group of species, and there may be intra-specific differences that make identification problematic (Martinez & Daniélsdóttir, 2000 and Figure 6). Sequencing is by far the most reliable but also expensive and time consuming, therefore its use as a routine technique by most laboratories is prohibitive at present. All these techniques demand some previous knowledge of the sample under analysis and make the identification procedure longer and more complex and expensive. However, they are the only methods that can be used when the DNA is severely degraded during processing.

In order to protect endangered fisheries, not only the species but also the location of capture will soon have to be documented and, as previously mentioned, important supermarket chains are interested in introducing the "ecolabelling" of some products. At this point, it is important to note that there is not yet a clear definition of what "stock" means. Taylor & Dizon (1999) argued that "management units" should be defined on a case-by-case basis and that the objectives of the policy established for the unit should be taken into account early in the design and execution of the scientific approach to preserving the unit. In the case of identifying the origin of samples of marine mammals, Taylor (see page 14 of Dizon *et al.,* 2000) suggested that the most efficient manner to answer the question of "where a sample came from" would be to take a hierarchical approach. This would identify first the species, then the ocean basin (for migratory animals), allocate the sample to a highly distinct population and then, finally, to the stock. This approach demands a exhaustive genetic analysis of each of the segments in the hierarchical level with the corresponding collection and analysis of a certain number of samples. The number of samples being dependant on how different the differ-

ent groups being compared are (see page 17 of Dizon *et al.,* 2000). In the case of minke whale we have found three genetic markers (Figure 7) that seem to be able to discriminate animals captured in the northeast and central Atlantic from those of the northwest Pacific (Martinez & Pastene, 1999). Similar markers will have to be found for other species.

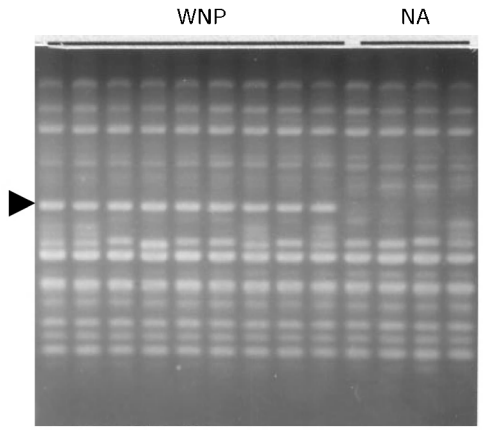

Figure 6 *RAPD fingerprinting of minke whale (*Balaenoptera acutorostrata*) from the Western North Pacific (WNP) and the North Atlantic (NA). The arrowhead indicates a stock-specific candidate marker. Each lane is one individual minke whale*

Targeting resistances

One issue of major concern at present is the increase in the number of bacterial strains that are developing resistance against antibiotics, especially if the resistance is against several antibiotics simultaneously (multi-resistance). Resistance to antimicrobials existed before the introduction of antibiotics to treat human and animal infections. The increase in the number of resistant strains, as well as in the number of antibiotics to which they show resistance, is attributed to their being selected, and their dissemination enhanced, primarily due to the misuse and abuse of these drugs for treatment and prophylactic purposes. There is a genetic basis for the resistance to antimicrobial substances, and susceptible strains may acquire the genes coding for the resistance mechanisms by conjugation (between bacteria of the same or similar species, see Gomis-Rüth *et al.*, 2001) transformation (uptake of DNA from unrelated species), or transduction (the transfer of DNA via a bacteriophage) (European Commission, 2000 and references therein).

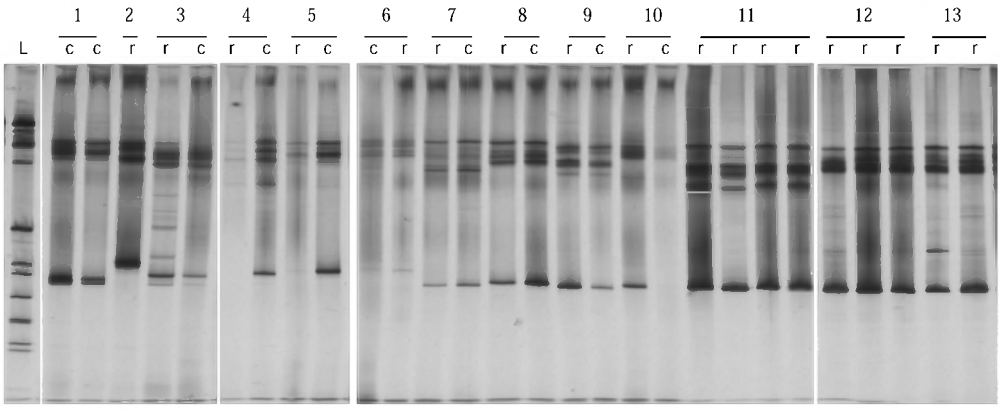

Figure 7 *Speciation analysis by Single Strand Conformation Polymorphism (SSCP) analysis of a 358 bp amplicon from the mitochondrial cytochrome b gene (Bartlett & Davidson, 1992). The samples are: 1, cod (*Gadus morhua*); 2, saithe (*Pollachius virens*); 3, hake (*Merluccisu merluccis*); 4, ling (*Molva molva*); 5, whiting (*Merlangius merlangus*); 6, haddock (*Melanogrammus aeglefinus*); 7, turbot (*Psetta maxima*); 8,* Onchorhynchus gorbuscha*; 9,* Onchorhynchus *keta; 10,* Salmo trutta*; 11, harp seal (*Phoca groenlandica*); 12, minke whale (*Balaenoptera acutorostrata*) and 13, fin whale (*B. physalus*). The processing conditions are: r, raw; c, cooked at 85°C. L, 1kb molecular mass standard ladder*

For the food processing industry the development of resistance to disinfectants, which may render their cleaning and disinfection procedures futile, is of concern. Disinfectants based on quaternary ammonium compounds (QAC) are commonly used in the food industry because they are non-corrosive and have low toxicity. It seems that there may be similarities between bacterial resistance to antibiotics and to biocides. Thus, gram-negative bacteria that have developed resistance to cationic biocides may also be unsusceptible to some antibiotics (Russell, 1997, 2000). Outer membrane changes are believed to be responsible for the non-specific increase in resistance; but efflux - another important resistance mechanism -, is associated with the *qacA/B* gene system in staphylococci and confers low-level resistance to cationic agents including chlorhexidine salts and QACs (Russell, 2000).

Gene transfer both between different genera and between species within a genus may occur with the possible spread of resistance genes to pathogenic strains (see Im *et al.,* 1996). Thus, clinical strains of QAC-resistant *Staphylococcus aureus* have been isolated in several countries from clinical environments (Leelaporn *et al.,* 1994) and from food products and food processing environments (Heir *et al.,* 1995, 1998). One of the plasmids that confer QAC-resistant is called *smr* (small multidrug resistance) which has been found in enterococci and staphylococci (Sasatsu *et al.,* 1995). The spread and maintenance of QAC resistance genes in staphylococci isolated from clinical environments and the food industry may be due to the selective pressure caused by the use of cationic biocides (Stickler & King, 1992). In order to design sensible disinfection procedures, Langsrud & Sundheim (1997) advocated the convenience of alternating the use of QACs with chlorine, phenolics, and alkylaminoacetate, to avoid the building-up of resistant strains.

Consequently, it seems that the acquisition of genes providing resistance to disinfectants may also help the bacteria to tolerate some antimicrobial substances. The opposite, on the other hand, may not always be the case: Kucken *et al.* (2000) and Rutala *et al.* (1997) found that multiple antibiotic-resistance does not appear to be correlated with increased resistance to the disinfectants phenol and a QAC.

The *Opinion of the Scientific Steering Committee on Antimicrobial Resistance - 28 May 1999* (European Commission, 2000) indicates very clearly the need to reduce the overall use of antimicrobials, which would involve the elimination of their unnecessary and improper use, more precise diagnoses of infectious agents, and monitoring of antimicrobial resistances. These goals call for the development of more rapid and accurate microbiological diagnostic tools to identify the resistance genes for the different antimicrobials carried by the strain under examination in order to proceed to the most satisfactory treatment.

Once the different genes conferring resistance to antimicrobials and/or disinfectants are sequenced, the PCR (particularly multiplex PCR, to detect several resistances simultaneously) can be the technique of choice to detect those targets in a fast and specific manner.

Simultaneous detection of many targets: DNA micro-arrays

DNA micro-arrays are miniature high density arrays that can have from a few hundreds to several hundred thousand oligonucleotides (probes) capable of taking part in hybridisation reactions (Figure 8). The probes are bound to a solid support (nylon membrane or glass). The sample to analyse is labelled (usually with a fluorophore) and applied to the array under hybridisation conditions (see Southern 1997, 2000; Southern & Elder, 1995; Southern & Maskos, 1995). Although the terms DNA micro-arrays and DNA chips are sometimes used indistinctly, Jordan (1998) made a distinction. He used the term micro-array to refer to sets of DNA targets deposited on a solid support (generally a glass microscope slide) with a spot density of several hundred individual spots per cm^2, while DNA chips (or oligonucleotide chips) refers to large sets of oligonucleotides synthesised *in situ,* usually at much higher densities.

The arrays are usually created by computer controlled robots that spot the probes onto precise locations on the support. The sample under analysis is labelled with a fluorogenic dye and added to the array, where it hybridises

to its complementary probe if it is present in the array. After ultraviolet irradiation, the bound sequences fluoresce and their intensity is measured by a detector. In addition to the three patents of Southern (Southern 1997, 2000; Southern & Maskos, 1995), Gerhold *et al.,* (1999), Jordan (1998) and Ramsay (1998) give a review and a description of the technology, operating principles and its applications. These include: detection of single nucleotide polymorphisms, linkage analysis, gene expression and the effects of stress, drugs or diseases on gene expression. DNA arrays have also been used for the simultaneous species identification, genotyping and assessment of antibiotic (rifampin) resistance of *Mycobacterium* spp (Gingeras *et al.,* 1998; Troesch *et al.,* 1999).

Quantification of the targets

One of the problems that the molecular techniques based on PCR have not been able to solve in the past was the quantification of the targeted sequence. Yet, this information about some components of food matrices is critical: from the number of colony forming units in the case of certain pathogenic bacteria, to the amount of gene modified ingredients or the amount of each ingredient (species) included in mixed products. Quantification of a target by PCR can be achieved by competitive PCR, and more recently by the application of real-time quantitative PCR (Holland *et al.,* 1991).

Application of proteomics to food safety and authenticity

Genomics has provided the whole DNA sequence of many organisms, and now there is a need to relate the nucleotide data to proteins. Proteins determine the cellular structure and activity, but while their sequence is dictated by genes, their activity is regulated in most cases by modifications suffered after being synthesised (called post-translational modifications) and by interactions with other proteins. The information obtained from the DNA is insufficient to predict protein modifications - which proteins and how are they going to be modified - how the modifications affect their activity and protein interactions. However, this information is crucial to understand how a cell will behave. Thus, a new field of research has emerged, called proteomics, to study all the proteins in a cell, tissue or organism.

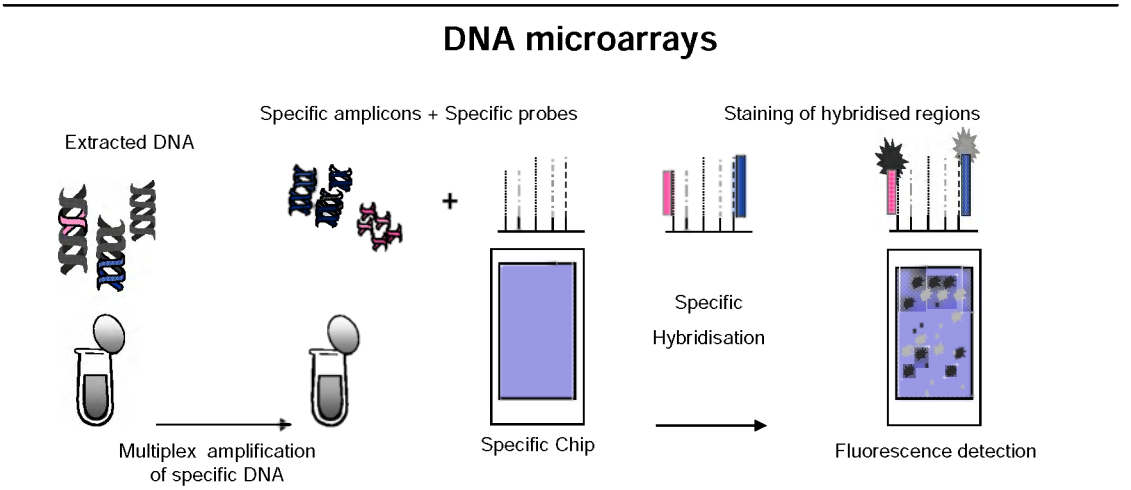

Figure 8 *Schematic representation of the DNA-microarray technology. In these arrays or chips, the hybridisations are carried out under conditions where the reaction rates and stringency are controlled by the temperature, salt concentration of the hybridisation solutions and washes, and the concentration of the target DNA. A later development in this technology exploits the use of electric fields (active microelectronic chips) to transport and concentrate the negatively charged nucleic acid molecules to the selected (positively charged) microlocations on the array (Heller* et al., *2000).*

Identification of the proteome - and the individual proteins of interest - usually starts by 2D-electrophoresis, followed by selecting the proteins under study, cutting the protein-spot from the gel (manually or by using robotics and automated systems), digesting them with proteases and then identifying the size, sequence, and post-translational modifications of the fragments by mass spectrometry. By comparison with sequences of known proteins contained in databases it is then possible to estimate as well the 3D structure of the peptides or proteins analysed.

There is not an absolute definition of the term "pathogenicity", and there are difficulties in distinguishing bacterial "virulence" factors from determinants permissive for pathogenicity, for example those promoting general fitness (Moxon & Tang, 2000). Yet, the availability of whole genome sequences, together with techniques for identifying key virulence genes, and the use of microarrays and proteomics will aid in the investigation of molecular basis of bacterial pathogenicity, as well as the patterns of gene expression needed to understand the host-microbe interactions *in vivo* (Moxon & Tang, 2000). Proteomics has provided new tools and information that complement the genomic-based information obtained about the epidemiology and taxonomy of human microbial pathogens, the identification of novel pathogenic mechanisms and the analysis of drug resistance (Cash, 2000; Moxon & Tang, 2000). The same principles and techniques applied to bacterial pathogens can be applied to parasite systematics, taxonomy, pathogenicity, susceptibilities and resistances (Barret *et al.,* 2000).

Proteomics has many relevant applications of direct interest to pharmaceutical companies since it permits the effective screening and design of drugs - and vaccines - for the treatments of cancer and genetic disorders (Kramer, 1998), infectious diseases (caused by viruses, bacteria or parasites, Barrett *et al.,* 2000; Gutierrez, 2000) and the study of the effect of toxic agents among others. Using the same techniques, it should be possible to apply proteomics to food safety for the characterization of the interactions pathogen-food matrix, and for the development of food-grade bacterial inhibitors. One interesting example could be *L. monocytogenes.* As mentioned above, about a quarter of the fishery products tested, including products ready to eat without further cooking, have been shown to contain *L. monocytogenes*, and yet, there has been only a few outbreaks in which the source was suspected to be fish products. Proteomics may help to clarify whether there might be some substances in fish products that either inhibits the growth of the bacterium or limits its pathogenicity. These type of studies can be important in the implementation of HACCP-plans in order to assess the real risk that the different hazards represent under different processing conditions and in different food matrices.

In addition, techniques fundamental to proteomics (2D-electrophoresis and mass spectrometry) have already been successfully applied to the authentication of hake species in fishery products by identifying species-specific protein fragments (Piñeiro *et al.,* 2000).

Summary and recommendations

Seafood may carry the risk of transmitting bacterial and/or parasitic infections. It is therefore essential to implement strict control systems such as HACCP, to avoid and/or eliminate the presence of these undesirable organisms in the seafood. Consumers in relatively rich countries are, apparently, better protected from food-borne diseases than in poorer countries, where the fish they eat may not have been assessed by the health control laboratories.

On the other hand, many countries' fisheries are severely depleted and alternative raw materials will have to be provided either from aquaculture or from different species. Interestingly, the main producers of aquacultured species and the ones with exploitable fishing grounds for alternative species are the poorer regions in the world - Asia, Africa and South America.

It is very important that when these countries introduce their products in new markets, the "new" consumers are properly

informed about the identity and properties of their purchases, which means ensuring that the products are safe, with characteristic organoleptic properties and that they are correctly labelled and identified. This will help the consumers to become familiar with a new product that they can come to appreciate and be willing to pay for. If, on the other hand, a new species is introduced as a substitute for another species or, even worse, is deceitfully labelled as something else, consumers will automatically assume that the new species is of lower quality than the "original" one. Since it is more difficult to restore a ruined reputation than to acquire one *de novo*, it is critical to avoid situations that can lead to negative perceptions from the very beginning.

The issue of safety is of major relevance. Seafood poisoning and diseases of bacterial origin have received relatively high attention and there are methods available to detect the micro-organisms or the toxins. This has not been the case with viruses, mainly because techniques for their detection have not been available. With improvement in the sensitivity, accuracy and capacity of diagnostic techniques, it is reasonable to expect an increase in the number of documented viral infections.

While viruses are mainly a problem in filter feeding organisms, parasites are primarily a problem in freshwater crustaceans and fish species. Aquaculture in infected areas demands the implementation of strict controls on each of the links of the chain. The waters and all inputs used to raise the fish must be free of potential human pathogens and parasites and the use of antibiotics must be reduced to the absolute minimum level. Many seafood consumers are not even aware of the fact that fish may carry potentially harmful parasites and there are not many studies published regarding the treatment necessary for their elimination from the flesh of the fish or crabs. Moreover, the tools for identification of parasites in clinical, veterinary and, especially food samples are troublesome, fastidious and require expert personnel. It is therefore not surprising that they often remain misdiagnosed. Faster and accurate techniques are required to identify these organisms, as well as the design of processing conditions that kill them if they are present in the product. Under present circumstances where these strict practices are not applied and with the lack of suitable methods for the detection of trematode metacercaria, the export of trematode-infested fish and crabs from endemic regions to other countries, may be only a matter of time. What it is particularly necessary is a long-term realistic plan to eradicate the parasites from populations where it is endemic and from the food chain. If local producers only aim to produce "safe" fish for export, the importing countries will still view with scepticism the quality and safety of a product coming from region where the parasite is endemic.

With advances in the fields of genomics and proteomics, together with increased concern about the quality and safety of foodstuffs, it is reasonable to assume that there will be an increase in the number of kits and equipment to conduct identity and safety tests on foodstuffs. Thousands of analyses will then be automatically handled in a much shorter time than required at present. This will impose more stringent conditions on the producers but will also help to diminish the risk associated with seafood consumption, increase the trust of the consumers in the safety of these products, and aid in improving public health by increasing the consumption of seafood.

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Notes

1) Iciar Martinez, the corresponding author, is Principal Researcher at the Centre of Industrial Processing of the Norwegian Institute of Fisheries and Aquaculture Ltd, and a Professor in Food Safety at the Norwegian College of Fishery Science, University of Tromsø; David James has recently retired from the post of Senior Fisheries Officer at the Food and Agriculture Organization if the United Nations, Rome.
2) The technique called Polymerase Chain Reaction (PCR) permits the billion-fold amplification of a template DNA. This technique has been made possible thanks to the availability of purified thermostable DNA polymerases (so that the enzyme does not losses its activity after each cycle) and chemically synthesized DNA oligonucleotides. The essential requirement to perform PCR is to have two oligonucleotides (called *primers*) whose sequence must be complementary to the sequence of the two fragments flanking the DNA region that one wishes to amplify (the *target*). The thermostable DNA polymerase is mixed together with a large excess of the two primers, the four deoxyribonucleoside triphosphates (dNTPs) and the purified DNA that one wishes to amplify. Each amplification cycle consists of three steps: the first step, called *denaturation*, is a brief heat treatment (92-96°C) to separate the two DNA strands; the second step, *annealing*, is performed at the temperature optimal for the hybridising of the primers to their complementary sequences in the template DNA strands (usually between 50-65°C); the third step, called *synthesis*, *extension* or *elongation,* is performed at the temperature at which the DNA polymerase has optimal activity, at about 72°C. Each cycle doubles the amount of targeted DNA and after *n* cycles, one has 2^n copies for each initial copy. The product of the PCR amplification is called *amplicon*. It is usual to perform somewhere between 25 and 40 cycles. A 40-cycle amplification may take from 15 min to about 4 hours, depending on the equipment used to heat and

cool-down the reactions. RNA can also be amplified in the same manner provided that the RNA is first "transcribed" into DNA, a reaction catalyzed by the enzyme called *reverse transcriptase*.

3) An example of this, a "classical" bacteria with novel and highly undesirable properties, is *E. coli* O157:H7, which causes haemorrhagic colitis and haemolytic uremic syndrome. The O157:H7 clone is closely related to a clone of O55:H7 strains that has been frequently associated with outbreaks of infantile diarrhoea. It is believed that the O157:H7 and O55:H7 clones have recently radiated from a common O55:H7-like ancestral cell. After horizontal transfer and recombination, plus the addition of Shiga-like toxin genes and adhesion genes to an *E. coli* genome,the end result was an organism preadapted for causing diarrhoeal disease (see Musser, 1996 and references therein).
4) The concept of the "Label of origin" which seems to be easily understandable when referring to raw materials becomes more complicated for manufactured products. Consider a fish captured in Norwegian waters, transported to China for filleting and then to Denmark where it is portioned and packaged - with packing material made from Russian wood, pulped in Finland, processed into paper in Sweden and printed in Finland . The packed portioned fillets are then transported to England where they are sold to a French company which is semi-processing the product and selling it to an Norwegian-owned cruise liner registered in Bermuda, operating out of Miami, where it is finally cooked and finally served, by a waiter from the Philippines, with vegetables grown in USA and a gravy based on dairy products from New Zealand. Now, where does the product that the consumer is served in the ship restaurant comes from?
5) 1 µl (microliter) equals 10^{-6} liter