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Detecting virus contamination in seafood

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12.1 Introduction: viruses and shellfish contamination

Viruses are obligate intracellular parasites depending on living cells for replication and usually infecting only a restricted range of hosts. Thus viruses cannot multiply in dead or processed food and therefore are not responsible for food spoilage. Human viruses may contaminate seafood but since the commercially exploited species (fish and shellfish) are widely divergent from humans, there is no evidence that they can act as a replication vector. Viral problems are thus limited to the role of seafood in passive transfer of viruses to humans. The viruses most adapted and likely to be carried in this way are those transmitted by the fecal-oral route. These include viral agents causing gastrointestinal disease in humans but also agents such as hepatitis A virus and polio virus which although being transmitted by the fecal-oral route, and often having a growth phase in the gut, exhibit their classical clinical symptoms elsewhere in the body. Such viruses can contaminate seafood at source through fecal pollution of the aquatic environment, or through poor hygiene during seafood processing (Fig. 12.1). Many viruses transmitted by the fecal-oral route are widely prevalent in the community and infected individuals can shed many millions of virus particles in their feces. Consequently viruses of many types occur in large numbers in municipal sewage and may also occur in other sources of human fecal contamination (Bosch, 1998). Sewage treatment processes are generally only partially effective at virus removal (depending on the treatment level) and may also be bypassed during periods of heavy rain or during emergencies (see Chapter 13). Other sources of fecal pollution, for example

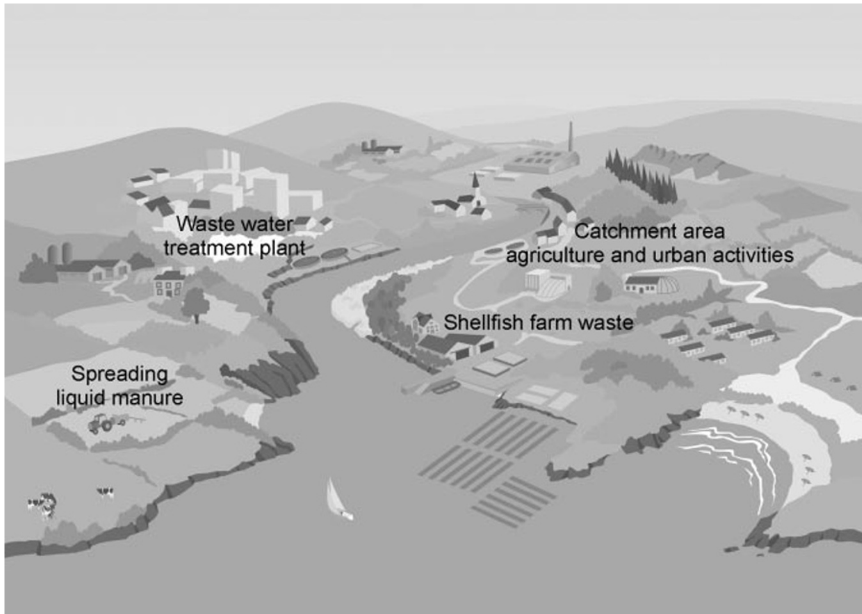

Fig. 12.1 Possible sources of contamination for shellfish growing in coastal area (source: Ifremer, www.ifremer.fr/envlit/).

septic tank overflows or boat discharges, may also contribute to marine contamination. Following marine discharge from such sources, viruses are capable of survival for long periods (Callahan *et al.*, 1995; Gantzer *et al.*, 1998; Nasser, 1994). Thus, seafood harvested from coastal locations is vulnerable to contamination with enteric viruses of potential health significance for man (Fig. 12.1). However, of the many harvested seafood species, only the filter-feeding bivalve molluscan shellfish (bivalve molluscs) have consistently proven to be an effective vehicle for the transmission of human viral diseases (Lees, 2000).

Bivalve molluscs are a type of shellfish that have two shell halves which hinge together. Species commonly commercially exploited in Europe include the native or flat oyster (*Ostrea edulis*), pacific oyster (*Crassostrea gigas*), common blue mussel (*Mytilus edulis*) and Mediterranean blue mussel (*Mytilus galloprovincialis*), cockles (*Cerastoderma edule*), king scallops (*Pecten maximus*) and queen scallops (*Chlamys opercularis*), and various clams including the native clam or palourde (*Tapes descussatus*), the hard shell clam (*Mercenaria mercenaria*), the manila clam (*Tapes philippinarum*), and the razor shell clam (*Ensis* spp.). The bivalve molluscs are an effective vehicle for transmission of enteric disease agents for several reasons. A principal factor is that these animals obtain their food by filtering small particles from their surrounding water. In the process of filter-feeding, bivalve molluscs may also concentrate and retain human pathogens derived from sewage contamination of growing waters. Many bivalve molluscs are harvested from sheltered in-shore coastal locations, such as river estuaries, which are often also susceptible to fecal

pollution (see Chapter 13). Important additional compounding factors are that a number of species of bivalve mollusc are traditionally consumed raw, or only lightly cooked, and are also consumed whole, i.e. including the viscera, which contains the bulk of contaminants. Disease incidents are most commonly associated with species consumed raw (or lightly cooked) and whole, such as oysters and clams and infrequently, or not at all, with species that are well cooked and where the viscera is not consumed, such as scallops. Disease incidents associated with bivalve molluscs have been extensively reported and have been reviewed by several authors (Richards, 1985; Rippey, 1994; Jaykus *et al.*, 1994, Lees, 2000).

The potential of bivalve molluscs to transmit enteric pathogens acquired through sewage pollution of growing areas first became recognised in the late 19th and early 20th century with numerous outbreaks of typhoid fever in several European countries, the US and elsewhere (Allen, 1899). Since this time there has been increasing recognition of the importance of human enteric viruses as the predominant aetiological agent in human illness incidents associated with consumption of bivalve molluscs. It is now well recognised that the most common illness associated with bivalve mollusc consumption is gastroenteritis caused by Norovirus (NoV). Other gastro enteric viruses, such as astroviruses and parvoviruses, have also occasionally been implicated in shellfish-related outbreaks, although their true epidemiological significance is not clear. NoV causes a relatively ‘mild’ gastroenteritis, often including nausea, diarrhoea, vomiting, fever and abdominal pain. The incubation period is 1 to 4 days with a duration of about 2 days and generally followed by complete recovery. NoV has previously been known as Norwalk-like virus or as small round structured virus (SRSV).

Human NoV cannot be propagated using cell culture (Duizer *et al.*, 2004) therefore characterisation and classification has been achieved largely using molecular techniques. It is now known that the *Norovirus* genus belongs to the *Caliciviridae* family and comprise a genetically diverse group of viruses which can be separated beneath this level into genogroups, clusters or genotypes, and individual strains (Zheng *et al.*, 2006; Hansman *et al.*, 2006). NoVs infecting humans group into genogroup one (up to 8 clusters), and genogroup two (up to 17 clusters) (Zheng *et al.*, 2006). Genetically related animal NoV strains have also been described (Oliver *et al.*, 2006); however, currently there is no evidence that they are capable of directly infecting man. The genetic diversity of NoV strains presents a difficult challenge for the design of molecular diagnostics capable of detecting all strains of health significance. It is now generally accepted that NoV is one of the most common causes of infectious intestinal disease in both outbreaks and in the community (Evans *et al.*, 1998; Tompkins *et al.*, 1999). Infections occur in all age groups including older children and adults. NoV is highly transmissible and often becomes noticeable through epidemic spread of diarrhoea and vomiting in closed communities such as hospitals, cruise and military ships and old people’s homes. NoV appears to be prevalent throughout the world. Infected individuals shed large amounts of virus in their

faeces thus domestic sewage and other polluted waters can normally be expected to be heavily contaminated with this virus (Lodder and Husman 2005), with the obvious risks for impacted bivalve molluscs. There are numerous reports in the scientific literature documenting the occurrence of NoV gastro enteric illness outbreaks associated with consumption of bivalve mollusc. These have been reviewed by several authors (Jaykus *et al.*,1994; Lees, 2000) and continue to occur (Doyle *et al.*, 2004). The need for measures to more adequately protect the consumer against viral infection are widely noted in such outbreak reports. US FDA risk assessments estimate cases of NoV mediated gastroenteritis related to seafood consumption at some 100,000 per year (Williams and Zorn, 1997). In addition to these direct health consequences, bivalve molluscs may also present a potent vector for emergence of recombinant NoV strains of enhanced virulence following contamination with multiple strains from human (and potentially animal) sources. Mixed human infections following consumption of bivalve molluscs with multiple contaminating NoV strains has been commonly reported (Gallimore *et al.*, 2005; Prato *et al.*, 2004; Kageyama *et al.*, 2004).

The other fecal-oral transmitted virus of major significance in bivalve molluscs related outbreaks is hepatitis A virus (HAV). HAV is a positive-strand RNA virus classified in its own genus of *Hepatovirus* within the *Picornaviridae*. There is only a single major serotype of HAV with three human antigenic variants and a number of genotypes identified by sequence analysis (Costa-Mattioli *et al.*, 2003). Compared to other enteric viruses HAV has an extended incubation period of about 4 weeks (range 2 to 6 weeks) and causes a serious debilitating disease progressing from a non-specific illness with fever, headache, nausea and malaise to vomiting, diarrhoea, abdominal pain and jaundice. HAV is self-limiting and rarely causes death but patients may be incapacitated for several months. Age has an important bearing on the severity of the infection with young children frequently experiencing only mild illness whereas overt hepatitis develops in the majority of infected adults. Recovery is complete and leads to long-term immunity from reinfection. HAV is a common endemic infection in developing countries with most children being seropositive by six years of age. However improving sanitary conditions in developed countries have lead to declining prevalence and resulted in large sectors of the population being susceptible to infection. HAV can be readily demonstrated in stools by molecular techniques (Yotsuyanagi *et al.*, 1996) and has also been demonstrated in sewage effluents and polluted receiving waters (Tsai *et al.*, 1994). Thus bivalve molluscs have frequently been implicated as food vehicles in outbreaks of hepatitis A (Klontz and Rippey 1991; Conaty *et al.*, 2000; Bosch *et al.*, 2001).

With the advent of molecular diagnostic methods in more recent years the polymerase chain reaction (PCR) has been used to study the contamination of molluscan shellfish with NoV and HAV at the low concentrations found in field samples. Various studies have shown rather high rates of viral contamination of commercially produced bivalve shellfish placed on the market in a number of different countries (Costantini *et al.*, 2006; Cheng *et al.*, 2005; Chironna *et al.*, 2002; Formiga-Cruz *et al.*, 2002; Nishida *et al.*, 2003; Boxman *et al.*, 2006)

illustrating the potential health hazards and the urgent need for diagnostic methods capable of identifying the risk and better protecting the consumer. The REFHEPA project within the SEAFOODplus consortium is aimed at the development of standardised procedures for the detection of HAV and NoV in bivalve molluscan shellfish to the point at which they could be used successfully in a routine diagnostic context to increase the safety of shellfish delivered to the consumer. The methodologies developed in the REFHEPA project have been successfully applied in another project, REDRISK, to detect viruses in naturally contaminated shellfish from France and Spain.

12.2 Methods for detecting viruses in shellfish

Viruses are present in shellfish in very low numbers; however, in sufficient amounts to pose a health risk (Bosch *et al.*, 1994; Sánchez *et al.*, 2002, Le Guyader *et al.*, 2003, 2006a). This low contamination makes necessary the development of highly sensitive viral extraction methods ensuring the virus recovery from shellfish tissues. The hypothesis made in the 1980s, that viruses are concentrated in digestive diverticulum tissues (Metcalf *et al.*, 1980), represented a major step for the progress of the extraction methodologies. This hypothesis was later confirmed by detection of HAV using an *in situ* system in oysters artificially contaminated following virus bioaccumulation (Romalde *et al.*, 1994) as well as through the tissue-specific quantification of infectious enteric adenoviruses and rotaviruses in mussels previously contaminated by bioaccumulation of such viruses (Abad *et al.*, 1997a). Additionally, a very interesting result has recently been described: the occurrence of a specific binding of Norwalk virus to oyster digestive tissues through the interaction with a N-acetylgalactosamine-containing receptor (Le Guyader *et al.*, 2006b). Analysis of digestive tissues provides several advantages, including increased sensitivity, decreased processing time and decreased interference with RT-PCR (Atmar *et al.*, 1995). Focusing the analysis of shellfish on the digestive tissues, where many of the viruses are concentrated, enhances assay performance by eliminating tissues (i.e. adductor muscle) that are rich in inhibitors but contain relatively little virus (Abad *et al.*, 1997a). As a matter of fact, the digestive tissues represent about one tenth of the total animal weight for oysters and mussels. Except for small species, such as clams or cockles, because dissection may be technically difficult, most of recent methods are based on dissected tissues and thus will be discussed here. Methods cited in the literature are diverse, complex, poorly standardised and restricted to a few specialist laboratories. It seems obvious that quality control and quality assurance issues must be solved, as well as simplification and automatation, of molecular procedures before they could be adopted by routine monitoring laboratories. All these latter issues have been addressed in the REFHEPA project of SEAFOODplus.

Extraction of enteric viruses from shellfish is based on several steps: virus elution from shellfish tissues, recovery of viral particles, and then virus

concentration. The weight analysed generally ranges from 1.5 to 2 g of digestives tissues. Some recent methods propose larger weights for the first step but thereafter analysing only part of them (Boxman *et al.*, 2006). Viruses are eluted from shellfish digestive tissues using various buffers (i.e. chloroform-butanol or glycine) before being concentrated either by polyethylene glycol precipitation or ultracentrifugation (Atmar *et al.*, 1995; Nishida *et al.*, 2003; Myrmel *et al.* 2004). Those approaches used in the analysis of whole shellfish meat, such as acidic adsorption prior to virus elution, are not applied to dissected tissues (Shieh *et al.*, 1999; Mullendore *et al.*, 2001). When working on dissected tissues, and applying molecular techniques, direct lysis of virus particles can also be used. For example, proteinase K, or Trizol and lysis of shellfish tissues using Zirconia beads and a denaturing buffer have all been used for virus and/or nucleic acid elution (Lodder-Verschoor *et al.,* 2005; Jothikumar *et al.,* 2005). A disadvantage of this direct approach is that a lower quantity of shellfish tissue is analysed in the RT-PCR assay.

Since the most relevant shellfish-borne viral pathogens, enteric hepatitis viruses A and E and noroviruses, are non-culturable RNA viruses, RT-PCR and now real-time RT-PCR are the methods of choice to set up sensitive protocol for their detection. The methods used for nucleic acid extraction are dependent on those used for virus elution and concentration. Most methods are based on guanidium extraction either using the methods described by Boom *et al.* (1990) or using a kit, based on similar chemistry (QIAamp or RNeasy kit by Qiagen®) (Shieh *et al.*, 1999; Loisy *et al.*, 2000; Schwab *et al.*, 2000). Capsid lysis by proteinase K and then purification of nucleic acid using phenol-chloroform and CTAB precipitation is a more labour-intensive but was one of the first successful methods described (Atmar *et al.*, 1993). One of the goals of the extraction methods is to remove inhibitors of the RT and PCR reactions sufficiently to allow detection of viral nucleic acids. Polysaccharides present in shellfish tissue are at least one substance that can inhibit the PCR reaction (Atmar *et al.*, 1993). Several reported methods eliminate inhibitors to varying degrees, although no systematic evaluation of the efficiency of inhibitor removal has been performed, and only a few of them have been applied on naturally contaminated shellfish. Inhibitor elimination is difficult to evaluate and depending on the time of the year and shellfish life, different compounds may be present (Di Giralimo *et al.*, 1977; Burkhardt and Calci, 2000). Internal control standards have been used to detect the presence of significant sample inhibition, and the amount of sample inhibition has varied depending upon the shellfish tissue being analysed (Atmar *et al.*, 1995; Schwab *et al.*, 1998; Le Guyader *et al.*, 2000). Dilution of the extracted sample is the approach often used to overcome the inhibitor problem, leading to a smaller quantity of shellfish tissues being analysed. For most methods in the literature, the weight of digestive tissues analysed in each RT reaction varies between 0.01 g and 2.5 g. The method analysing the smallest shellfish tissue weight (0.01 g) is based on direct lysis of virus without a concentration step (Jothikumar *et al.*, 2005), while the method analysing the largest tissue weight (2.5 g) is based upon direct extraction of all nucleic acids

followed by purification of nucleic acid using a poly A capture (Goswami *et al.*, 2002). It is important to promote methods allowing the biggest tissue analysis as it helps to improve the detection sensitivity.

Beside the inhibitor problem, molecular analysis of viruses in shellfish samples includes other frequent difficulties such as low virus concentrations, and sequence variation. As the extraction-concentration procedure is not virus specific, the nucleic acid of several viruses can be extracted at the same time. RT-PCR must be performed under stringent conditions and confirmed by hybridisation. The first important step for sensitivity and specificity is the synthesis of the complementary DNA (cDNA) by reverse transcription (RT). Most assays utilise a virus-specific primer in the RT reaction (Atmar *et al.*, 1995; Le Guyader *et al.*, 2000; Kingsley *et al.*, 2002; Formiga-Cruz *et al.*, 2002; Sánchez *et al.*, 2002; de Medici *et al.,* 2004; Myrmel *et al.,* 2004; Boxman *et al.*, 2006) but random hexamers are also used in some assays (Chung *et al.*, 1996; Green *et al.*, 1998; Cheng *et al.*, 2005). PCR amplification is usually performed for at least 40 cycles; some methods use nested PCR formats with fewer than 40 cycles in the first amplification reaction. Probe hybridisation is then performed as a confirmation step and enhances both assay sensitivity and specificity (Atmar *et al.*, 1995; Chung *et al.*, 1996; Shieh *et al.*, 1999; Le Guyader *et al.*, 2000; Sánchez *et al.*, 2002; Costantini *et al.*, 2006).

Sometimes it is necessary to analyse the amplified sequence in order to characterise the viral strains, and virus-specific amplicons must be sequenced to obtain additional information about the virus(es) present in the sample. This is particularly important for NoV detection, due to its wide strain diversity. However, sequence analysis is hampered by the scarce product sometimes obtained after PCR amplification from shellfish tissues. One of the limitations in developing RT-PCR assays for the detection of NoV has been the selection of proper primer and probe combinations that allow the detection of most or all strains of concern. The high genetic diversity of NoV makes it necessary to use broadly reactive primers. Despite several improvements in the methodology, up to now no single primer set is able to amplify all strains (Atmar and Estes, 2001; Vinje *et al.*, 2003). In the absence of such a universal primer set, multiple sets increase the chance to detect a greater number of strains, and the homology of the primers with the NoV strain is important in terms of sensitivity (Le Guyader *et al.*, 1996a, 2000, 2003, 2006a). No single assay stands out as the best by all criteria, such as evaluation of sensitivity, detection limit and assay format, not even for the stool analysis being clearly more difficult in the case of shellfish samples with such very low contamination (Atmar and Estes, 2001; Vinje *et al.,* 2003). For example, in three outbreak reports, primer sets targeting different regions of the NoV genome were needed to be able to amplify the strain both in clinical or environmental samples (Shieh *et al.*, 2000; Le Guyader *et al.*, 1996b, 2003, 2006a).

For HAV, primer selection is easier since the degree of variation, particularly in the 5′ non-coding region, is significantly lower (Sánchez *et al.*, 2004; Costafreda *et al.*, 2006). However, when genotyping is required other regions

must be analysed such as the VP1X2A junction (Robertson *et al.*, 1992; Sánchez *et al.*, 2004) or even larger fragments. However, the low virus concentration very often hampers the amplification of such large fragments.

Real-time RT-PCR assays, in which the RT, PCR and hybridisation assays are combined in a single well, are being developed and used successfully to detect enteric viruses in shellfish (Nishida *et al.*, 2003; Loisy *et al.*, 2005; Jothikumar *et al.*, 2005; Costafreda *et al.*, 2006). This technology takes advantage of not merely detecting but also quantifying the viruses present in the sample. However, for this last purpose it is necessary not only to develop but also to standardise the methodologies by including several controls at those most critical steps, let's say the nucleic acids extraction and the RT reaction (Costafreda *et al.*, 2006). The efficiency of the virus nucleic acids extraction must be controlled by means of a model virus while the efficiency of the RT-PCR reaction must be traced by means of a RNA molecule amplifiable and detectable with the same combination of primers and probes as those used for the actual virus. When these two reagents are added at known concentrations their recovery can be measured. Costafreda and colleagues (2006) proposed the use of the Mengo virus to evaluate the nucleic acid extraction efficiency, in general for any enteric virus and in particular for HAV, while the RNA molecule should be specific for each assay. The use of such an internal RNA control for the evaluation of the molecular reactions inhibition has been extensively used even in qualitative assays (Schwab *et al.*, 1998; Le Guyader *et al.*, 2003).

Regarding other viral pathogens, such as rotaviruses and astroviruses, an interesting alternative exists based on their capability of replication in some tissue culture systems, such as the CaCo-2 cells, which represents a universal *in vivo* amplification system for the enteric viruses (Pintó *et al.,* 1994) combined with either molecular (Abad *et al.*, 1997b; Pintó *et al.*, 1994, 1996, 1999) or immunological (Abad *et al.*, 1998; Bosch *et al.*, 2004) detection methods. Other cell culture molecular integrated systems have been proposed for enteroviruses (Reynolds *et al.*, 1996). Interestingly, these combinations allow the quantification of infectious viruses (Abad *et al.*, 1997a). However, although these techniques have been satisfactory evaluated and used in water samples, their application in shellfish is not common due to the infrequent shellfishborn viral outbreaks other than enteric hepatitis and norovirus gastroenteritis.

In summary, the quantitative assays open a new view in terms of analysis of the sanitary risks associated to the consumption of virus contaminated shellfish.

12.3 Potential emerging virus problems

The gut as a 'factory' of viruses is strongly selective. On one side, the alimentary tract with strong salivary enzymatic activity together with large pH shifts followed by bile acids and pancreatic enzymes aiming at breaking down foodstuff into its smallest components sets harsh conditions for a virus to survive. Most of the enteric viruses are small, non-enveloped RNA-viruses

possessing an icosahedral capsid. However, an increasing number of enveloped viruses have also emerged that are capable of surviving the enteric route.

The abundance of viruses in the gut offers exceptionally favorable conditions for genetic modifications of enteric viruses. Not only mutations, but recombinations and reassortments may facilitate the appearance of new variants of the already recognised viruses. An example of this is the recent appearance of a new variant of the well-known GII.4 type NoV. Within weeks it was able to spread via a variety of epidemiological routes throughout the world causing extensive outbreaks. This pandemic also demonstrates how fast an enteric pathogen may spread. It emphasises the threat posed by a pathogen of high medical impact, too.

There is only a semantic difference between a virus called 'new' and just a genetically modified old one. For practical reasons, a virus is 'new' when the population immunity is missing totally or to a considerable part. An example would be the above mentioned GII.4 new variant NoV. Owing to their error prone polymerase, viruses that possess a ssRNA genome are constantly modified by mutations and may lead to strains or variants of high virulence. An example of this was the poliovirus type 3 that caused an outbreak in a vaccinated population in Finland (Hovi *et al.*, 1986). Human rotaviruses, having a segmented genome, can undergo genetic changes through interchange of RNA segments, i.e. give rise to reassortants. This has been demonstrated clearly among group A rotaviruses (Maunula and von Bonsdorff, 2002). Waterborne outbreaks caused by group A rotaviruses have been detected (Villena *et al.*, 2003; Divizia *et al.*, 2004) but it is not known to which extent that virus possibly was modified. Rotaviruses of group B cause extensive outbreaks among adults, which appear to be restricted almost exclusively to China (Hung *et al.*, 1984). Also in China new unclassified rotaviruses have emerged causing outbreaks that are still only poorly defined (Yang *et al.*, 2004).

Rotaviruses of group C have been involved in cases of gastroenteritis throughout the world, both in sporadic cases and in outbreaks (Jiang *et al.*, 1996; Brown *et al.*, 1989). However, in general, the rotavirus C infections seem to be rare (Abid *et al.*, 2007). Group C viruses are also found in animals, preferentially in pigs. The porcine strains are, however, not identical to the human ones. For both rotaviruses belonging to groups B and C there is the potential that they may undergo changes that could increase their pathogenicity.

Some zoonotical agents have caught a lot of attention due to their potential to spread emerging infections. One of these agents is the severe acute respiratory syndrome (SARS, Peiris *et al.*, 2005). The causative virus belongs to the family Coronaviridae and is able to overcome the harsh alimentary tract conditions and is excreted in stools. However, whether this observation indicates an effective infection route for SARS remains to be determined, and thus a risk for seafood safety seems presently rather remote.

Another group of emerging viruses that has evoked a lot of attention are the highly pathogenic avian influenza viruses (HPAI), preferentially the ones classified as H5N1 and H7N3, reviewed by Horimoto and co-workers (2005).

These viruses have evolved from viruses of low pathogenicity by a mutation in the cleavage site of the hemagglutinin protein. This site is affected both by the local protein and the carbohydrate moieties (Stieneke-Gruber *et al.*, 1992; Kawaoka and Webster, 1988). The trypsin specific cleavage is changed to a less specific protease cleavage (Li *et al.*, 2004; Glaser *et al.*, 2005). In seabird colonies, among which all known influenza A viruses reside, they seem to cause very little harm. Such pathogenic strains emerge occasionally, as is the case at the time of the writing of this chapter. In birds the viral infection is enteric, i.e. the virus is secreted in the chloaca. Especially waterfowl, such as ducks and other dabblers, that reside and excrete the virus in shallow waters are of importance (Markwell and Shortridge, 1982). The inactivation of the viruses in water is rather slow lasting from weeks to months, depending on the conditions (Stallknecht *et al.*, 1990). Thus the viruses in water pose an infection risk for humans, too. The HPAI viruses show a varying pathogenecity among bird species. In general they cause mass death among cultured fowl like chicken, geese and turkey. Among wild bird species the pathogenicity varies. The reason for additional concern is the fact that they may infect humans and that the infections concur with a high mortality, up to 50%. The infections in man are, however, rare due to the receptor distribution in the respiratory pathway. It appears, that the 'right' sialic acid construction is found only in alveolar cells, not in nasopharynx (2,3- vs 2,6-sialic acid bond) (Matrosovich *et al.*, 2004). Thus only directly inhaled viruses that reach the susceptible cells will lead to an infection.

Although an infected duck may excrete as much as 1,010 infectious doses per day, and the virus is able to survive in contaminated waters as long as 4 days at 22 ºC and 30 days at a 0 ºC, the risk to acquire the infection through bathing in contaminated waters has been estimated to be negligible (WHO, 2006). Like HAV, hepatitis E virus (HEV) replicates in the gut epithelium. It evolves, however, into a systemic infection mostly affecting the liver. The disease is similar to that of HAV-infection with the exception of its devastating effect on pregnant women. Up to 20–30% of them succumb as a consequence of the infection (Khuroo, 1980). It is still unclear what causes this high mortality. Large waterborne outbreaks caused by HEV have been reported originally from India, but the virus circulates widely in tropical and subtropical areas. HEV underwent several classification steps before it was placed into its present own family Hepeviridae (Reyes *et al.*, 1990; Tam *et al.*, 1991). Most of the human hepeviruses belong to one serogroup, although a genome-based division into four genotypes has been defined (Schlauder and Mushahwar, 2001). Apart from the human HEVs, they have also been broadly found among animals, most commonly among swine (Meng *et al.*, 1997; Tei *et al.*, 2003). The swine HEV appear in three clusters, in two of these, human cases have been identified. The swine farms when contaminated provide a rich source of HEV with direct close contact to man but will also enter the circulation via water. The detection of porcine HEV in cases of human disease (van der Pool *et al.*, 2001; Meng *et al.*, 2002; Tamada *et al.*, 2004; Li *et al.*, 2006) indicates that this threat is real.

Nipah virus is a newly recognised zoonotic virus. The virus was 'discovered' in 1999 (Chua *et al.*, 2000). It has caused disease in animals and in humans, through contact with infectious animals. The virus is named after the location where it was first detected in Malaysia. Nipah is closely related to another newly recognised zoonotic virus called Hendra virus, named after the town where it first appeared in Australia. Both Nipah and Hendra are members of the virus family Paramyxoviridae (Eaton, 2001). Although members of this group of viruses have only caused a few focal outbreaks, the biologic property of these viruses to infect a wide range of hosts and to produce a disease causing significant mortality in humans has made this emerging viral infection a public heath concern. In symptomatic cases, the onset is usually with 'influenza-like' symptoms, with high fever and muscle pains (myalgia). The disease may progress to inflammation of the brain (encephalitis) with drowsiness, disorientation, convulsions and coma. Fifty percent of clinically apparent cases die. It is unlikely that Nipah virus is easily transmitted to man, although previous outbreak reports suggest that Nipah virus is transmitted from animals to humans more readily than Hendra virus. Pigs were the apparent source of infection among most human cases in the Malaysian outbreak of Nipah, but other sources, such as infected dogs and cats, cannot be excluded. Human-to-human transmission of Nipah virus has not been reported. The low stability of the paramyxovirus virions makes the shellfishborne transmission of Nipah virus an unrealistic possibility.

Advances in the detection tools for the 'classic' enteric virus pathogens (rotavirus, astrovirus, adenovirus and norovirus) also evidenced the occurrence of a variety of other agents such as Aichi virus, belonging to genus *Kobuvirus* within the Picornavirus family, and picobirnavirus in the Birnaviridae family. Their apparent rather limited circulation or low pathogenicity for man may be just temporary. With the increasing spread and efficiency by which especially food- and waterborne viruses are propagated all over the world, one can foresee the emergence of some of them as pathogens with more serious impact on the disease burden.

Another important issue in the emergence and re-emergence of viruses is their potential implication in bioterrorism. Apart from the airborne route of infection, the most damaging spread of a pathogen is achieved if (drinking) water can be contaminated. This also applies for possible contamination of molluscs. For this purpose, viruses normally not transmitted through water or food may be employed, smallpox being an obvious candidate. However the potential of poliovirus as a bioterrorism weapon in a future immunologically naïve population if poliomyelitis is finally eradicated should not be underestimated.

12.4 Conclusions

One key element in reducing foodborne spread of viruses is the implementation of surveillance, controls on the products before the commercialisation and awareness. Additionally, consumer-information campaigns must be

strengthened, including the promotion of suitable procedures of food preparation and consumption.

The REFHEPA project (SEAFOODplus) has produced methods for the detection of HAV and NoV in bivalve molluscan shellfish to the point at which they could be included in regulatory standards for viruses in molluscan bivalves which would greatly increase the safety of these products for public consumption.

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