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Viruses and bivalve shellfish

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1. Introduction

Two fundamental properties of viruses limit the extent to which they present a seafood safety problem. Firstly, viruses are obligate intracellular parasites depending entirely on a living host cell for the essential elements of replication. Although viruses can be extremely hardy in the external world, for instance hepatitis A virus has been shown to survive for more than a month in seawater (Callahan et al., 1995), they are inherently unable to multiply in the environment or in dead cells. Viruses are not therefore associated with food spoilage issues. They may however survive well on, or in, food following a contamination event. Indeed, the common seafood processing procedures of icing and freezing are likely to enhance survival of viruses as these are widely used laboratory preservation techniques for viruses. Secondly, the obligate intracellular nature of viruses tends to make them species specific for their host cell. Although viruses can jump species, or can have evolved a life cycle involving multiplication in different species, infection of widely divergent species is uncommon. So although indigenous marine viruses are the most abundant life form in the sea, typically numbering ten billion per litre (Fuhrman, 1999), only contaminating human viruses have ever been associated with illness in seafood consumers. Viral problems are therefore limited to the role of food in recycling human viruses back to humans. The viruses most adapted, and likely, to be carried in this way are those transmitted by the faecal–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 faecal–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 either through contamination at source, principally through sewage pollution of the marine environment, or in association with seafood processing through inadequate hygiene practices of operatives or systems. This review concentrates on seafood contamination through pollution of the marine environment. Contamination during food processing or handling shares common features with contamination of other food stuffs and is covered elsewhere.

Many viruses transmitted by the faecal–oral route are widely prevalent in the community and infected individuals can shed millions of virus particles in their faeces. Consequently viruses, of many types, occur in large numbers in sewage. Sewage treatment processes, if present, are only partially effective at virus removal (Sorber, 1983) therefore coastal dis-

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charges constantly release human viruses into the marine environment. Following shed into the environment viruses can survive for weeks to months (Nasser, 1994; Callahan et al., 1995; Gantzer et al., 1998) either in the water column or by attaching to particulate matter and accumulating in sediments. Thus seafood species harvested from sewage polluted areas may potentially be contaminated with human enteric viruses. However, a number of factors determine whether such potential contamination constitutes a health risk. Major factors include whether viral contamination remains on the surface or becomes internalised, and if so whether such contaminated organs are consumed or are removed during food preparation, and how thoroughly the seafood is cooked before consumption. Of the various seafood species although some, such as shrimp (Botero et al., 1996), have occasionally been shown to be contaminated with enteric viruses only the bivalve molluscan shellfish have consistently proven to be an effective vehicle for the transmission of viral disease.

2. Bivalve molluscan shellfish

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.*). With the exception of scallops these are normally static animals that attach themselves to, or bury themselves in the sea bed or other submerged surface. Dense beds of the animals can occur in productive areas and have been an important source of food since prehistoric times.

Indigenous species such as cockles, mussels and the native oyster continue to be harvested from natural populations, however, the characteristics of bivalve molluscs also make them suitable for cultivation. Nowadays the cultivation of indigenous species such as mussels and oysters is supplemented by breeding and farming introduced species such as pacific oysters and manila clams. Bivalve species vary greatly in their characteristics and habitats. Those adapted to drying conditions close their shells tightly when out of the water to retain a marine environment around their fleshy internal parts. Such species (oysters, mussels and clams) can survive for extended periods out of the water and are historically traded, and consumed, as whole live animals. Other species such as cockles are less hardy and are normally processed soon after harvest, however, they may also be traded as live animals if carefully handled. Scallops and other species not adapted to drying conditions soon die out of water and are normal handled as chilled or processed fishery product.

Contamination of bivalve shellfish with viruses and other agents occurs principally because these animals obtain their food by filtering small particles, such as algae, from their surrounding water. Many of the commercial species are common in inshore estuaries or similar shallow or drying areas where nutrient levels are high and the waters are sheltered. Unfortunately such shallow, in-shore, growing waters are also frequently contaminated with human sewage. In the process of filter-feeding bivalve shellfish may also concentrate and retain human pathogens derived from such sewage contamination. Bivalve shellfish can also accumulate naturally occurring toxic algae and pathogenic bacteria through filter-feeding activity. The hazards posed by bioaccumulation of harmful micro-organisms are compounded by the traditional consumption of certain shellfish species raw, or only lightly cooked, and by the consumption of the whole animal including the viscera. These circumstances are largely unique to bivalve shellfish and they therefore represent a special case among the microbial hazards associated with food. Human health problems associated with bivalve shellfish are well recognised internationally and have been recorded since medieval times. The association of shellfish-transmitted infectious disease with sewage pollution became well documented in the late 19th and early 20th century with numerous outbreaks of typhoid fever in several European countries, the USA and elsewhere (Allen, 1899). In more recent years the epidemiological evidence (reviewed below) suggests that human enteric viruses

are the most common aetiological agents transmitted by bivalve shellfish.

Among bivalves the oyster predominates as a disease vector in clinical statistics for the UK and Australia. In the USA both the oyster and the clam are important vehicles. A large variety of oyster and clam species have been associated with transmission of viral disease (Jaykus et al., 1994). The cockle and mussel have also been responsible for some large outbreaks both in the UK and elsewhere (see Section 4). The association of infectious disease incidents with these species probably reflects their traditional consumption raw, or only lightly cooked, and by the consumption of the whole animal including the viscera. Scallops which are both harvested from less polluted off-shore waters, and are generally eviscerated and cooked, do not present the same infectious disease hazard as oysters, mussels, cockles and clams. The filter-feeding nature of scallops however means they may still be associated with health incidents relating to contamination with marine biotoxins and naturally occurring bacterial pathogens. Other bivalve species such as razor clams (*Ensis spp*.) are not commonly associated with outbreaks possibly because they currently occupy a specialised, and small, share of the commercial market. Gastropods (e.g. winkles and whelks) are not filter feeders, are extensively cooked before consumption, and therefore do not present the same infectious disease hazards as the bivalve shellfish. However, like scallops, these species may present a hazard from bioaccumulation of marine biotoxins.

3. Viruses associated with bivalve shellfish

A number of human viruses transmitted by the faecal–oral route have been associated with shellfish either by the isolation of virus from shellfish samples or from epidemiological evidence linking them with disease in shellfish consumers.

3.1. Caliciviruses

Human enteric caliviruses are a group of related viruses with a positive sense single stranded nonsegmented RNA genome, a generally round morphology and small at about 30–35 nm diameter. Classical morphology caliciviruses have characteristic cup shaped depressions on their surface known as the ''Star of David'' morphology. These surface features are much less distinct in a large number of related strains with a generally amorphous surface with a ragged outline described collectively as Small Round Structured Viruses (SRSV) (Caul and Appleton, 1982). This group contains the prototype Norwalk virus, other characterised strains such as Southampton virus, Hawaii agent, Snow Mountain agent, and many other less well characterised strains. Recent taxonomic proposals classify the human enteric caliciviruses into two genus, Norwalk-like viruses (NLV) having a generally undefined SRSV type surface morphology and Sapporo-like viruses with the more classical caliciviruses morphology (Pringle, 1998). Hepatitis E virus, which like hepatitis A virus is transmitted by the faecal–oral route and is characterised by causing liver damage, also has calicivirus like features. However recent taxonomic proposals remove hepatitis E virus from the Caliciviridae and leave it not formally assigned to any virus group (Pringle, 1998). These human caliviruses are difficult to work with in the laboratory and cannot be cultured by conventional virological techniques. This has impeded progress in understanding of these agents until fairly recently with the advent of molecular techniques. Both NLV (see 5.2), Sapporo virus (Liu et al., 1995) and hepatitis E virus (Bradley, 1995) have now been cloned and sequenced. These molecular advances have paved the way for virus characterisation (Clarke and Lambden, 1997) and for important diagnostic advances through deployment of molecular techniques such as the polymerase chain reaction (PCR) and have lead to a better understanding of taxonomic relationships (Berke et al., 1997).

Sapporo viruses are genetically distinct from the NLVs (Liu et al., 1995) and, although studies are at an early stage, may comprise several distinct strains (Jiang et al., 1997). Seroepidemiological studies have shown a world-wide distribution for these viruses with a high seropositivity rate in adults (Nakata et al., 1996). Sapporo viruses cause sporadic individual cases and occasional outbreaks of diarrhoea illness mainly in infants and young children less than 5 years old, and also in the elderly. Clinical symptoms are similar to those following NLV infection (described below). Sapporo viruses have not been

documented as causing infectious disease following seafood consumption.

NLV (Kapikian, 1994) comprises a genetically diverse group of virus strains separated into genogroup I, containing the prototype Norwalk virus, and genogroup II, containing Snow Mountain and other strains (Green et al., 1994; Wang et al., 1994; Ando et al., 1994). Over recent years NLV genogroup II strains have tended to be more prevalent (Fankhauser et al., 1998; Maguire et al., 1999). Until recently NLVs had only been recognised as human viruses. However, similar diarrhoea causing viruses, closely related by morphological and molecular criteria, have now been recognised in cattle (Dastjerdi et al., 1999; Liu et al., 1999) and also possibly in pigs (Sugieda et al., 1998). Human NLV is now recognised to be a major cause of epidemic gastrointestinal illness occurring in families or in community-wide outbreaks. Infections occur in all age groups including older children and adults. The evidence clearly suggests that NLV is the most common cause of non-bacterial gastroenteritis in adults (Kaplan et al., 1982b; Kapikian, 1996; Vinje and Koopmans, 1996; Caul, 1996a; Fankhauser et al., 1998). Furthermore recent data from the UK suggests that NLV is the most significant cause of outbreaks of infectious intestinal disease, causing 43% of outbreaks during 1995 and 1996 compared with 15% for salmonellas (Evans et al., 1998), and also appears to be the most important cause of infectious intestinal disease in the community (Tompkins et al., 1999). Clinical symptoms include nausea, vomiting, diarrhoea, fever, and abdominal pain with an incubation period of 1–4 days generally followed by complete recovery with no complications. As far as is known these viruses are prevalent throughout the world. Unlike common causes of viral gastroenteritis in children, such as rotavirus, astrovirus and enteric adenovirus, NLV also frequently causes symptomatic infection in adults. This may be linked to the immunological response to these agents which is complex, and not well understood, but appears to be short term with individuals becoming susceptible to reinfection within six months to a year (Greenberg and Matsui, 1992). Consequently much of the adult population appears susceptible to infection with these agents and epidemic spread can ensue. NLV 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 (Khan et al., 1994; Sharp et al., 1995; Jiang et al., 1996b). The generally susceptible nature of the adult population becomes particularly noticeable in outbreaks where attack rates can be as high as 90–100%. NLV has been frequently linked with diarrhoea and vomiting (gastroenteritis) following seafood, and in particular bivalve molluscan shellfish, consumption. Patterns of immunity may influence this association since adults are generally susceptible and also tend to be the primary consumers of seafoods. This is particularly so for species such as the bivalve molluscs (oysters, mussels etc) known to be most at risk from viral contamination. The epidemiological features associated with NLV associated seafood outbreaks are described in more detail below (4.2).

Hepatitis E virus (Bradley, 1995) shares morphological and biophysical characteristics with caliciviruses however there are significant genomic differences. Recent taxonomic proposals leave hepatitis E virus not formally assigned to a virus group (Pringle, 1998). Like NLV, characterisation of hepatitis E virus has been achieved largely through molecular methods. Currently a number of aspects of the natural history of the virus are not well defined however it does appear to have a wide distribution and to infect all age groups. Although a number of genotypes can be differentiated, linked to geographical isolation, there may only be a single serotype (Tsarev et al., 1999). The clinical presentation of hepatitis E as an acute self-limiting liver disease shares many features with hepatitis A (see 3.6) with some differences notably the high mortality rate in pregnant women. Hepatitis E is endemic in many developing countries, particularly in Asia, causing both outbreaks and sporadic cases and has been recognised as a major cause of enterically transmitted non-A, non-B hepatitis. In developed countries, such as the UK, cases are usually associated with a history of travel to endemic areas. Most hepatitis E outbreaks are associated with the consumption of contaminated drinking water (Cromeans et al., 1994). The propensity for water borne transmission suggests shellfish would be at risk of contamination and could act as a vector for transmission of hepatitis E. Although direct evidence is not

currently available the association of shellfish with transmission of non-A, non B hepatitis (see 4.1) is highly suggestive.

3.2. Astroviruses

Astroviruses (Kurtz and Lee, 1987) have a positive sense single stranded (ss) non-segmented RNA genome. Virus particles are small, at 28 nm diameter, and round. A proportion of virus particles display a characteristic five or six pointed star-shape in their centre (hence astro). Astroviruses do not grow readily in cell culture however some strains have been adopted to grow in a continuous cell culture (Lee and Kurtz, 1981) and more recently specialised cell lines have been used to grow astroviruses directly from stool samples (Willcocks et al., 1990; Taylor et al., 1997). These advances have helped the molecular characterisation of astroviruses (Carter and Willcocks, 1996). Immunological and molecular techniques for diagnosis and characterisation are available in specialised laboratories (Cubitt, 1996). There are seven recognised human serotypes. Astroviruses cause sporadic individual cases and occasional outbreaks of diarrhoea illness mainly in infants, young children and the elderly. Astrovirus infections occur throughout the world and in temperate zones are most common during the winter months. Until recently astroviruses had been considered as fairly rare causes of enteric illness however it is now becoming clearer that astroviruses are common agents of childhood diarrhoea (Glass et al., 1996b). Clinical symptoms are similar to those following NLV infection and include vomiting, diarrhoea, fever, and abdominal pain. The incubation period may be longer than for NLV at 2–4 days with illness typically lasting another 2–3 days but occasionally 10–14 days (Kurtz and Lee, 1987; Caul, 1996a). Recovery is normally complete with no complications. Immunity following natural infection is solid, however, it may wane with age and the elderly are also a high risk group for astrovirus infection. It has been suggested that the use of diagnostic electron microscopy may tend to underestimate astrovirus prevalence in outbreaks since the classical astroviruses morphological features are not always readily visible in stool specimens. Astroviruses have been implicated in outbreaks where food and water (Kurtz and Lee, 1987; Oishi et al., 1994) and seafood (oyster) (Yamashita et al., 1991; Anon, 1998) were vehicles of infection. However, direct epidemiological links with food consumption are not well documented and there are few such reports compared with NLV. The importance of astroviruses as causative agents of gastroenteritis following seafood consumption is therefore currently unclear. The increasing availability of molecular diagnostic techniques for both outbreak investigation and food surveillance (see 5.2) should help elucidate the true prevalence of astroviruses as a cause of diarrhoea illness following seafood consumption.

3.3. Rotaviruses

Rotaviruses (Mattion et al., 1994; Bishop, 1996), established as a genus of the family Reoviridae, are spherical viruses with cubic symmetry. Complete virions are about 72 nm diameter with a smooth outer edge. They are named after their wheel like morphology in which the double icosahedral shell of protein subunits forms a rim round the nucleic acid core. Rotaviruses have a double-stranded RNA genome consisting of 11 separate strands. These strands can be readily demonstrated by polyacrylamide gel electrophoresis and provide characteristic information on virus strains. The majority of human rotavirus isolates share a common group antigen, with at least seven serotypes existing within this group, and are known as group A rotaviruses. However other viruses have been isolated that are morphologically identical but serologically unrelated to group A. These are known as group B to G rotaviruses. Only groups A, B and C have been associated with disease in humans. Rotavirus strains can be adapted to grow in cell culture which has considerably facilitated characterisation. The culturable properties of rotavirus, and the presence of large amounts of virus in stool, has lead to the development of a variety of diagnostic tests and assays (Yolken and Wilde, 1994) and consequently rotaviruses have been thoroughly investigated. Group A rotaviruses are ubiquitous infections with antibodies detectable by the age of 5 years in virtually all children. Group A rotaviruses have been frequently identified as the most important viral pathogen in diarrhoea disease requiring treatment or hospitalisa-

tion in children under 2 years of age. In developing countries they are very important pathogens accounting for some 20% of all diarrhoreal-associated deaths in children under five years of age. Rotaviruses are also important causes of diarrhoea requiring hospitalisation of children in industrialised countries. However, group A rotaviruses are not considered to cause any significant degree of disease in adults. By contrast non-group A rotaviruses (Bridger, 1994) are much less common and tend to cause sporadic family and community outbreaks of, sometimes severe, gastroenteritis in all age groups. Antibody prevalence levels in the community are correspondingly low and adults can get symptomatic infections.

The group A rotaviruses therefore normally present as paediatric infections occurring in infants or early childhood. Group A rotaviruses are common infections and viruses are shed in large numbers in stools ($>10^{12}$ particles/g faeces) leading to readily detectable virus presence in sewage effluents and polluted receiving waters (Gajardo et al., 1995; Dubois et al., 1997). Consequently rotavirus presence has also been demonstrated in bivalve shellfish grown in contaminated waters (see 5.2). However rotaviruses have not been linked with infectious disease following seafood consumption. The general absence of symptomatic disease in the adult population probably influences this lack of association. Adults, rather than children, tend to be the primary consumers of seafoods and adults tend not to demonstrate clinical disease following rotavirus infection. Age related resistance to severe infection may be due to active immunity reinforced by repeated infection throughout life (Bishop, 1996). Non-group A rotaviruses could pose a potential threat via contamination of seafood, particularly bivalve shellfish, however the author is not aware of any such documented incidents.

3.4. Adenoviruses

Adenoviruses (Wadell et al., 1994) have double stranded nonsegmented DNA genome. The virus particle is a symmetrical nonenveloped icosahedron with a diameter of 80 nm. Adenoviruses were originally isolated from adenoids, hence the name, and are common viruses among both humans, birds and other animals causing respiratory, ocular, gastroenteric and other infections. In humans a large number of serotypes are grouped into six main subgenera. Adenoviruses are generally readily culturable except for the fastidious enteric adenovirus, serotypes 40 and 41, predominantly associated with gastroenteritis (Wadell et al., 1994). Enteric adenoviruses are reported to cause about 10% of infantile gastroenteritis and are second only to rotavirus as a cause of diarrhoea related hospitalisation in children. Clinical symptoms in children include diarrhoea, vomiting and fever. In comparison with rotavirus infections enteric adenoviruses generally cause a less severe disease but with a more prolonged course. Diarrhoea may persist for several weeks infection among children seems common since serological studies show antibody in about 50% of older children and young adults. Both enteric adenoviruses causing diarrhoea, and non-enteric adenoviruses associated with infection elsewhere such as in the respiratory system, may be shed into the gut and be isolatable from faeces. Although adenoviruses can be detected in polluted sewage effluents, in seawater and in shellfish (Girones et al., 1995; Vantarakis and Papapetropoulou, 1998; Pina et al., 1998b) no seafood related outbreaks have been reported. This is presumably because enteric adenoviruses are not generally associated with gastroenteric disease in adults and, for the most part, young children are not primary consumers of seafood.

3.5. Enteroviruses

The enteroviruses (Minor et al., 1990) are a genus of the family Picornaviridae which comprises a large family of viruses. Enteroviruses have a positive sense single stranded nonsegmented RNA genome. The virion is a largely featureless smooth round nonenveloped particle of about 27 nm diameter. About 66 immunologically distinct serotypes are known to cause infections in humans including the polioviruses, group A and B coxsackieviruses, echoviruses, and the more recently designated enteroviruses serotypes 68 to 71 (Muir et al., 1998). All age groups can become infected. The normal site of replication is the intestinal tract where infection may often be mild or clinically inapparent. However in a proportion of cases the virus can spread to other organs and can cause serious or even fatal disease. The clinical characteristics of which, as for example with poliomyelitis, may be characteristic of par-

ticular enterovirus types. In addition to poliomyelitis enteroviruses can cause acute myocarditis, aseptic meningitis, hemorrhagic conjunctivitis, congenital infection of neonates and other non-specific febrile illnesses. There is also concern that enteroviruses may cause or contribute to a range of common chronic diseases such as diabetes mellitus. Commonly such acute symptoms occur only in a small proportion of those suffering an enterovirus infection. It should be noted that although enteroviruses replicate in the intestinal tract, and are transmitted by the faecal–oral route, they do not commonly cause classical gastroenteritic symptoms such as diarrhoea and vomiting. They are however shed in large numbers in faeces and most types can be readily cultured from stool samples. Because of their prevalence and ease of culture enteroviruses have a long history of use as markers of viral pollution of the environment. Many reports have documented their isolation in sewage effluents, polluted receiving waters and in bivalve shellfish (reviewed by Jaykus et al. 1994). However despite numerous demonstrations of contamination bivalve shellfish have not been linked to transmission of enterovirus disease. The epidemiological considerations are discussed in more detail below (4.3).

3.6. Hepatitis A virus

Hepatitis A virus (Cromeans et al., 1994) was formerly a member of the genus Enterovirus (serotype 72) and shares morphological and general genomic properties, and transmissibility by the faecal–oral route, with the enteroviruses as described above. However it is atypical with regard to its nucleic acid sequence, its extreme stability and its site of replication, currently thought to be the liver, and is now placed in a separate genus, the Hepatovirus (Minor, 1991), within the Picornaviridae. Compared to other enteric viruses hepatitis A virus has an extended incubation period of about 4 weeks (range 2–6 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. Hepatitis A 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. Hepatitis A is a common endemic infection in developing countries with most children being seropositive by 6 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 (see 4.1).

During infection virus is released from liver cells into the bile and is then excreted in faeces. Although laboratory adapted hepatitis A virus strains can be grown in culture, wild type hepatitis A virus is more fastidious. However hepatitis A virus 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) and in bivalve shellfish using PCR (see 5.2). Bivalve shellfish have frequently been implicated in outbreaks of hepatitis A infection the epidemiological features of which are described in more detail below.

4. Epidemiology of shellfish associated illness

Despite the variety of viruses transmitted by the faecal–oral route, many of which can be shown to contaminate shellfish or their harvesting areas, only a few have been epidemiologically linked to disease following consumption of bivalve shellfish or other seafood. Illness has been related to viruses causing gastroenteritis and viruses causing hepatitis. These are further discussed below.

4.1. Infectious hepatitis

Hepatitis A is the most serious virus infection linked to shellfish consumption causing a serious debilitating disease and even, occasionally, death. The first documented infectious hepatitis outbreak occurred in Sweden in 1955 when 629 cases were associated with raw oyster consumption (Roos, 1956). Since this time many hepatitis A virus outbreaks world-wide have been linked to bivalve mollusc consumption and have been reviewed by several authors (Richards, 1985; Rippey, 1994; Jaykus et al., 1994). Occasionally such outbreaks assume an epidemic scale and are worthy of special

note. The most graphic illustration being an outbreak of Hepatitis A in Shanghai, China in 1988 when almost 300 000 cases were traced to the consumption of clams harvested from a sewage polluted area (Halliday et al., 1991; Tang et al., 1991). This outbreak ranks as the largest viral food poisoning outbreak reported. In the outbreak the estimated attack rates in those who had, and had not, eaten clams were about 12% and 0.5% respectively with a very high overall hepatitis attack rate during the epidemic of 4083 per 100 000 population (1 person in 25) (Halliday et al., 1991). The USA has also reported a number of shellfish associated hepatitis A outbreaks (Richards, 1985; Rippey, 1994) as has the UK (Bostock et al., 1979; Sockett et al., 1985) and other countries such as Italy (Mele et al., 1989; Malfait et al., 1996) and Japan (Fujiyama et al., 1985). A hepatitis A epidemic in western France in 1991/92 involving around 800 cases was considered to be associated with shellfish consumption although definitive evidence was not available (Apairemarchais et al., 1995). Over 1000 cases of oyster and clam associated hepatitis A occurred in several US States during several major outbreaks from 1961 to 1964 (Richards, 1985). Since then further sizeable episodes occurred in 1973 due to Louisiana oysters (Glass et al., 1996a) and in a multistate outbreak in 1988 due to oyster from Florida (Desenclos et al., 1991). The Louisiana outbreak was attributed to sewage pollution of harvesting areas through flooding of the Mississippi and the Florida outbreak to illegal harvesting from contaminated areas. In the Louisiana outbreak hepatitis A virus was suspected to have been retained in shellfish for at least 6 weeks following the contamination event (Glass et al., 1996a). At the time of harvesting oysters were fully compliant with the USA sanitation program standards. These reported hepatitis A outbreaks have generally been linked to shellfish consumption by epidemiological data although hepatitis A virus was detected by molecular techniques in the harvesting areas associated with the Florida outbreak (Desenclos et al., 1991) and by isolation in primates in the Shanghai outbreak (Li et al., 1992).

Despite the incidents described above hepatitis outbreaks are less frequently reported than outbreaks with other clinical presentations, particularly gastroenteritis. It is also notable that many of the hepatitis A virus outbreaks reported involve large numbers of cases. The fairly protracted (average 4 weeks) incubation period of hepatitis A virus makes association with a particular food vehicle in individual or sporadic cases very difficult. Normally the food is not available for testing and consumption histories inconclusive. Generally therefore reports of shellfish associated hepatitis A virus disease fall into two groups: the large outbreaks where a common vehicle of infection is more obvious, and the smaller clusters or sporadic infections where a food vehicle involvement may only become evident through diligent epidemiological investigation. Since such investigation is the exception rather than the rule the association of shellfish with sporadic hepatitis A is probably considerably underreported (Rippey, 1994).

However, retrospective seroepidemiological investigation can help identify the respective risk factors for hepatitis A virus infection and place shellfish consumption in the larger disease context. Several such studies have been published. In an early study in the USA among infectious hepatitis patients in 10 Boston hospitals consumption of raw shellfish or steamed clams was as significant a risk factor as was exposure to a jaundiced person (Koff et al., 1967). In a German study between 1968 and 1971 shellfish consumption was linked to infectious hepatitis in 19% of patients attending a clinic in Frankfurt (Stille et al., 1972). Similar data was reported in a UK study which showed a significant correlation between infectious hepatitis and cockle consumption with as many as 25% of cases attributed to shellfish (O'Mahony et al., 1983). In a Japanese study 651 patients with acute viral hepatitis identified serologically between 1976 and 1985 were examined for risk factors occurring within 6 months of the onset of infection (Kiyosawa et al., 1987). Of the patients with hepatitis A, ingestion of raw shellfish was the highest identified risk factor (11%) slightly exceeding familial contact with a patient (10%), the other main risk factor identified. Studies in Italy have consistently identified shellfish consumption as one of the most important risk factors (Mele et al., 1990; Mele et al., 1991). Recent estimates suggest that shellfish may be responsible for 70% of all hepatitis A cases in Italy (Salamina and D'Argenio, 1998). These various studies suggest that shellfish may be significant vehicles of hepatitis A virus in sporadic cases as well as identified outbreak cases. However a large case control study of sporadic hepatitis A in

England carried out from July 1990 to June 1991 did not support these findings (Maguire et al., 1995). Although shellfish consumption appeared to be significant at the 5% level in a simple model it failed to retain significance when confounders were examined. It may be significant that during this study period there was only a single reported outbreak of shellfish associated hepatitis A virus in England (Sockett et al., 1993). Possibly recognisable outbreaks reflect a larger burden of sporadic shellfish-borne disease in the community. It is noticeable that earlier seroepidemiological studies in the UK were performed in years when several large outbreaks were reported.

A common feature of hepatitis A in many developed countries is the generally declining incidence of infection as shown by seroepidemiological surveys (Mele et al., 1990; Koff, 1995; Gdalevich et al., 1998). Seropositivity rates in the older population may now be much higher than in young adults. In endemic areas hepatitis A is generally acquired in childhood when the disease may be mild or subclinical and generally acquired by social contact. In many developed countries declining endemic exposure of children leaves the adult population exposed to the potential for hepatitis A virus epidemics from vectors such as food or water or to acquiring infection from travel to an endemic area (Armigliato et al., 1986; Pebody et al., 1998). Although shellfish associated hepatitis A virus infections may currently be uncommon in some developed countries, such as the UK, continuing vigilance is important as shellfish have demonstrated on many previous occasions their potential to act as vectors for hepatitis A virus epidemics and the adult population is now largely susceptible. Australia suffered such an explosive outbreak of oyster mediated hepatitis A during 1997. Although official reports are not yet available reports in the media and from subsequent court proceedings suggest this was the first such hepatitis A virus epidemic for a number of years and that over 400 people were infected (Anon, 1999b). The impact of such an epidemic on both the oyster industry, the health services and the regulatory authorities should not be underestimated. In Australia expanding coastal populations in fragile estuarine locations with inadequate sewage systems were highlighted as the probable cause.

Cases of non-A non-B hepatitis have also been linked to shellfish consumption (Jaykus et al., 1994; Torne et al., 1988) and shellfish have been possibly implicated in transmission through seroepidemiological studies (Mele et al., 1986). It is probable that such cases are caused by faecal–orally transmitted hepatitis E virus although this has not yet been formally established. Hepatitis E is not endemic to the UK and other developed countries and most cases are travel associated. However, shellfish may be vectors for hepatitis E in endemic areas such as India and it is possible that globally traded shellfish could cause infections in non-indigenous areas. Recently hepatitis E has been demonstrated in sewage samples from a non-endemic area (Barcelona, Spain) raising the concern that shellfish could act as vectors for this virus even in non-endemic areas (Pina et al., 1998a).

4.2. Viral gastroenteritis

Gastroenteritis has been recognised as a clinical consequence of the consumption of contaminated shellfish for many years (Richards, 1985; Rippey, 1994). The clinical symptoms in such cases are often similar and are typically described as relatively ‘mild’ gastroenteritis, often including diarrhoea and vomiting, with an onset of 24–28 h, a duration of about 2 days and rarely requiring care from a physician. Since the last cases of typhoid fever in the fifties bacteria have rarely been identified as the cause of shellfish-vectored enteric illness and certainly the majority of cases of gastroenteritis appeared to be non-bacterial in nature. Indeed until the 1970s laboratory investigation generally failed to reveal a causative agent in the large majority of such incidents. However the clinical symptoms were characteristic of viral gastroenteritis, such as caused by NLV, and it is now widely recognised that these viral agents are the major cause of shellfish-vectored gastroenteritis.

The first linkage of viruses with shellfish-borne gastroenteritis infection was made in the winter of 1976/77 in the UK when cooked cockles were epidemiologically linked to 33 incidents effecting nearly 800 people (Appleton and Pereira, 1977). Small round virus like particles, like those seen in outbreaks of ‘winter vomiting disease’, were observed by EM in a high proportion of patients faeces. Subsequently the same author examined clinical

samples associated with 9 separate shellfish-vectored gastroenteritis outbreaks and showed similar small round virus like particles in about 90% of samples. This evidence indicated for the first time the probable broad significance of viruses in shellfish transmitted infections in the UK (Appleton et al., 1981). Meanwhile in Australia a large oyster associated gastroenteritis outbreak involving some 2000 persons occurred during the summer of 1978 (Murphy et al., 1979). Laboratory investigations failed to show a bacterial aetiology but 'parvovirus-like' virus particles were seen by EM in patients faeces and development of a related antibody response shown by immune electron microscopy. Further examination identified the virus particles as Norwalk virus demonstrating for the first time the association of this virus with shellfish-vectored disease. This outbreak was linked to sewage contamination of the oyster harvesting area near Sydney caused by heavy rainfall. Frozen oysters from the same harvesting area caused a related outbreak in Darwin, Australia in December 1978 and Norwalk virus was again confirmed by both serology and by EM in patients stools (Linco and Grohmann, 1980). Subsequent efforts in Australia to improve the food safety of oysters again demonstrated the importance of Norwalk virus as a cause of gastroenteritis when a number of volunteers in consumption trials became ill with this agent (Grohmann et al., 1981). Again illness was associated with heavy rainfall, and hence probable sewage contamination, in the oyster harvesting area.

Since these early reports numerous publications have documented shellfish associated outbreaks of gastroenteritis caused by NLV and incidents up to about 1990 have been reviewed by several authors (Richards, 1985; Rippey, 1994; Jaykus et al., 1994). In the USA the first cases of shellfish-associated gastroenteritis shown to be caused by NLV occurred in 1980 among individuals consuming oysters harvested in Florida (Gunn et al., 1982). Since this time a succession of suspected or confirmed shellfish associated viral gastroenteritis incidents have been reported in the USA (listed up to 1985 by (Richards, 1985). During 1982 the situation was judged to have taken on epidemic proportions with 103 well documented outbreaks in which more than 1000 persons became ill from eating clams or oysters in the State of New York alone (Morse et al., 1986). Norwalk virus was implicated as the predominant aetiological agent based on the clinical features of disease, on seroconversion to Norwalk virus and by demonstration of Norwalk virus contamination of shellfish by radioimmunoassay. Shellfish originated from several north-eastern states with faecal contamination caused by runoff due to heavy spring rains the most likely cause. A further large incident suspected to be caused by Norwalk virus occurred in Louisiana in 1982 with 472 cases linked to oysters (Richards, 1985). These and other outbreaks during the early 1980s prompted dealers to import purified (depurated) English clams during 1983. These were responsible for a further wave of at least 14 separate suspected viral gastroenteritis outbreaks involving some 2000 consumers in New York and New Jersey over a 3 month period during 1983 (Richards, 1985). In one outbreak alone over 1100 persons reported ill after consumption of clams at a picnic. In the UK oysters were implicated in an outbreak in London in 1983 causing illness in over 300 people (Gill et al., 1983). The attack rate was 79% among oyster eaters and NLV was confirmed as the aetiological agent by detection in stools by electron microscopy and by a rise in antibody to small round viruses. Oysters had been depurated for 72 h prior to consumption. Further gastroenteritis outbreaks associated with depurated shellfish (oysters) were reported in the UK in 1986 (Heller et al., 1986), 1993 (Chalmers and Mcmillan, 1995), 1994 (Perrett and Kudesia, 1995) and 1997 (Ang, 1998). Investigation of two incidents in New York State during 1983 provided the first documented shellfish associated outbreak of Snow Mountain agent, another NLV strain (Truman et al., 1987). Attack rates in those who eat clams were 55% and clam eaters were more likely to seroconvert to Snow Mountain Agent than controls. Clams were harvested in Massachusetts during a period of heavy rainfall and high runoff. In 1984 and 1985 further outbreaks of shellfish-associated probable viral gastroenteritis were reported in New York State (Richards, 1985; Guzewich and Morse, 1986). In 1990 another major incident involving oysters from the same area as previous outbreaks occurred in Australia (Bird and Kraa, 1995). Fifty-seven separate outbreaks of oyster-associated viral gastro-enteritis occurred over an 18 day period: the incident followed a period of heavy rainfall with the resultant flooding of the sewage system and discharge of large amounts of crude sewage. In late 1991 an outbreak

of gastroenteritis following the consumption of raw oysters provided the first documented shellfish associated NLV outbreak in Canada (Pontefract et al., 1993). Some 200 persons were effected. NLV was demonstrated by electron microscopy in stools and an antibody response to these virus particles demonstrated by immune electron microscopy. The source of oyster contamination could not be determined. Gastroenteritis outbreaks connected to oysters have been reported from a number of districts in Japan. In Kyushu district four of five oyster related outbreaks between 1987 and 1992 were shown to be caused by NLV (Otsu, 1999). In Gifu prefecture 2 oyster outbreaks occurring in 1989 and 1991 were shown to be caused by NLV (Kawamoto et al., 1993). Attack rates among oyster eaters were 68% and 92% for the outbreaks. Western blotting immunoassay provided additional information and showed the NLV strain detected to be antigenically related to Hawaii agent (genogroup II) but not to Norwalk virus (genogroup I). Investigation of a further four outbreaks were reported in Shizuoka district between 1987 and 1994 with both genogroup I and genogroup II NLV strains demonstrated by molecular techniques (Sugieda et al., 1996). During recent years this application of molecular techniques to NLV (see 5.2) has raised new possibilities for the investigation of outbreaks. Virus genome characterisation by sequencing enables the definitive linkage of patient and vector, the linkage of isolated episodes in a large single-source outbreak, and potentially the tracing back of contaminated product to the responsible harvesting area. A good example of the application of these new molecular epidemiological tools occurred in 1993 when a large multistate outbreak of gastroenteritis occurred in the USA in Louisiana (Kohn et al., 1995b), Maryland, North Carolina, Mississippi, Texas and Pennsylvania (Dowell et al., 1995). The outbreak was linked to oysters harvested in a small area in Louisiana over a 4 day period in November 1993. Twenty five separate clusters of illness were identified with some 200 cases identified probably representing only a small proportion of the total number of persons effected. With over 4 million oysters harvested and an attack rate among oyster eaters of 62% the authors calculate that as many as 186 000 people may have become ill (Dowell et al., 1995). NLV was confirmed as the causative agent by both electron microscopy and PCR on stool samples and by seroconversion to Norwalk virus in patients (Kohn et al., 1995b). The oysters were harvested by boat from a remote bed thought to be free of any sewage pollution. However the investigation showed oyster harvesters routinely disposed of faeces overboard and furthermore that one harvester, with a high level of antibodies to Norwalk virus, had experienced gastroenteritis just prior to the responsible harvesting period. It is remarkable that faeces from a single individual may have been responsible for contaminating such a large quantity of oysters and powerfully illustrates the bioaccumulative potential of bivalve shellfish. Molecular epidemiological approaches were applied, possibly for the first time, to the investigation of this NLV outbreak. PCR followed by sequencing was applied to stools from a number of cases from different clusters of illness and this clearly showed that clusters were linked and caused by a single unique NLV strain. This approach was also able to distinguish this large outbreak from a smaller co-incident cluster of cases associated with Florida oysters, an outbreak in a nursing home, and a case in an oyster packer, which were all shown to be caused by unrelated NLV strains (Ando et al., 1995a). The cluster of cases in Florida were separated investigated and shown to be caused by locally harvested oysters and to be unrelated to the main Louisiana outbreak (Davis et al., 1994). A further large gastroenteritis outbreak also linked to overboard dumping of faeces by oyster harvesters occurred in Florida in January 1995 (Mcdonnell et al., 1997). Illness was reported in 38 gatherings state-wide and was reported to be the largest oyster-borne outbreak yet identified in Florida. A cohort epidemiological study among 223 people exposed confirmed oysters as the vehicle and showed an attack rate of 58% among oyster eaters. The aetiological agent was confirmed as NLV by electron microscopy and by seroconversion of patients to Norwalk virus. However during this outbreak PCR was not found useful possibly because the primers used were not appropriate for the outbreak strain. The majority of oysters were restaurant or home cooked prior to consumption however this appeared to offer little protection. During the investigation it became apparent that an outbreak of diarrhoea illness had occurred in two bayside communities just prior to oyster harvesting for the New Year celebrations. This resulted in an increase in the reportedly com-

mon practice of overboard dumping of faeces by harvesters with the consequential contamination of the beds in December 1996 through to January 1997 yet further outbreaks of viral gastroenteritis were linked to oysters harvested in Louisiana (Farley et al., 1998). Sixty clusters of cases comprising 493 persons were reported from 5 USA States. NLV was found in stools by electron microscopy and by PCR. However unlike previous outbreaks sequencing identified 3 different NLV strains occurring in the clusters examined which implicated 3 separate harvesting areas in Louisiana. Again overboard dumping of sewage by oyster boats was implicated as the probable cause of oyster contamination. Molecular techniques have also been applied to investigation of 4 outbreaks of shellfish-associated gastroenteritis occurring in Japan between 1987 and 1994. Both genogroup I and genogroup II NLV strains were demonstrated in both stool and shellfish samples by PCR and sequencing confirming NLV as the causative agent in these outbreaks (Sugieda et al., 1996). Recently PCR has been used to document NLV contamination of oysters associated with viral gastroenteritis outbreaks in the UK (Lees et al., 1995b; Green et al., 1998) and the USA (Le Guyader et al., 1996b) illustrating the increasing potential of this technique for determination of virus contamination in shellfish as well as stool samples (see 6.2). In Europe a major outbreak, involving some 350 identified cases, occurred in Denmark and neighbouring Scandinavian countries in January 1997 (Christensen et al., 1998). The outbreak was caused by imported oysters. NLV was identified as the causative agent of the gastroenteritis by electron microscopy and PCR in stool samples and by PCR in shellfish. During this outbreak unusual secondary complications were seen which may have indicated infection or intoxication by an agent additional to NLV. Enterovirus was detected by PCR in both stools and shellfish and low, sub-regulatory, levels of domoic acid (a naturally occurring marine algal biotoxin) were also found.

Suspect or confirmed viral gastroenteritis outbreaks associated with oysters and other bivalve shellfish continue to be reported in the UK and many other countries up to the present date (epidemiological surveillance data is reviewed below). Incidents vary in size and significance however when appropriate laboratory tests are conducted NLV is almost invariably identified as the responsible pathogen. An increasingly frequent aspect highlighted in reports, in all countries, is the compliance of both harvesting areas, treatment processes, and products sold to the consumer, with all regulatory requirements. It seems therefore to be increasing widely recognised that current regulations are apparently unable to guarantee protection of the oyster consumer or to prevent further outbreaks. In the UK, Australia, and some other countries, purification (depuration) is widely practised for oysters and other shellfish consumed raw. However a number of reports show that gastroenteritis outbreaks can occur following consumption of oysters purified according to all legal requirements (Grohmann et al., 1981; Gill et al., 1983; Chalmers and Mcmillan, 1995; Perrett and Kudesia, 1995; Ang, 1998) and therefore that this process as currently practised also cannot guarantee consumer protection. The need for measures to more adequately protect the consumer against viral infection are widely noted in outbreak reports.

Recent molecular data has shown that patients may suffer from a mixed infection of both genogroup I and genogroup II NLV strains following shellfish consumption (Ando et al., 1995a; Sugieda et al., 1996). Recent studies in this laboratory concur with these findings and suggest that mixtures of NLV strains appear fairly common in shellfish harvested from contaminated sites. Likewise it is interesting to note that in some outbreaks patients may experience gastroenteritis followed by hepatitis (Richards, 1985; Halliday et al., 1991) suggesting a mixed NLV/hepatitis A virus contamination in shellfish causing such outbreaks. In a similar fashion in an oyster associated outbreak involving navel officers two episodes of gastroenteritis occurred the first caused by NLV and the second seemingly by astrovirus (Caul, 1996b). It seems possible therefore that shellfish harvested from contaminated areas could often contain a cocktail of viruses and that patients may consequently be infected simultaneously with a number of virus strains. This could contribute to the high attack rates, sometimes as high as 100%, noticed in some shellfish mediated NLV outbreaks. NLV immunity is generally regarded as short term and given the emerging data showing antigenic diversity between strains (Jiang et al., 1996a) may also prove to be fairly strain specific. In a mixed infection even where short term immunity is present it is unlikely to protect against a flux of contaminating strains. These findings may also emphasis the potential significance of shellfish as a vehicle for

spreading NLV strains particularly given the increasingly global trade in food commodities such as shellfish.

Retrospective seroepidemiological studies can help establish high risk factors, and hence the importance of particular vehicles of transmission, and have been used to investigate the significance of food-borne hepatitis A virus. As immunological reagents become available for the NLVs it will be interesting to carry out similar studies to help establish the contribution of shellfish as a food vector to the community disease burden with NLVs. Such data is an important aspect of modern risk assessment techniques. Currently however NLV proteins expressed by molecular techniques appear too narrow in their serological reactivity to be particularly useful reagents for such seroepidemiological studies (Noel et al., 1997).

Although NLV is clearly the predominate cause of gastroenteritis linked to shellfish consumption other viruses have also been identified in stool specimens. Virus particles morphologically similar to parvoviruses (small round and featureless) have been reported in stool samples from shellfish and other outbreaks (Appleton, 1994). However establishing their clinical significance is complicated by their observation also in patients with no gastroenteric symptoms and by their occurrence in stool specimens alongside more established gastroenteritis agents such as NLV and rotavirus. As EM is currently the only detection method for these agents progress has been slow and their significance as agents of foodborne disease remains to be established. A few reports link astrovirus to shellfish consumption (Yamashita et al., 1991; Caul, 1996b; Anon, 1998) however this seems comparatively rare. Such incidents may be associated with less common serotypes as astroviruses are now generally regarded as ubiquitous infections of the young with consequently high levels of adult immunity (see 3.2). Rotaviruses are a similarly common infection of the young and although they have been demonstrated as contaminants of polluted shellfish they have not been reported as a cause of shellfish-vectored disease outbreaks (see 3.3).

4.3. Other viral diseases

Human enteroviruses are commonly isolated from sewage and sewage polluted waters and can readily be demonstrated as contaminants of bivalve shellfish by conventional virus isolation and by molecular techniques such as PCR (see 5.1 and 5.2). Indeed for many years methods for detection of ‘viruses’ in shellfish targeted the culturable enteroviruses. Enteroviruses detected include poliovirus, originating from the live oral vaccine, coxsackievirus, echovirus and other strains. It is now clear that enteroviruses are not generally responsible for clinically apparent gastroenteritis following shellfish consumption. Enteroviruses transmitted by the faecal–oral route are however clinically associated with a wide variety of other illness ranging from aseptic meningitis to chronic diseases such as myocarditis. Such systemic illness often has a protracted incubation period following initial faecal–oral transmission and may only occur in a percentage of those infected. Enteroviruses have only rarely been associated with food-borne infections (Cliver, 1994b) and have not generally been associated with shellfish (or seafood) vectored infection. This is intriguing given the ready demonstration of enterovirus contamination in shellfish harvested from polluted, and even relatively clean, sites (reviewed by Jaykus et al., 1994) and hence the probable exposure of shellfish consumers. Possibly the epidemiological presentation of many enterovirus infections (i.e. their long incubation period and unpredictable appearance of clinical symptoms) does not favour their identification and linkage to a particular food vehicle. Other authors have commented that the public health impact of enteroviruses transmitted by shellfish is probably not fully appreciated (Lipp and Rose, 1997). In a recent oyster associated outbreak of NLV gastroenteritis in Scandinavia unusual secondary symptoms, possibly reminiscent of enterovirus infection, occurred in some infected consumers (Christensen et al., 1998). Enterovirus was detected by PCR in both shellfish and patients stool with isolations from stool showing a common sequence identity suggestive of a common source infection. It is not clear whether an enterovirus infection caused the secondary symptoms however further consideration of the possible role of enteroviruses in shellfish transmitted infection is probably warranted.

Two epidemiological case control studies have reported, amongst other significant factors, statistically significant or nearly statistically significant, relationships between consumption of raw bivalve shellfish and Creutzfeldt-Jakob disease (Bobowick et al., 1973; Davanipour et al., 1985). This is a surprising linkage given that a route of transmission

is not obvious. However these studies were fairly small with evaluation of multiple risk factors therefore this association remains speculative at present.

4.4. Disease surveillance data

Investigations of specific disease outbreaks linked to shellfish consumption have been reported in the scientific literature in many countries however few countries systematically collate and report such data through a disease surveillance system. In the UK epidemiological data is collected for England and Wales by the Public Health Laboratory Service Communicable Disease Surveillance Centre and published periodically (Sockett et al., 1985, 1993; Anon, 1992, 1993c, 1998; PHLS Viral Gastroenteritis Sub-Committee, 1993). Between the years 1941 to 1970 more than 80% of reported shellfish-associated food poisoning outbreaks were of unknown cause. However since the late 1970s with the recognition of viruses as significant agents of infection increasing numbers of outbreaks were recognised as viral in origin. Between the years 1965 and 1983, of 60 reported outbreaks associated with molluscan shellfish, 22 (37%) were caused by 'Small Round Viruses', 10 (17%) by hepatitis A virus and 26 (43%) were of unknown cause (Sockett et al., 1985). No outbreaks were identified as bacterial in origin. During the decade 1981–90 a further 99 outbreaks of gastroenteritis related to molluscan shellfish were reported (PHLS Viral Gastroenteritis Sub-Committee, 1993) and this trend has continued with the number of reported outbreaks in subsequent years ranging between 5 and 15 a year. The latest available data shows that 6 shellfish-associated gastroenteritis outbreaks were reported in 1996 and 12 in 1997 (Anon, 1998). Of these incidents 13 were due to oysters with NLV identified as the causative agent in 5 outbreaks. The number of individual cases associated with each outbreak varies considerably from <10 to hundreds depending on the particular circumstances. Gastroenteritis accounts for the large majority of clinical symptoms reported with NLV the most frequently identified cause. In the UK during recent years NLV has accounted for up to 40% of reported incidents. Other viral causes of gastroenteritis, such as astroviruses, also feature occasionally in disease reports. Shellfish associated outbreaks caused by hepatitis A virus continue to be reported occasionally in England and Wales however over the last decade such outbreaks have become less frequent now constituting $<5\%$ of reported outbreaks. It is interesting to note that several large gastroenteritis and hepatitis outbreaks reported in the 1970s and 1980s were associated with commercially cooked cockles (Appleton and Pereira, 1977; Sockett et al., 1985). Following review and strengthening of UK controls on commercial cooking procedures (see 6.2) cockle associated outbreaks markedly decreased. Outbreaks associated with authorised commercial processors of cooked cockles or mussels have not been reported for a number of years suggesting these improvements were effective. Bacterial causes of infection associated with bivalve shellfish have only occasionally been reported during the last decade (Anon, 1998) probably reflecting the success of depuration processes (see 6.3) at removal of these contaminants. Of the bivalve species oysters now predominate as the shellfish vehicle of most concern in the UK. Non-filter feeding shellfish have not been reported as outbreak vectors for viral gastroenteritis in the UK. Incidents of food poisoning associated with these species are less common than those associated with filter-feeders and often of a bacterial aetiology suggesting post-processing contamination rather than contamination at source (Sockett et al., 1985).

Data on infectious diseases associated with molluscan shellfish consumption has also been collated, from a variety of sources, for the USA (Richards, 1985; Rippey, 1994). Until the 1980s up to about 50 disease outbreaks per decade were documented in the USA. This increased dramatically to 217 outbreaks in the decade 1980 to 1990 with nearly 7000 associated cases (Rippey, 1994). Oysters and clams were responsible for most of these reported cases with soft clams, mussels and scallops only infrequently implicated. Most illness outbreaks were associated with organisms derived from faecal pollution of shellfish harvesting areas. Over the period 1905 to 1990 some 80% of reports were ascribed to gastroenteritis with no causative agent isolated or identified. Excluding typhoid, which last occurred in 1954, of outbreaks with an identified cause 17% were of viral origin with only 4% bacterial in nature. Among identified viral causes hepatitis A virus predominated being responsible for 42 reported outbreaks, with some 1800 cases, between the turn of

the century and 1990. However, shellfish associated hepatitis A virus outbreaks were not recognised in the USA until the early 1960s and over 1000 of these cases occurred during several major outbreaks from 1961 to 1964 (Richards, 1985). A further major outbreak occurred in 1973 and shellfish vectored hepatitis A virus cases continue to be reported in the USA although at reduced levels (Rippey, 1994). Until recently NLV was positively identified in only a relatively small percentage of outbreaks in the USA. However, the symptoms of disease reported in most outbreaks of unknown cause were similar and were described as 'mild' gastroenteritis with clinical features consistent with NLV gastroenteritis as described below. During the last decade with increased awareness and improved diagnostic techniques many more outbreaks in the USA have been ascribed to NLV (Dowell et al., 1995; Mcdonnell et al., 1997; Farley et al., 1998).

Outbreaks caused by NLV are characterised by mean incubation periods of 24–48 h, mean illness durations of 12–60 h, and a high percentage of patients with diarrhoea, nausea, abdominal cramps and vomiting and a lower percentage with fever (Kaplan et al., 1982a). This combination of clinical symptoms is highly characteristic for viral gastroenteritis and unlike those reported for enteric infections with bacteria or parasites or for chemical intoxication. In particular outbreaks of Salmonella are characterised by longer duration of illness and more fever than vomiting (Hedberg and Osterholm, 1993). These characteristics, along with stool cultures negative for bacterial pathogens, have been proposed as epidemiological criteria for considering an outbreak to be viral in origin (Kaplan et al., 1982a). The available epidemiological data for both the UK and the USA show that an aetiological agent is not identified in the majority of molluscan shellfish associated outbreaks. Of the causative agents identified viruses predominate. However, unlike other forms of food poisoning, bacterial causes of gastroenteritis are rarely linked to molluscan shellfish consumption. It is now widely accepted that the clinical symptoms reported in the large majority of outbreaks of unknown cause are consistent with the epidemiological criteria for viral gastroenteritis, such as caused by NLV, and therefore that these agents probably constitute the bulk of the disease problem both in the UK (Sockett et al., 1985; PHLS Viral Gastroenteritis Sub-Committee, 1993), the USA (Richards, 1985; Rippey, 1994) and elsewhere. In many outbreaks the failure to identify a cause probably relates mainly to the absence of appropriate virological investigation. Statutory hygiene controls or shellfish are based on bacterial indicators of faecal pollution, such as *E. coli* or faecal coliforms. The relative paucity of outbreaks confirmed as bacterial in origin is generally attributed to the success of these controls for containing contamination of shellfish by bacterial pathogens. By contrast many reports have shown that viral contamination of shellfish is not readily identified or controlled using bacterial indicators of faecal pollution.

It is interesting to note that UK epidemiological data shows a pronounced seasonal effect for illness associated with shellfish consumption with outbreaks occurring predominantly in the winter months (PHLS Viral Gastroenteritis Sub-Committee, 1993). Data for the USA also shows seasonal variation but here disease peaks occur in both late spring and late fall (Rippey, 1994). In the UK NLV circulation in the community has historically been associated with winter months being known in early literature as 'winter vomiting disease'. However circulation in the community is not always consistent with peaks of shellfish associated gastroenteritis. As an example in the UK during 1995 greatest numbers of NLV infection were reported during spring and summer months with the fourth quarter being unusually low (Anon, 1996). In the UK the large majority of oysters are purified (depurated) prior to sale to the consumer. Recent data from this laboratory suggests that the seasonal pattern of shellfish associated illness in the UK may also be influenced by failure of oysters to clear viral contamination in purification systems during the winter months when shellfish metabolic activity is reduced (Dore et al., 1998).

Disease surveillance authorities in the UK (PHLS Viral Gastroenteritis Sub-Committee, 1993) and elsewhere (Rippey, 1994) recognise that officially reported cases considerably underestimate the actual burden of disease from eating shellfish. There are a number of reasons for this including the absence of mandatory requirements for reporting gastroenteritis and the relatively mild nature of the illness which, although unpleasant, is of short duration and followed by complete recovery with no longer term sequel. Patients frequently do not present to their

doctor and even when they do the illness is not further investigated or reported. Reported incidents therefore relate principally to large functions where multiple illness is more apparent and where classical statistical correlations between illness and a food vehicle can be made. Individual cases are rarely if ever investigated and do not feature in the disease statistics of either the UK or the USA. It is likely however that the majority of oysters and other shellfish are consumed in the individual or small group setting. It is clear that public awareness through media reports (Davis et al., 1994) and aggressive investigation and case finding by authorities (Rippey, 1994) can substantially effect both the number of outbreaks identified and the volume of illness reported. This highlights the probable major underreporting by passive surveillance systems. When immunological assays become available case controlled seroepidemiological studies may help establish more accurately the true incidence of shellfish transmitted viral gastroenteritis. Such data is an important aspect of modern risk assessment techniques and would help inform the debate over expenditure on remediation of sewage pollution in shellfish harvesting areas. USA FDA risk assessments estimate cases of Norwalk viral gastroenteritis related to seafood consumption at some 100 000 per year (Williams and Zorn, 1997). Considering the numbers that are potentially at risk during major episodes (Dowell et al., 1995) this estimate may be realistic. Similar estimates have not been performed in the UK.

5. Detection of viruses in shellfish

The development of methods for investigation of seafood contamination with enteric viruses has concentrated on the bivalve shellfish because of their proven association with disease outbreaks. Until this decade such developments focused almost entirely on detection of the enteroviruses. This reflected the availability of conventional culture techniques for these viruses and their recognition as contaminants of the marine environment (Sellwood and Dadswell, 1991; Sellwood, 1992). Early literature is sometimes confused regarding the epidemiological significance of enterovirus detection in shellfish. It is now clear that although enteroviruses are common viral contaminants of shellfish they are not responsible for disease symptoms, such as diarrhoea and vomiting, following consumption of contaminated shellfish. With the recognition of NLV as the main aetiological agent responsible for shellfish associated viral gastroenteritis more recent developments have focused on developing methods for these and other agents causing gastrointestinal illness and for hepatitis A virus.

5.1. Detection of culturable enteric viruses

Until recently methods for detecting human enteric viruses from shellfish had as their goal the production of extracts suitable for inoculation into cell cultures. Crude shellfish extracts are highly cytotoxic and therefore not suitable for direct inoculation onto cells. Simple dilution produces unfeasibly large volumes of extract therefore methodologies were required for the separation of virus from shellfish tissue and their subsequent reconcentration. During the seventies and eighties numerous workers tackled this difficult and complex task. Such structures have been comprehensively reviewed (Gerba and Goyal, 1978; Williams and Fout, 1992; Jaykus et al., 1994) and a detailed treatise is outside the scope of this review. Key objectives were to develop processing procedures that resulted in a low volume, non-cytotoxic extract with good virus recovery. The plethora of reported methods, and the lack of consensus on an ideal candidate, testifies to the difficulty of the task. Methods are generally separated into two alternative approaches, the extraction-concentration methods and the adsorption-elution-concentration methods (Sobsey, 1987; Jaykus et al., 1994). However, within the literature there are many variations on detail within these general schemes. Both extraction-concentration (Landry et al., 1980; Richards and Goldmintz, 1982; Lewis and Metcalf, 1988) and adsorption-elution-concentration (Sobsey et al., 1975, 1978; Johnson et al., 1981; Sullivan et al., 1984; Jaykus et al., 1994) methods have been reported for a variety of bivalve species including oysters, clams, mussels and cockles. Following extraction and reconcentration final elluents produced by either approach are normally treated with antibiotics (to reduce bacterial and fungal overgrowth) prior to inoculation into cell culture. Cell lines commonly used for culturable enteric viruses include buffalo

green monkey kidney (BGM), Vero, MA-104, HeLa, BSC-1, LLC-MK2 and others. No one cell line supports the growth of all enteric viruses however BGM cells have proved useful for the growth of a variety of enteroviruses from environmental samples, such as water and shellfish, and are used by a number of workers (Morris and Waite, 1980). The lack of standardisation in method development and reporting makes direct comparison of method efficacy difficult. Comparative evaluations of different methods have been performed (Cole et al., 1986; Riordan, 1991; Speirs et al., 1987; Millard et al., 1987) however such studies have often reported conflicting results. It seems clear that no one procedure is clearly superior in all attributes and certainly a universal consensus on methodology has not been reached. Given these difficulties the extraction and culture of enteric viruses from shellfish is likely to remain a task performed only in specialised laboratories.

The extraction methods described above for detection of the culturable enteroviruses have also been adapted for the detection of rotavirus (Speirs et al., 1987; Lewis and Metcalf, 1988; Boher et al., 1991) and hepatitis A virus (Millard et al., 1987; Lewis and Metcalf, 1988; Alouini and Sobsey, 1995) in shellfish. These viruses however require more specialised methods for virus growth and titration following extraction. To date most studies have been performed on shellfish experimentally contaminated in the laboratory with virus strains adapted for growth in culture. Astroviruses and enteric adenoviruses are also difficult to culture although specialised or primary cell culture systems are available. However, being predominantly paediatric infections these viruses are rarely linked to seafood consumption. Consequently no work has been reported on the application of culture methods for their detection in shellfish.

5.2. Detection of non-culturable enteric viruses

NLV and hepatitis A virus are the most significant viral pathogens associated with shellfish consumption. Although experimental work can be performed on laboratory adapted strains of hepatitis A virus, wild type strains can only be cultured with great difficulty. NLV is even more problematical. Culture methods for this virus have never been reported and they are currently regarded as non-culturable. For both of these viruses therefore methods for their detection in shellfish cannot be based on growth in cell culture. Animal tests are also not generally available for detection of these viruses, with the possible exception of hepatitis A virus. Following a large epidemic in China hepatitis A was shown to contaminate clams by experimental exposure of marmosets (Li et al., 1992). Although this proved useful in this major epidemic the use of primates for diagnostic tests is not generally feasible or desirable. For many years electron microscopy was the only technique available for study of the nonculturable gastroenteritis viruses and this remains a key technique for examination of clinical samples. However electron microscopy is relatively insensitive requiring in the order of a million particles for virus visualisation (Hedberg and Osterholm, 1993). Whilst such numbers are obtainable in clinical samples, such as stools, they are probably rarely present in contaminated foods. There are only a few reports of visualisation of enteric viruses in shellfish by electron microscopy (Murphy et al., 1979; Appleton et al., 1981) and certainly this method has not proved practical for routine application.

During recent years non-culture detection methods, such as immunoassays and molecular based techniques, have become available for a number of gastroenteritis viruses and for hepatitis A virus. Laboratory adapted hepatitis A strains grown in culture have provided antigen preparations for the development of diagnostic immunoassays (Coulepis et al., 1985). Immunoassays are also available for the astroviruses (Moe et al., 1991; Herrmann et al., 1990), the enteric adenoviruses (Vizzi et al., 1996), and for NLV (Herrmann et al., 1985, 1995; Monroe et al., 1993). The development of immunoassays for NLV in particular has been greatly aided by the advent of molecular cloning and in-vitro protein expression (see below). Immunoassays have proved valuable for clinical investigations, including foodborne outbreaks (Fleissner et al., 1989), and are helping researchers understand the epidemiological significance of these viruses (Moe et al., 1991; Gray et al., 1993; Smit et al., 1997). However although attempts have been made to apply immunoassays (such as enzyme linked immunosorbant assay) to detection of viruses in water (Grabow et al., 1993), food (Hernandez et al., 1997) and shellfish (Giachino

et al., 1985), reports are very limited and not always successful (Pietri et al., 1988). The limited success of this approach is probably because immunoassay, like electron microscopy, is relatively insensitive requiring a thousand or more virus particles for a positive reaction (Kogawa et al., 1996). Whilst such titres are common in clinical samples, contaminated food will contain much lower numbers since contamination is passive with no virus multiplication. Viral infections generally have a low infectious dose with NLV thought to be as low as 10–100 virions (Caul, 1996b). Clearly therefore low virus titres in food may constitute an infectious hazard and detection methods need to be correspondingly sensitive.

The cloning and sequencing of hepatitis A (Cohen et al., 1987) and E (Bradley, 1995), Norwalk (Jiang et al., 1993) and related NLVs (Lambden et al., 1993; Lew et al., 1994; Dingle et al., 1995), and other gastroenteritis viruses, revolutionised possibilities for the development of sensitive virus detection methods. Initial studies utilising molecular methods employed gene probing and hybridisation techniques and were applied to detection of hepatitis A, rotavirus and enterovirus in shellfish (Zhou et al., 1991; Le Guyader et al., 1993; Van Cuyck-Gandre et al., 1994). However probe hybridisation suffered, like previous methods, from sensitivity problems requiring in the region often thousand or more virus particles for a positive reaction (Zhou et al., 1991; Kogawa et al., 1996). Like many other areas of biological science the major breakthrough came with the development of the powerful and sensitive gene amplification polymerase chain reaction (PCR) procedure. A further major benefit of PCR is the added potential for virus strain characterisation by PCR product probe hybridisation or sequencing. The availability of genomic sequence for NLV quickly lead to the development of sensitive PCR based assays for clinical samples (De Leon et al., 1992; Jiang et al., 1992; Moe et al., 1994; Green et al., 1995a,b).

Similarly clinical diagnostic PCR assays have been reported for hepatitis A (Jansen et al., 1990; Apairemarchais et al., 1995) and E viruses (McCaustland et al., 1991; Turkoglu et al., 1996), astroviruses (Saito et al., 1995; Jonassen et al., 1995), rotavirus (Gouvea et al., 1990; Xu et al., 1990) and the enteric adenoviruses (Tiemessen and Nel, 1996). These powerful new techniques have been utilised for clinical investigations and, particularly for NLV, have provided data of public health significance that could not have been provided by classical epidemiological methods alone (Ando et al., 1995a; Green et al., 1995a,b; Beller et al., 1997). However, such studies have also demonstrated the diversity of NLV strains (Moe et al., 1994; Norcott et al., 1994; Jiang et al., 1995) which has made construction of universal PCR primer sets difficult. Recent studies have divided strains into two broad genogroups (Ando et al., 1994; Wang et al., 1994) and proposed regions with sufficient commonality to allow broadly reactive primer design (Green et al., 1995a,b; Ando et al., 1995b; Le Guyader et al., 1996a). The two genogroups reflect antigenic clusters of strains with related, but distinguishable, properties (Noel et al., 1997). Currently combinations of PCR primers seem able to detect most NLV strains in clinical samples but expressed antigen based immunoassays lag behind (Noel et al., 1997). By contrast hepatitis A virus has a relatively conserved genome with a single serotype. PCR primer design has therefore been more straight forward and a number of sets have been proposed (Birkenmeyer and Mushahwar, 1994).

Progress with clinical PCR assays for NLV and other enteric viruses prompted the exploration of this technology to detection of viruses in food. Several initial studies utilised culturable viruses, such as poliovirus, simian rotavirus or laboratory adapted strains of hepatitis A virus, in laboratory seeding studies to develop methods and assess the feasibility of PCR based assays for detection of enteric virus in shellfish (Atmar et al., 1993; Goswami et al., 1993; Jaykus et al., 1993, 1995, 1996; Yang and Xu, 1993; Le Guyader et al., 1994; Lees et al., 1994, 1995a; Cromeans et al., 1997). These studies generally showed that the approach is feasible but also that crude shellfish extracts are inhibitory to the PCR. Method development has concentrated on refining virus extraction and or nucleic acid extraction and purification techniques to overcome this inhibition problem. A common approach has been to base methods for virus extraction on those previously developed for detection of culturable enteroviruses in shellfish (Lees et al., 1994; Jaykus et al., 1995). A later modification targeted extraction of the shellfish digestive organ (Atmar et al., 1995), since these are known to harbour most of the contaminating virus (Romalde et al., 1994), thus minimising contamina-

tion by other shellfish tissues. Following virus extraction a variety of subsequent nucleic acid extraction and purification protocols have been employed. Since PCR reaction volumes are small (typically <100 ul) these protocols generally need to incorporate concentration steps. Commonly used approaches include the purification of nucleic acid by virus lysis with guanidine and nucleic acid recovery with a silica matrix (Lees et al., 1994; Hafliger et al., 1997), and purification with organic solvents followed by selective precipitation of nucleic acid using the cationic detergent cetyltrimethyl ammonium bromide (Atmar et al., 1993; Jaykus et al., 1995). With the advent of useable PCR primers for NLV similar shellfish seeding studies were also performed for these viruses using stool preparations as source of virus (Gouvea et al., 1994; Atmar et al., 1995; Lees et al., 1995b; Jaykus et al., 1996; Hafliger et al., 1997). These studies showed that virus extraction and nucleic acid purification methods developed for other enteric viruses were generally equally applicable to NLV. A multi-centre collaborative study also demonstrated that NLV detection techniques could be reliably applied in a number of laboratories (Atmar et al., 1996).

Seeding studies have demonstrated the successful application of PCR to detection of virus in shellfish in the laboratory. However, fewer studies have reported the application of these methods to detection of virus in naturally contaminated samples. Contamination of shellfish obtained from polluted harvesting areas with hepatitis A, enterovirus and rotavirus has been documented (Desenclos et al., 1991; Yang and Xu, 1993; Le Guyader et al., 1994; Lees et al., 1994, 1995a; Chung et al., 1996) and in one small survey rotavirus was detected in three oyster samples from a market (Hafliger et al., 1997). Detection of NLV in naturally contaminated shellfish from polluted harvesting areas, or in shellfish associated with disease outbreaks, has currently been documented in only a few reports (Lees et al., 1995b; Le Guyader et al., 1996b; Sugieda et al., 1996; Green et al., 1998; Henshilwood et al., 1998). Experience in our laboratory has been that although presence of NLV can be fairly readily demonstrated in polluted harvesting areas reliable detection in processed products sold to the consumer, or associated with outbreaks, is more difficult. In the UK depuration is routinely performed on shellfish, such as oysters, sold live. Although this processing may not completely remove viruses it does seem to reduce virus levels (see 6.3). Residual low virus levels whilst still a health hazard may be difficult to detect by conventional single round PCR (Lees et al., 1995b). We and co-workers have recently described a nested PCR for NLV that helps overcome these sensitivity problems and has allowed more reliable detection of NLV in shellfish associated with outbreaks and sold for consumption (Green et al., 1998; Dore et al., 1998, 2000) and has allowed reliable monitoring of NLV contamination in polluted harvesting areas (Henshilwood et al., 1998). This development has also facilitated post PCR characterisation by sequencing which provides major benefit, for epidemiological investigation (see 4.2). Other workers have reported failure of PCR to detect NLV in oysters associated with outbreaks of NLV gastroenteritis in the USA (Kohn et al., 1995; Mcdonnell et al., 1997). However, further work did detect NLV in oysters using PCR primers specific to the virus strain associated with one of these outbreaks (Le Guyader et al., 1996b). This report confirmed NLV presence by nucleotide sequencing of cloned PCR amplicons. However the sequence was not found to be identical to that obtained from the stools of patients from the outbreak. A recent report from Japan has documented the coexistence of two NLV genogroup strains in both faecal samples and shellfish associated with an outbreak (Sugieda et al., 1996). Recent experience in our laboratory has confirmed this finding in outbreaks in Europe and indeed suggests that mixed cocktails of NLV strains are common in shellfish from polluted harvesting areas (Henshilwood et al., 1998). These findings suggest that definitive linking of shellfish and clinical samples through sequence comparison may prove problematical in some cases. Following similar developments PCR based methods have also been described for the detection of astroviruses and adenoviruses in environmental samples such as polluted water and shellfish (Marx et al., 1997; Girones et al., 1995; Pina et al., 1998b). A complicating factor for NLVs which has emerged recently is the existence of closely related bovine and porcine strains cross-reactive with PCR primers used for human NLV detection (Sugieda et al., 1998; Dastjerdi et al., 1999; Liu et al., 1999). If these veterinary pathogens are wide spread it could complicate the interpretation of NLV positive results obtained from environmental samples subject to agricultural run-off. It remains to

be seen whether this is a significant problem and if so whether primer design can be refined accordingly.

An uncertainty with the use of PCR is whether test results necessarily indicate the presence of viable infectious virus. PCR amplifies nucleic acid which could originate from either viable virus or damaged non-infectious virus. It is not clear whether this is likely to be a significant problem effecting detection of viruses in shellfish. The enteric viruses of concern all have RNA genomes. Although DNA is remarkably stable RNA is not, being inherently susceptible to digestion by widely prevalent cellular enzymes (RNAses). It is debatable whether free virus genome, or virus genome unprotected by a complete protein coat, would remain intact for long in the RNAse rich environment of sewage or in the hostile environment of the shellfish digestive tract. Very little data is available however a laboratory seeding study on cooked shellfish showed that in some samples feline calicivirus, a possible model for NLV, was detectable by PCR but not by culture (Slomka and Appleton, 1998). Clearly therefore this may be a significant problem in applying PCR to the quality assessment of cooked shellfish. It is not clear whether similar problems would arise when testing live shellfish. To address these possible concerns some workers have proposed an initial round of culture in cells followed by detection of propagated virus with molecular methods. This approach has been reported for detection of viable astrovirus (Abad et al., 1997b) and enterovirus (Murrin and Slade, 1997) in water however it is not applicable to the unculturable NLV. An alternative approach has combined the selective properties of antibodies with the sensitivity of PCR in an antibody capture PCR. This has been applied to detection of hepatitis A virus in seeded shellfish samples and shown to be both sensitive and to remove PCR inhibitors (Deng et al., 1994; Lopez-sabater et al., 1997). An advantage of this approach is that whole virions are recovered which may help ensure that the PCR is detecting viable virus. This approach may prove useful for other enteric viruses however immunological reagents for the most important group, the NLVs, are not yet sufficiently advanced or available for general use. In the absence of definitive human volunteer studies more extensive correlation of NLV detection in shellfish sold for consumption with illness in consumers should help establish whether PCR positive results always indicate the presence of viruses capable of causing illness.

Since removal of PCR amplification inhibitors is a major factor effecting successful application of PCR to shellfish most authors agree the need for good documentation of method capability in this respect. Inadequate removal of inhibitors can lead to false negative reactions. The use of internal virus RNA standards has been proposed (Atmar et al., 1995; Nairn et al., 1995) and these should assist result interpretation. Internal standards may also prove useful for tackling another problematical aspect of PCR, the lack of quantitation, through the development of a competitive PCR approach. However such standards are not yet readily available for all enteric viruses, and strains, of interest. Good quality control procedures for amplification inhibition, and other aspects, remain an important area to be addressed.

Despite these uncertainties PCR has proved to be a major step forward in the development of methods for detection of enteric viruses in shellfish. However, most data has currently been generated from laboratory experiments with only a few applied studies yet reported. Further developments can be anticipated in this area over the next few years. PCR methods are clearly beneficial for the epidemiological investigation of outbreaks, for investigation of commercial shellfish processing procedures (such as depuration) for virus removal (see 6.3), and for investigation of the mechanisms of virus uptake and elimination in shellfish (Romalde et al., 1994; Schwab et al., 1998). They may also prove useful for investigation and surveillance of virus contamination of shellfish harvesting areas and for surveys of virus contamination in shellfish. Such applications are beginning to be reported (Dore et al., 1998; Henshilwood et al., 1998). However, further work and improvement is required on method simplification and standardisation, internal and quality controls, cost, quantitation and method availability before PCR can be considered for more routine applications.

6. Controls on the production and processing of bivalve shellfish

The infectious disease hazards associated with consumption of bivalve shellfish have been recognised for many years. Consequentially most coun-

tries have enacted sanitary controls on the production of bivalve shellfish. In the European Union these were drawn together into a European Directive 91/492/EEC (Anon, 1991a) to enable operation of the single European market in 1993. In the United States by interstate trading agreements set out in the FDA National Shellfish Sanitation Program Manual of Operations (Anon, 1993b). These regulations cover similar ground on the requirements for clean growing areas, the controls and processing requirements for more contaminated areas, the hygiene conditions for processing and dispatch establishments, requirements for marketing documentation etc. Third country imports into the EU and USA have to be produced to the same standard as domestic products. Major exporting nations have therefore developed programs for compliance with the regulations of their target export markets A major feature of these controls is the use of traditional bacterial indicators of faecal contamination, such as the faecal coliforms or *E. coli*, to assess contamination and hence implement the appropriate control measures. Faecal indicators are either measured in the shellfish themselves (EU approach) or in the shellfish growing waters (US FDA approach). Historically it has been widely accepted internationally that harvested shellfish which meet a microbiological standard of less than 230 *E. coli*, or 300 faecal coliforms, in 100 g of shellfish flesh can be placed on the market for human consumption. This standard, together with standards for specific pathogens (such as salmonella), chemical; and algal biotoxins, has been adopted as an 'end-product' standard in EU Directive 91/492.

It is generally accepted that the most effective and reliable approach to controlling the contamination of shellfish is to harvest from areas with good water quality. Control of contamination through mollusc processing procedures tends to be less effective although providing a practical option for many countries where waters may be subject to sewage contamination. There are, conventionally, two different forms of commercial processing available for reducing microbial contamination of shellfish. Contamination may be reduced by heat treatment (cooking) or by extending the natural filter-feeding processes in clean seawater to purge out microbial contaminants. This can be performed in tanks (depuration) or in the natural environment (relaying). Additionally, shellfish harvesting may be prohibited in areas with unacceptable levels of pollution. The EU and USA sanitary legislation controls these various options primarily through the use of faecal indicator monitoring which determines the appropriate treatment in accordance with the level of contamination.

6.1. Classification of shellfish harvesting areas

Both EU and USA legislation employ a grading or classification approach with standards set for categories varying from waters with very low contamination levels to those where harvesting is prohibited. A synopsis of the EU and USA legislative standards for bivalve shellfish is set out in Table 1. In the EU faecal indicators are measured in shellfish flesh whereas in the USA indicators are measured in

Table 1
Synopsis of EU and USA legislative standards for live shellfish[a]

Shellfish treatment required	US FDA classification	Microbiological standard per 100 ml water	EU classification	Microbiological standard per 100 g shellfish
Non required	Approved	GM < 14 FCs and 90% < 43 FCs	Category A	All samples < 230 E. coli or All samples < 300 FCs
Depuration or relaying	Restricted	GM < 88 FCs and 90% < 260 FCs	Category B	90% < 4600 E. coli or 90% < 6000 FCs
Protected relaying (> 2 months)			Category C	All samples < 60 000 FCs
Harvesting prohibited	–	Above levels exceeded	–	Above levels exceeded

[a] FCs = faecal coliforms, GM = geometric mean, 90% = 90%-ile compliance.

the shellfish growing waters. Both the EU and the USA systems base standards on a 5-tube 3-dilution most probable number (MPN) test. Point source pollution inputs are generally identified through a shore line survey, or similar identification, and quality monitoring programs designed accordingly. USA FDA 'approved' and EU 'category A' standards describe the cleanest growing areas from which shellfish can be taken for direct human consumption without further processing. All shellfish from EU category A areas must contain less than 30 *E. coli*, or 300 faecal coliforms in 100 g of shellfish flesh. The USA FDA program gives a choice of using either total or faecal coliforms to establish a classification. It further expresses standards in two components, a geometric mean (GM) count of results and an upper standard which not more than 10% of results can exceed. Approved areas must comply with a total coliform GM of 70 per 100 ml water with not more than 10% of samples exceeding 230 per 100 ml. Alternatively they can comply with a faecal coliform GM of 14 per 100 ml water with not more than 10% of samples exceeding 43 per 100 ml (see Table 1).

In both EU and USA legislation shellfish cannot be harvested for direct human consumption from growing areas exceeding the above levels of contamination. They may however be placed on the market following commercial depuration, relaying or heat processing. However because these processes are known not to be completely effective an upper threshold is placed on the degree of contamination beyond which such procedures may not be used. Shellfish from EU 'category B' and USA FDA 'restricted' classifications may be placed in the market following depuration or relaying (see 6.3). Shellfish from EU category B may also be heat treated by an approved method (see 6.2). EU category B areas must contain less than 4600 *E. coli*, or 6000 faecal coliforms per 100 g of shellfish flesh in 90% of samples. USA FDA restricted areas must comply with a total coliform GM of 700 per 100 ml water with not more than 10% of samples exceeding 2300 per 100 ml. Alternatively they can comply with a faecal coliform GM of 88 per 100 ml water with not more than 10% of samples exceeding 260 per 100 ml (see Table 1).

In EU legislation shellfish from harvesting areas exceeding the limits of category B may only be placed on the market following protracted relaying (a minimum period of 2 months is specified) or commercial heat treatment by an approved method. Shellfish from category C areas must contain less than 60 000 faecal coliforms per 100 g of shellfish flesh (see Table 1). Relayed shellfish may, if necessary, be depurated before being placed on the market. USA FDA controls do not incorporate an equivalent to EU category C. Shellfish growing areas exceeding these prescribed levels of contamination, or areas for which harvesting area survey and classification has not been conducted, cannot be harvested for human consumption in either USA or EU legislation.

If areas meet the above standards only for certain periods because of predictable pollution events authorities may classify them for a restricted period. In the USA FDA program such areas are defined as 'conditionally approved' or 'conditionally restricted' and may be harvested during periods when they meet the standards subject to a management plan. In the UK such areas are described as 'seasonally classified' with the period when the classification applies defined. In addition to criteria for harvesting area classification both EU and USA legislation set out requirements for other aspects such as bivalve transport, wet storage, depuration, relaying, analytical methods, movement documentation and provisions for suspension of harvesting from classified areas following a pollution or public health emergency.

6.2. Heat treatment (cooking)

For shellfish sold as a processed product commercial heat treatment (cooking) is used to reduce levels of microbiological contamination. Various heat treatment processes have been described for shellfish varying from pasteurisation through to sterilisation by canning. However it is important to ensure that cooking processes are properly regulated and controlled. In the UK during the 1970s and 1980s a number of outbreaks of gastroenteritis and hepatitis were linked to commercially cooked cockles (Appleton and Pereira, 1977; Sockett et al., 1985). Investigation suggested that the batch cooking procedures in use at the time were probably undercooking shellfish particularly when environmental temperatures were low and shellfish were insufficiently warmed prior to cooking. Research following these outbreaks showed that hepatitis A virus could be inactivated by more than 4 $\log_{10}$ infectious units by

raising the internal temperature of shellfish (cockle) meats to 85–90°C for 1 min (Millard et al., 1987). Subsequent recommendation by the UK Ministry of Agriculture for commercial cooking operations was that internal shellfish meat temperatures should be raised to 90°C and held at that temperature for 1.5 min. However such heat cook parameters may be difficult to reliably achieve for shellfish cooked in large batches without rendering some shellfish unpalatable. Consequently continuous flow machinery was designed for high throughput operations capable of reliably delivering the above heat cook parameters to all shellfish. Since this review and strengthening of UK controls on commercial cooking procedures, cockle associated illness outbreaks markedly decreased. In the UK viral outbreaks associated with authorised commercial processors of cooked cockles or mussels have now not been reported for a number of years suggesting these improvements were effective. EU Directive 91/493/EEC (Anon, 1991c) requires that commercial heat treatment methods undertaken directly on EU category B or C shellfish must be approved. Several commercial heat treatment processes used in the EU have been officially approved including the UK heat cook parameters of raising the internal temperature of shellfish meats to 90°C for 1.5 min (Anon, 1993a). These heat cooked parameters were based on data obtained for hepatitis A virus in cockles. However, the most frequently reported illness associated with consumption of bivalve molluscs is gastroenteritis caused by NLV. Since NLV cannot be cultivated heat inactivation data for this virus is not available. However similar studies carried out on feline calicivirus, a possible model for NLV, showed this virus to be more readily inactivated than hepatitis A virus (Slomka and Appleton, 1998). It is probable therefore that currently approved commercial heat treatment processes used in the UK are effective for NLV. This view is certainly supported by the available epidemiological evidence.

Although modern carefully regulated commercial heat treatment processes appear to have proved effective in controlling enteric virus continuation in shellfish sold processed, such as cockles, the same cannot be said for shellfish sold live and cooked in the home or restaurant. A major factor limiting the effectiveness of such home or restaurant cooking appears to be the limited nature of the processing applied. Overcooking results in an unpalatable product with low consumer acceptance. In the UK mussels are not eaten raw, but are frequently lightly cooked in dishes such as moules marinière. Mussels sold live have to conform to hygiene standards set for species, such as oysters, consumed raw. Despite this, home or restaurant cooked mussels continue to feature occasionally in the UK disease statistics as a cause of outbreaks of gastroenteritis (Anon, 1993c; Anon, 1998). However, the low incidence of such reporting compared to species commonly eaten raw, such as oysters, suggests home or restaurant cooking may be having at least some protective effect. The success of commercial heat processing for controlling health risks in cockles and mussels shows that commercially achievable heat cook parameters will reliably inactivate enteric viruses. It seems likely therefore that viral problems associated with home and restaurant cooked mussels are a consequence of under or inconsistent cooking. Only a few scientific studies relevant to this are available. An epidemiological investigation into an epidemic of hepatitis A in China associated with clam consumption showed that within the population investigated attack rates were highest in those who eat raw clams (18%), but also higher among those who ate cooked clams (7%) than among those who did not eat clams (1%) (Wang et al., 1990). Similarly a recent study in the USA following a well researched multi-state outbreak of NLV gastroenteritis associated with oysters showed that home or restaurant cooking offered little or no protection. In this study the authors suggest that the degree of cooking required to reliably inactivate NLV would probably render oysters unpalatable to consumers (Mcdonnell et al., 1997). These epidemiological findings are supported by a recent laboratory study showing that rotaviruses and hepatitis A virus could still be recovered in steamed mussels 5 min after the opening of the mussel valves (Abad et al., 1997a). It seems likely therefore that home and restaurant cooking as currently performed is, at best, only a partially effective control measure. Cooking may possibly offer more protection for smaller species, such as mussels, than for larger species such as oysters and clams.

6.3. Purification (depuration) and relaying

Although commercial heat processing seems effective it is not however applicable for shellfish sold live which constitute the bulk of the infectious

disease hazard. Purification (depuration) procedures were first developed in the UK during the 1920s (Dodgson, 1928) as a means of extending the natural bivalve filter-feeding processes in clean seawater to purge out microbial contaminants. Tank-based depuration is now widely practised in many countries including Australia, the UK, France, Italy, Spain and elsewhere, it is however less widely used in the USA (Otwell et al., 1991). Depuration periods may vary from 1 to 7 days, however around 2 days is probably the most widely used period. Depuration systems also vary and include processes where water is static or changed in batches through to systems where seawater is flushed through continually or recycled through a steriliser. Water sterilisation processes include ozone, chlorination, UV irradiation and iodophores (Otwell et al., 1991; Roderick and Schneider, 1994; Poggi and Le Gall, 1995). Depuration has been applied to most bivalve shellfish species sold live including oysters, clams, mussels and cockles.

Commercial depuration, when used as a treatment process to reduce microbial contaminants, is subject to legal control in both the EU, the USA and in other countries. The National Shellfish Sanitation Program Manual of Operations (Anon, 1993b) sets out FDA requirements in the USA. In the EU Directive 91/492/EEC (Anon, 1991a) details requirements for approval of shellfish purification centres covering such aspects as tank construction and operation, laboratory testing, packaging, labelling and transportation. In addition purified shellfish are required to comply with the end-product standard for shellfish sold live which includes the faecal coliform parameter of less than 230 *E. coli*, or 300 faecal coliforms, in 100 g of shellfish flesh. The regulatory principles relating to purification plant construction and operation set out in the EU Directive are implemented by the 'competent authority' in each member state. In practice compliance with the end-product faecal coliform standard is frequently seen as evidence of satisfactory design and operation of depuration plants. However evidence from various sources suggests that depuration plants functioning satisfactorily by faecal coliform criteria may still fail to fully remove human enteric viruses. Perhaps most significant is the epidemiological evidence demonstrating that infection can occur following consumption of depurated shellfish. This was documented in Australia during volunteer trials to assess the safety of depurated shellfish (Grohmann et al., 1981) and has also been documented in outbreaks in the UK (Gill et al., 1983; Heller et al., 1986; Chalmers and Mcmillan, 1995; Perrett and Kudesia, 1995; Ang, 1998) and the USA (Richards, 1985). The epidemiological evidence therefore strongly suggest that depuration may fail to eliminate enteric viruses from contaminated shellfish and further that compliance with *E. coli* (or faecal coliform) end-product standards does not provide a guarantee of virus absence. The success of depuration in removing bacterial contaminants may account for the very low incidence of such infections following shellfish consumption (see 4.4) however it is clear that this process, as currently undertaken, is considerably less effective for virus elimination. This observation is supported by numerous laboratory studies which have examined elimination rates of human enteric viruses (such as poliovirus), or possible models for the behaviour of human enteric viruses (such as various bacteriophage species), from shellfish during the depuration process (Richards, 1988; Power and Collins, 1989, 1990a,b; Sobsey and Jaykus, 1991; Jaykus et al., 1994; Dore and Lees, 1995). Although elimination rates in individual studies vary significantly the overwhelming finding from these studies is that viruses are eliminated from bivalve shellfish at a slower rate than faecal coliforms. These findings are confirmed by recent laboratory seeding studies using NLV detected by PCR. NLV was found to efficiently accumulate in shellfish (oysters and clams) however it was only poorly removed by depuration compared to *E. coli* (Schwab et al., 1998). This laboratory has recently evaluated virus elimination in commercially depurated shellfish (oysters) as judged by both NLV and male specific RNA (F+) bacteriophage (a potential viral indicator) content (Dore et al., 1998, 2000). Processed shellfish were found to be routinely compliant with faecal coliform end-product standards however significant numbers were contaminated with both NLV and F+ bacteriophage. Viral contamination was found to be highly correlated with the degree of harvesting area pollution and to the known incidence of disease outbreaks linked to the site. This data supports previous laboratory findings and confirms that compliance with faecal coliform end-product standards does not provide a guarantee of the absence of enteric viruses in depu-

rated shellfish. A further important finding was that virus contamination, as judged by both NLV and F + bacteriophage content, in commercially depurated oysters was much more prevalent during colder winter months. The dramatic effect of season on viral, but not bacterial content suggests that physiological requirements for elimination of viruses during shellfish depuration may be significantly different to those required for effective elimination of faecal coliforms. Laboratory studies have suggested that process temperature (Power and Collins, 1990a,b; Jaykus et al., 1994; Dore et al., 1998) may be an important factor in virus removal during depuration. This is supported by these seasonality findings since seawater used in commercial shellfish depuration plants is generally not heated except in extreme conditions. The effect of temperature on virus elimination during depuration requires further study as do other physiologically important parameters such as food availability, salinity, dissolved oxygen, and shellfish condition. Recent studies in this laboratory suggest that a seawater temperature of 18–20°C is optimal for removal of F + bacteriophage from oysters (*C. gigas*) but also that successful elimination within a 2–3 day period is critically dependent on initial contaminant on level (Dore et al., 1998). Heavily contaminated shellfish failed to clear bacteriophage within 7 days even at elevated temperatures. It is probably the case that many other aspects of depuration plant design and operation, optimised to ensure faecal coliform removal, require reevaluation in the light of methods for detection of viruses.

Relaying involves the transfer of harvested animals to cleaner estuaries or inlets for self-purification in the natural environment. This process can be used as an alternative to depuration for lightly polluted shellfish. Shellfish can only be held for relatively short periods in depuration tanks but can obviously be maintained for much longer periods in the natural environment. This makes relaying also suitable for treating more heavily polluted shellfish where longer periods (a minimum of two months is specified in EU Directive 9 1/492 for category C shellfish) are required to remove heavy contaminant loads. The main disadvantages of relaying are the limited availability of suitable unutilised clean coastal areas and of obtaining ownership rights to those areas the difficulty of controlling water quality and other water parameters and the susceptibility of stock to poaching. Combinations of these processes may also be used, for instance in France traditional practices include holding molluscs in ‘claires’ (man-made tidally submersible ponds) for several months and then in ‘degorgeoirs’ (wet storage ponds) for two days. Relatively little information exists on the removal of viruses during shellfish relaying although again factors such as seawater temperature and initial contamination levels appear critical (Cook and Ellender, 1986; Jaykus et al., 1994). Data from this laboratory on NLV and F + bacteriophage (Dore et al., 1998) and unpublished data suggests that removal of viruses from heavily contaminated shellfish by a combination of relaying for 4–6 weeks followed by depuration can be effective but again is critically dependant on seawater temperature. In these studies differences were also seen between virus clearance in native (*O. edulis*) and cultured (*C. gigas*) oysters suggesting that species specific factors should also be considered. Other workers have reported similar data using bacteriophage studies (Humphrey et al., 1995).

6.4. The prospects for viral standards

Viral disease outbreaks associated with oysters and other bivalve shellfish continue to be reported in many countries (see Section 4). An aspect highlighted in many outbreak reports is the compliance of both harvesting areas, treatment processes, an products sold to the consumer, with the various regulatory requirements summarised above. It seems therefore to be increasing recognised that current regulations are apparently unable to guarantee protection of the shellfish consumer or to prevent further outbreaks. In many countries depuration is widely practised for oysters and other shellfish consumed raw. However a number of reports have shown that gastroenteritis outbreaks can occur following consumption of oysters purified according to all legal requirements (see 6.3) and therefore that this process as currently practised also cannot guarantee consumer protection. The consequential requirement for measures to better protect the consumer from virus contamination are widely noted in outbreak reports. During a recent outbreak in Scandinavia oysters complying with the relevant European faecal coliform standards (Anon, 1991a) were shown to cause NLV gastroenteritis in at least 356 patients (Christensen et al., 1998). This and previous similar

outbreaks have prompted the formal establishment within the EU of a network of reference laboratories tasked with the introduction of better systems for the monitoring and control of viral contamination of bivalve molluscs (Anon, 1999a). This laboratory has been designated as the co-ordinating European Community reference laboratory. Recent outbreaks in other countries (Anon, 1999b) have also prompted reevaluation of approaches for the control of viral contamination of shellfish. In the USA a proposed extensive reevaluation of approaches to shellfish sanitation, the National Indicator Study, has published a comprehensive literature review (Hackney and Pierson, 1994) prior to further initiatives. USA workers have also proposed a novel quantitative risk assessment approach (Rose and Sobsey, 1993).

Clearly PCR based detection methods for viral pathogens (see 5.2) could form an element of improved regulatory controls for viral contamination of shellfish. This laboratory has recently demonstrated that NLV monitoring by RT-PCR in a polluted harvesting area is possible and that virus contamination can both be readily detected and was consistent with subsequent outbreaks in products harvested from the study area (Henshilwood et al., 1998). French workers have also recently applied RT-PCR to detection of enteric viral (NLV, hepatitis A, rotavirus and enterovirus) contamination in shellfish harvesting areas and have similarly shown that these techniques can readily detect virus contamination (Le Guyader et al., 1998). Both studies have demonstrated that enteric virus contamination can be detected in areas judged suitable for commercialisation by faecal oliform criteria. However currently published methods are complex, not yet standardised, and not widely available. It is probable therefore that further work on these aspects is required before their introduction on a widespread basis for regulatory control could be considered practical. These methods do however have important current applications for research and for epidemiological investigations.

The current regulatory controls in most countries rely heavily on the concept of faecal pollution indicator organisms (as described above) to assess microbiological hazard. These methods are cheap, standardised and widely available. However the organisms currently used, the faecal coliforms, have been shown to inadequately reflect the presence of viral contaminants. This is probably because viruses are more hardy than bacteria and therefore survive better in the marine environment (see 6.5) and are more resilient to inactivation or removal during depuration or relaying (see 6.3). In this context it is likely that other possible bacterial indicators, such as the faecal streptococci, would share similar disadvantages to the faecal coliforms. For these reasons a number of workers have proposed alternative indicators for better assessment of viral contamination in the marine environment. Some of the most promising candidates are various species of bacteriophage because of their physical and genomic similarity to human enteric viruses, their abundance in sewage effluents and their ease of assay (IAWPRC Study group on health related water microbiology, IAWPRC, 1991). Male specific RNA (F +) bacteriophage have been proposed as candidate viral indicators for water pollution (Havelaar, 1987) and specialised hosts have been developed for their specific assay (Debartolomeis and Cabelli, 1991; Havelaar et al., 1993). F + bacteriophage share many physical and genomic properties with the enteric viruses of concern however, like the faecal coliforms, their distribution is not restricted to human effluents. To address this issue other workers have proposed the use of bacteriophage of the obligate anaerobe *Bacteroides fragilis* as a potential indicator of human specific pollution (Tartera and Jofre, 1987). It may also be possible to speciate F + bacteriophage in order to ascribe contamination to animal or human sources (Hsu et al., 1995; Beekwilder et al., 1995). A number of studies have been performed using these bacteriophage and in general they have been shown to hold promise as indicators of enteric virus presence in sewage effluents and polluted receiving waters (Lucena et al., 1994; Lee et al., 1997). General somatic bacteriophage of coliforms have also been suggested (Humphrey et al., 1995), however since this constitutes a diverse grouping standardisation and reproducibility are problematical (Havelaar, 1987; Vaughn and Metcalf, 1975). An alternative to bacteriophage may be the detection of other human viruses which are either more widely prevalent in polluted waters, or easier to detect, than the viral pathogens of concern. Human enteroviruses have a long history in this context (see 5.1) however extraction and culture of enterovirus from shellfish is a specialised and expensive task and most workers have now abandoned this approach.

Recently human adenoviruses, detected by a molecular approach, were suggested as a possible index of human specific pollution (Pina et al., 1998b). Adenoviruses are widely prevalent in sewage effluents and easily detectable by PCR. This is an interesting proposal however further evaluation is required both of adenovirus prevalence compared with the enteric pathogens of concern and of the current practicality of molecular assays in a regulatory context.

The majority of studies on alternative indicators have been performed in the context of water pollution. However during recent years studies relating to bivalve shellfish have also been performed. This laboratory has investigated the potential of F+ bacteriophage in commercially depurated market ready oysters and demonstrated that F+ bacteriophage is a much better indicator of virus contamination (as judged by degree of harvesting area pollution, association of the harvesting area with gastroenteritis outbreaks, and presence of NLVs in marketed shellfish) than is *E.coli* (Dore et al., 1998, 2000). F+ bacteriophage may have particular advantages as a 'viral' indicator for depurated shellfish as several studies have shown its elimination kinetics during this process appear to reflect those of enteric viruses (Power and Collins, 1989; Dore and Lees, 1995). Recently other workers have evaluated the performance of F+ bacteriophage and phages of *B. fragilis* as indicators of virus pollution in shellfish harvesting areas and similarly concluded that F+ bacteriophage is a promising indicator of human enteric virus pollution in oysters (Chung et al., 1998). By comparison conventional bacterial indicators did not adequately reflect virus presence and phages of *B. fragilis* were present in insufficient numbers to be useful. Other workers have suggested phages of *B. fragilis* may prove useful for indication of remote pollution effecting shellfish (Lucena et al., 1994). Perhaps most persuasively, analysis in this laboratory of shellfish associated with gastroenteritis has shown that of 14 outbreak samples 10 were negative for *E. coli* with only 2 above the required bacteriological standard (<230 *E. coli* per 100 g), whereas all samples were positive for F+ bacteriophage with average levels of 2500 pfu per 100 g (range 60–17 500) (unpublished data).

Given these various advances it is now becoming pertinent to consider how best to utilise potential alternative indicators, and advances in virus detection methods, for the further development of public health controls for bivalve shellfish. Given the far reaching implications of adoption into legislation it is important that potential viral controls for shellfish are well researched and shown to perform adequately in practice. Adoption of appropriate measures will also be facilitated by a scientific consensus on the most appropriate methods and approaches. Issues to be considered are the respective roles of alternative indicators versus pathogen detection, on the merits of the various candidate indicator organisms, on the selection of methodological procedures, and on numerical standards that might be applied. Currently however little has been published on this topic. This laboratory has recently suggested a possible management strategy for bringing the various elements together (Dore et al., 1998). However much further work remains to be done before the most appropriate approach can be identified and the implications fully understood.

6.5. Control of harvesting area pollution

It may seem obvious to say that the most effective way to tackle shellfish vectored virus disease is to prevent sewage pollution of shellfish harvesting areas, prevention generally proving better than cure. However growing coastal populations and the high investment cost of sewage treatment processes have proven difficult obstacles to overcome. In some countries, such as in the UK, the shellfish industry is widely dispersed whereas in others, such is The Netherlands, it is concentrated into a few geographical areas. Clearly focusing on the difficult and expensive task of achieving and maintaining high standards of water quality is easier where the shellfish industry is recognised as a major factor in the local economy. In other situations expenditure on adequate sewage treatment can seem disproportionate to the value of the shellfish industry and this has hampered investment. However it is not clear that agencies have ever attempted to factor in the wider public health and other costs of shellfish transmitted infections. Where assessments have been performed they suggest that such costs may not be trivial (Dalton et al., 1996). However in recent years the economic arguments have become balanced, in many countries, by growing environmental concerns. In the EU environmental quality legislation has become a

major factor effecting expenditure on sewage infrastructure. Water quality standards for bathing beaches (Anon, 1976) and for minimum levels of sewage treatment prior to marine discharge (Anon, 1991b) have dictated high levels of expenditure in many European countries. Of direct relevance to shellfisheries Directive 79/923/EEC (Anon, 1979) on the quality required of shellfish waters stipulates a guideline faecal coliform standard approximately equivalent to category A (quality suitable for direct consumption) under the sanitary controls Directive 91/492. In many EU countries this has become an important driver for maintenance or improvement of water quality in molluscan shellfisheries. Whilst these various initiatives can bring demonstrable, and welcome, improvements it should be recognised that they also rely heavily on conventional bacterial pollution indicators for measuring performance against set water quality standards. Like sanitary controls for shellfish such water quality standards cannot necessarily be relied upon to deliver the necessary improvements to the virological quality of water. Periodic monitoring programs provide better protection against continuous discharges than against intermittent spills associated with storm water discharges in combined sewer and rainfall systems. However the latter may be heavily contaminated with untreated effluent, particularly during the 'first flush'. Rainfall associated outbreaks of shellfish vectored disease have indeed been reported on many occasions (see 4.2) demonstrating the importance of this factor. Indeed although the antiviral efficiency of sewage treatment can be questioned it is interesting to note that fully treated effluents have not yet been shown to have contributed to foodborne or waterborne viral disease (Cliver, 1994a). An additional problem is that virus can survive for weeks to months in the marine environment (Nasser, 1994; Callahan et al., 1995; Gantzer et al., 1998) which is appreciably longer than for bacterial indicators (Solic and Krstulovic, 1992). Sewage schemes designed for compliance with bacterial standards are generally modelled using standard bacterial T90 seawater decay rates. It remains to be seen whether such designs will prove adequate for protection against viral contamination, In a similar manner disinfection of sewage effluents designed for simple compliance with bacterial standards may distort bacterial indicator to virus ratios and achieve at best cosmetic improvements to water quality (Tree et al., 1997). To maximise public health gains from expenditure on sewage infrastructure it is therefore wise for agencies responsible for water quality to adopt a holistic approach considering, in addition to the details of the sewage treatment scheme, the appropriate discharge location and the adequacy of arrangements for storm water storage and treatment. Over reliance on simple numerical compliance with bacterial water quality standards at a set monitoring point is unlikely to yield the optimum protection from viral contamination for vulnerable bivalve shellfisheries. Clearly meaningful tests for virological quality of seawater would bring similar benefits in this area as it would to sanitary assessments of shellfish quality. A number of workers are actively pursuing such goals (Metcalf et al., 1995).

7. Summary

The epidemiological data clearly demonstrates that filter feeding bivalve shellfish can, and do, act as efficient vehicles for the transmission of enteric viruses transmitted by the faecal–oral route. This identified hazard has been documented as a cause for concern by various international agencies and has a long history. Disease outbreaks can occur on an epidemic scale as graphically illustrated by an outbreak of Hepatitis A in Shanghai, China in 1988 involving about 300 000 cases. Improvement of harvesting area water quality offers the most sustainable route to improvement in the virological quality of bivalve shellfish sold live. However there is growing awareness, and concern, that current regulatory standards based on faecal coliform monitoring do not fully protect the shellfish consumer from viral infection. New viral test methods based on PCR, and the development of alternative more reliable faecal pollution indicators, offer new approaches for the further development of public health controls. However, further work is required to build a scientific consensus and to understand the implications of their introduction into legislation.

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