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OPINION OF THE

SCIENTIFIC COMMITTEE ON VETERINARY MEASURES RELATING TO PUBLIC HEALTH

ON

NORWALK-LIKE VIRUSES

(adopted on 30-31 January 2002)

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ACKNOWLEDGEMENTS

This opinion of the Scientific Committee on Veterinary Measures relating to Public Health is substantially based on the work of an ad hoc working group of the Committee. The working group included members of the Committees and external experts.

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1. BACKGROUND

Council Directive 91/492/EEC in Annex I – Chapter I laid down the conditions for production areas and in Chapter V – point 8 states that "in the absence of routine virus testing procedures and the establishment of virological standards, health checks must be based on faecal bacteria counts…"

The German authorities drew the attention of the Commission to the increased findings of contamination of food and in particular of bivalve molluscs, which might constitute a major food hazard. It has to be highlighted that a few numbers of communications via the Rapid Alert System have been made.

2. TERMS OF REFERENCE

The Scientific Committee on Veterinary Measures relating to Public Health is requested to assess the risk of contamination of food, particularly seafood, by Norwalk Like Viruses (NLVs). The risk assessment should provide an overview of their role as a possible health hazard for the consumer.

The Committee should in particular consider and give advice on:

- The methods of detection and inactivation of the pathogen;
- Possible methods or measures that could be used to reduce or eliminate the risk to human population.

Considering the common field of interest, the Committee is invited to set up a joint working group including external experts and experts from both the Scientific Committee on Veterinary Measures relating to Public Health and from the Scientific Committee on Food.

3. INTRODUCTION

NLVs represent an increasingly well-recognised and described virus group that can cause gastroenteritis in humans. In 1972, Kapikian *et al.* identified a 27 nm virus in the faeces of subjects involved in an epidemic outbreak of acute non-bacterial gastroenteritis in an elementary school in Norwalk, Ohio. The "Norwalk agent" was the first virus to be conclusively associated with diarrhoea in man. Subsequently, researchers began to recognise viruses as causative agents of gastroenteritis. Research progressed slowly, however, mostly due to the unavailability of cell culture systems for virus amplification and the insensitivity of electron microscopy for detecting the virus in clinical samples. In addition to the Norwalk agent, other morphologically similar but antigenically distinct viruses (e.g., Hawaii, Snow Mountain) were reported in the following years. Based on their morphology, these viruses were originally described as "small round structured viruses (SRSV)" and "classic human caliciviruses". Modern taxonomical analysis has confirmed two groups of viruses - either the NLV (Norwalk-like viruses) or SLV (Sapporo-like viruses) genera of the Caliciviridae. The public health significance of these agents

has been largely underestimated because of the self-limiting nature of the illness and the lack of diagnostic tests.

During the early 1990s, the cloning and sequencing of the Norwalk and Southampton viruses led to a rapid increase of knowledge in the field (Xi *et al.,* 1990), and prompted the development of sensitive molecular diagnostics (e.g., reverse transcription-polymerase chain reaction, probes) and tools for genome characterisation and viral strain comparison. Epidemiological links of apparently unrelated outbreaks, sometimes involving several countries, as well as tracing of the source of infection have been made possible by the development of techniques for viral strain genotyping and sequence comparison. The increasing data on virus diversity and the establishment of a database have facilitated phylogenetic analyses and comparison of the molecular epidemiology of NLVs between countries. Finally, substantial progress has been made towards the reliable detection of viral genome specific sequences in foods and waters (Beller *et al.,* 1997; Daniels *et al.,* 2000; Kohn *et al.,* 1995), which to date have only been implicated as the vehicle for NLVs on epidemiological grounds. With the application of these new diagnostics, NLVs have emerged as the most common pathogens associated with outbreaks of gastroenteritis in all countries where they were specifically sought (i.e., United States (Fankhauser *et al.,* 1998), United Kingdom (Lewis *et al.,* 1997), Japan (Iritani *et al.,* 2000), the Netherlands (Vinje and Koopmans, 1996), and Austria (Wright *et al.,* 1998).

Although virtually any food may be implicated in NLV transmission, bivalve molluscs present a particularly high risk because of their ability to concentrate viruses from contaminated waters in their tissues. Due to the low infectious dose and the high concentration of viruses in stools, fresh products including frozen raw products and ready-to-eat foods (e.g., salads and deli sandwiches) also appear to present a particular risk following contamination by food-handlers infected with NLVs. Another food group, which has emerged as a risk in regard to NLV infection, is fresh produce. Contamination of such products can occur at many stages including pre-, during and post-harvest

Human health problems associated with bivalve shellfish are well-recognised internationally. 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 US and elsewhere. In more recent years, the epidemiological evidence suggests that human enteric viruses, principally NLVs and hepatitis A virus, are now the most common aetiological agents transmitted by bivalve shellfish (Lees, 2000).

Until recently, humans were considered to be the only reservoir for NLVs. However, the characterisation of closely related viruses in pigs and cattle indicates interspecies transmission, and the potential for zoonotic transmission of NLVs is under investigation.

The following chapters will review, according to a risk assessment framework, the current knowledge on NLVs and food safety, particularly in relation to fresh produce and seafood, especially bivalve molluscs. Methods for detection and inactivation will also be reviewed, and possible preventive measures that can be applied in order to reduce the risk to the human population will be considered. Finally, conclusions and recommendations in regard to the above will be presented.

It is emphasised that the NLVs in relation to water will only be addressed as it affects the safety of other food products, and not as a food product as such or as a recreational/environmental hygienic issue.

4. RISK ASSESSMENT

4.1. Hazard identification

4.1.1. Introduction

In recent years, it has become clear that NLVs represent a significant cause of gastroenteritis in humans and are among the most important causes of gastroenteritis in all age groups (de Wit *et al.*, 2001; Wheeler *et al.*, 1999). Due to inadequate diagnostics, the self-limiting and short duration of the disease there is reason to believe that the incidence of NLV infection in most countries is underreported. NLVs are human pathogens transmitted by the faecal-oral route, and NLV infections occur both sporadically as well as in outbreaks. Foodborne and waterborne transmission of NLVs are well-documented. Foodborne NLV infections have been linked to a range of food products, including drinking water, fresh produce and bivalve molluscs.

4.1.2. Taxonomy

Caliciviruses are small, non-enveloped spherical viruses, measuring between 28 and 35 nm in size that contain a single stranded RNA (ribonucleic) genome of 7.3-7.6 kb. The genome is of positive polarity. It contains coding information for a set of non-structural proteins located at the 5'- end of the genome and for a major structural protein at the 3'-end. At present, viruses in the family Caliciviridae, based on their sequences, are grouped into four genera, named *vesivirus, lagovirus, Norwalk-like virus* (NLV) and *Sapporo-like virus* (SLV) (Green *et al.*, 2000). Viruses belonging to the first two genera mostly infect animals, although anecdotal reports suggest that zoonotic transmissions may be possible (Smith *et al*, 1998). The caliciviruses that infect humans thus mainly belong to two genera; the Norwalk-like viruses (NLVs), also known as small-round-structured viruses (SRSVs), for which the Norwalk virus (NV) is the prototype, and the Sapporo-like viruses (SLVs) or "typical" caliciviruses (Green *et al.,* 2000).

The genome organization of NLVs and SLVs is quite similar with some differences in the arrangement of the open reading frames (ORFs). In both genera, ORF1 encodes a set of proteins that is essential for replication and formation of new particles. The ORF2 region codes for the single major capsid protein, which assembles to form the virus particle (Jiang *et al.*, 1993). A third ORF encodes a minor structural protein. In SLV, the region encoding the capsid protein is found in the same reading frame and is contiguous with the non-structural proteins.

Sequence analysis of viruses from different outbreaks and different geographical locations have confirmed that NLVs found in humans can be divided into two major genetic groups (termed genogroups) based on capsid sequence and polymerase (POL) sequence data (Ando *et al.*, 2000). Norwalk

virus, Southampton virus, and Desert Shield virus are members of genogroup I. Snow Mountain virus, Hawaii virus, Toronto virus are members of genogroup II. In addition, stable lineages have been identified within these genogroups, based on phylogenetic analysis based on the complete capsid gene of at least two representatives per cluster (Green *et al.*, 2000; Vinjé and Koopmans, 2000; Vinjé *et al.*, 2000). To date, 15 distinct genotypes have been recognised, but as more strains are characterised, this number is likely to increase (Vinjé *et al.*, 2000).

4.2. Hazard characterisation

4.2.1. Clinical features

Following an average incubation period of 12-48 hours, infected persons may experience an acute onset of nausea, vomiting, abdominal cramps, and diarrhoea as prominent symptoms. In adults, projectile vomiting frequently occurs. Constitutional symptoms such as low-grade fever, headache, chills, and myalgia are frequently reported. The illness generally is considered mild and self-limiting, with symptoms lasting on average 12-60 hours (Kapikian *et al.*, 1996; Kaplan *et al.*, 1982). In outbreaks, the average attack rate is high- typically 45% or more (Vinje *et al.*, 1997). Data from a recently completed community-based cohort study in the Netherlands were surprising in that 20% of NLV infected persons reported symptoms for more than two weeks, suggesting that NLV infections may be more severe than previously recognised (Rockx *et al.*, 2002). Asymptomatic infections are also common. A total of 5% of healthy controls were found to shed NLVs in a community study, as compared with 16% of people with gastroenteritis (de Wit *et al.*, 2001). Similarly, in outbreak settings, 75% of people with gastroenteritis were found to shed NLVs compared with 20% of healthy contacts (Vinje *et al.*, 1997). Furthermore, since contaminated foods may contain multiple agents, multiple infections can occur and hence give rise to an even broader range of symptoms (Bettelheim *et al.*, 1999).

Sometimes hospitalisation and even parenteral fluid therapy due to severe dehydration are required in NLV infections, with up to 12% of cases hospitalised in a recent outbreak in military recruits (Arness *et al.*, 2000; Dolin *et al.*, 1972; Kaplan *et al.*, 1982b; Middleton *et al.*, 1977). Deaths associated with NLV infections have been reported, but the aetiological link needs to be confirmed (Djuretic *et al.*, 1996). No long term sequelae of NLV infections have been reported (CDC 2001).

NLV infections are highly contagious, resulting in a high rate of person-to-person transmission. Virus particles are shed via stools and vomit, starting during the incubation period, and lasting up to 10 days or, longer (Graham *et al.*, 1994; Greenberg *et al.*, 1979; Kaplan *et al.*, 1982) even in the absence of clinical illness (Rocks *et al.,* 2002).

<u>Transmission modes</u>

The infectious dose can probably be as low as 10-100 virus particles (Caul *et al*., 1996). NLVs are transmitted by direct person-to-person contact or indirectly via contaminated water (Beller *et al*., 1997; Brugha *et al*., 1999;

Gray *et al*., 1997; Hafliger *et al*., 2000; Kukkula *et al*., 1997, 1999), food (Berg *et al*., 2000; Daniels *et al*., 2000; Gaulin *et al*., 1999; Iversen *et al*, 1987; Kohn *et al*., 1995; Marshall *et al*., 2001; Parashar *et al*., 1998; Ponka *et al*., 1998; Sugieda *et al*., 1996) or contaminated environments (Cheesbrough *et al*., 2000; Green *et al*., 1998; Marks *et al*., 2000; Sawyer *et al*., 1988). Person-to-person transmission appears as the main route of infection. In the community survey in the Netherlands, 17% of NLV episodes were attributed to contact with a sick person within the same household, and 56% to contact with a case outside the household (de Wit, M., Koopmans, M., van Duynhoven, Y. Risk factors for gastroenteritis due to Norwalk-like viruses, Sapporo-like viruses, and rotaviruses, submitted for publication). However, many foodborne NLV outbreaks have been described often resulting from contamination by an infected food-handler. In addition several waterborne NLV outbreaks have been described, both directly (e.g. by consumption of contaminated water) or indirectly (e.g. via washed fruits, by swimming or canoeing in recreational waters) (Beller *et al*., 1997; Brugha *et al*., 1999; Gray *et al*., 1997; Hafliger *et al*., 2000; Kukkula *et al*., 1997, 1999). Of special interest is the finding that a substantial proportion of bottled mineral waters contained caliciviral RNA (Beuret *et al*., 2000), although this finding is still to be confirmed by others.

Since projectile vomiting is a common feature following NLV infection and viruses can be present in vomit (Greenberg *et al*., 1979; Patterson *et al*., 1997), aerosolised vomit is recognized as an important vehicle for transmission, both by mechanical transmission from the vomit-contaminated environment and even air-borne transmission (Caul, 1994; Chadwick and McCann 1994a; Chadwick *et al*., 1994b; Cheesbrough *et al*., 2000; Patterson *et al*., 1997; Sawyer *et al*., 1988). The most compelling evidence for airborne transmission came from a study by Marks *et al*. (2000); they described an outbreak of gastroenteritis following a meal in a large hotel during which one of the guests vomited, and found an inverse relationship between attack rate per table and the distance from the person who became sick.

It is important to note that contamination may occur not only at the end of the food distribution chain, but rather at almost any step from farm to table. Foodborne illness associated with consumption of oysters has been traced back to a crewmember of a harvesting boat (Kohn *et al*., 1995). From that same outbreak investigation, it was reported that 85% of oyster harvesting boats in the US routinely disposed sewage overboard. Little is known about the hygienic conditions in harvesting areas in other parts of the world, not only for shellfish, but also for products such as fresh fruits. Infected food-handlers may transmit infectious viruses during the incubation period and after recovery from illness (Gaulin *et al*., 1999; Lo *et al*., 1994; Parashar *et al*., 1998). Another aspect of NLV epidemiology is that food-handlers may unknowingly transmit viruses, e.g., when they have a sick child at home (Daniels *et al.*, 2000)

Besides person-to-person transmission via food vehicles, zoonotic transmission has been reported for some enteric viruses (rotavirus, coronavirus), although this appears to be of no significance for foodborne infections. This may change, however, based on new data for NLVs. NLVs were found in healthy pigs in Japan and in historic calf stool specimens from

the UK and from Germany (Liu *et al.*, 1999; Dastjerdi *et al.*, 1999; Sugieda *et al.*, 1999; Poel *et al.,* 2000). The calf viruses, named Newbury agent and Jena virus, are pathogenic for young calves. The two bovine enteric caliciviruses and the pig enteric calicivirus are genetically distinct from human strains, but cluster within the NLV genus (Liu *et al*., 1999, Sugieda *et al.*, 1999). Their close relationship to human NLVs suggests that interspecies transmission can occur. Using the calf calicivirus capsid as antigen in an ELISA, antibodies were detected in more than 30% of veterinarians tested (Koopmans, unpublished).

4.2.2. Epidemiology

Community cases

Data on the incidence of NLV gastroenteritis are available from different types of studies. The most accurate community incidence data have recently been determined by nationwide community surveys of infectious intestinal disease in the UK and in the Netherlands. These studies provide estimates of the total burden of illness due to NLV infections, including very mild cases, which will never be evaluated by a physician.

The *Sensor* study followed two cohorts of the Dutch population for six months each, from December 1998 to December 1999 (de Wit *et al.,* 2001). After standardisation, the incidence of infectious gastroenteritis was determined to be 283 cases per 1000 person-years. In the case-control component of the study, viruses accounted for 34% of all cases, with NLVs the most common viral pathogen, accounting for 11% of cases. SLVs were found in 6% of all cases.

Similarly, a community cohort of nearly a half-million was followed-up from 1993 to 1996 in England's Infectious Intestinal Disease (IID) study. The overall rate of 194 cases per 1000 person-years was lower than the overall rate in the Netherlands (Wheeler *et al.,* 1999). The rate of NLV infection was 13 cases per 1000 person-years (6% of all cases) and the rate of SLV infection 2.2 cases per 1000 person-years (0.01% of all cases) (IID Study Team 2000; Wheeler *et al.,* 1999). These lower rates in England may be because electron microscopy was the diagnostic tool as opposed to RT-PCR in the Dutch *Sensor* study (de Wit *et al.,* 2001). This may also have contributed to the large proportion of community cases of unknown aetiology (61%) in the IID study. Nevertheless, NLV was still the most common aetiological agent detected in both studies.

In Finland, a two year follow-up of approximately 2400 children (2 months to 2 years of age) in a randomised, double-blind, placebo controlled rotavirus vaccine trial confirmed the importance of caliciviruses (Pang *et al.,* 2000). Using RT-PCR to examine stool samples, caliciviruses were found to be as common as rotavirus among the cohort of children. NLVs were responsible for 20% of cases and SLVs for 9%. The course of illness appeared to be somewhat milder than rotavirus gastroenteritis (Pang *et al.*, 1999). This may explain the lower percentage of hospitalised children with NLVs that has been reported (Schnagl *et al.*, 2000).

Medical consultations

The IID study in England also showed that calicivirus infection rates, when measured by presentation to a general practitioner (GP), were approximately one-sixth of those in the community. However, there may be a substantial under-ascertainment of community cases since institutions where outbreaks may be disproportionately frequent (such as hospitals, residential homes, universities and prisons) were excluded from the study population (Wheeler *et al,* 1999). Nonetheless, 6.5% of the cases presenting to a GP tested positive for NLV and 1.5% tested positive for SLV.

In a similar GP-based study in the Netherlands, NLVs were detected slightly less frequently- 5.0% of cases (de Wit *et al.,* 2001b). SLVs were detected in 2.0% of cases. Since the Dutch study used RT-PCR to detect human caliciviruses (Hu CVs) and the English study used the less-sensitive electron microscopy, it is somewhat surprising that rates were lower in the Netherlands. This may be due to a real difference in NLV incidence between the two countries, but there is also evidence that consultation rates for gastroenteritis may be lower in the Netherlands due to an active deferral policy (de Wit *et al.,* 2001b). In a French survey, NLVs were detected by RT-PCR in 14% of stools of children consulting their GP. None of the detected agents were SLVs (Bon *et al.,* 1999).

Outbreaks

A system of general outbreak surveillance for infectious intestinal diseases in England and Wales has been operated from the Communicable Disease Surveillance Centre (CDSC) since 1992. From its origin until 1995, information on 2154 general outbreaks had been collected (Dedman *et al.,* 1998). Laboratory testing, primarily electron microscopy, confirmed that 709 (33%) of these outbreaks were caused by NLVs. SLV outbreak reports were far less common and accounted for only a few outbreaks a year (de Wit *et al.,* 2001).

Surveillance conducted in Sweden from 1994 to 1998 also points to the high fraction of gastroenteritis outbreaks attributable to NLVs (Hedlund *et al.,* 2000). Of 676 outbreaks analysed, 407 (60%) were attributed to NLVs and nine were attributed to SLVs (by electron microscopy). Caliciviruses were the predominant aetiological agent in every investigated outbreak and Hedlund *et al.* (2000) concluded that NLVs are the most common cause of gastroenteritis outbreaks in both hospitals and the community in Sweden. Similarly, caliciviruses were found as the primary cause of outbreaks in Finland (Maunula *et al.,* 1999), Germany (Schreier *et al.,* 2000), and the Netherlands (Koopmans *et al.,* 2000; Vinjé *et al.,* 1996, 1997).

In Finland, 55 % of the 117 outbreaks reported as food- or waterborne and from which stool samples, and foodstuff, in some instances, have been submitted for virological screening, were NLV-positive (Maunula *et al.*, 1999, Maunula and v. Bonsdorff, unpublished data). Among them, 15 outbreaks were related to imported frozen berries, mainly raspberries. A recommendation for all catering and other large-scale kitchens not to serve unheated frozen berries was implemented (Ponka *et al.*, 1999). Since then,

only a few raspberry-associated outbreaks have occurred, and these have been related to neglect of this recommendation. Whereas a single NLV genotype was recovered from the patients in each outbreak, a broad selection of different lineages was recovered from all the 15 outbreaks. This suggests that contamination of the batches of berries was not linked to a single common source (Maunula and v. Bonsdorff, unpublished). The findings indicate that frozen berries are a potential source for wide-spread international epidemics of NLV infections (Parashar *et al*., 2001).

Several outbreaks of NLV infections on cruise- and war-ships have been reported. These outbreaks may well be initiated as foodborne, but due to the crowded conditions on these ships, person-to-person spread is efficiently favoured (Fankhauser *et al.*, 1998, Sharp *et al.*, 1995, McCarthy *et al.*, 2000).

Nosocomial outbreaks of NLV infections are common (Hedlund *et al.*, 2000; Vinjé *et al.*, 1997; Traore *et al.*, 2000). In Finland, of the 48 hospital outbreaks in 1997 – 2001 (mostly on geriatric wards), 32 (66%) were caused by NLVs, although the reporting and sampling has not been systematic.

Estimates for foodborne NLV infections

For NLV infections, the most common mode of transmission is person-to-person, and it is difficult to provide a reliable estimate for the number of cases that are related to foodborne transmission, including waterborne transmission. The most conclusive evidence would be to find an identical virus in a patient and in a food item consumed by this person, but such evidence is rarely available. From assessment of risk factors addressed in a detailed questionnaire during the community survey in the Netherlands, 12-16% of NLV infections were attributed to food- or waterborne transmission (de Wit *et al.,* submitted for publication).

Data from national outbreak surveillance show that the proportion of outbreaks associated with NLV ranges widely (6-100% reported for foodborne outbreaks). This can be explained by lack of harmonisation in outbreak surveillance and laboratory testing between countries. Almost all surveillance schemes address a different sample of outbreaks, ranging from all outbreaks reported to municipal health services (highly biased towards institutional outbreaks), to selected foodborne outbreaks. Methodologies used for virus testing range from rather insensitive electron microscopy to highly sensitive RT-PCR methods (Lopman *et al.,* submitted for publication). Nevertheless, the data suggest that NLV is a common cause of foodborne infection around the world (Mead *et al.,* 1999). Drinking water as a source of NLV outbreaks has also been well-documented (e.g. Miettinen *et al*., 2001) emphasising its important role either as such or when contaminating foodstuffs.

Foodborne outbreaks have been linked with many different food items in case reports, but there is no overview of what are the highest risk categories. Based on the outbreak reports, two major food groups can be distinguished:

1. *filter-feeding shellfish*: Numerous outbreaks have been linked to consumption of faecally-contaminated shellfish (reviewed in Lees, 2000).

2. *any other food type that may be contaminated during production, handling, and preparation, provided it is not subjected to a virus inactivation step afterwards e.g., heating at 90°C for 2 minutes*: Outbreaks have been traced to, among others, contaminated vegetables, fruits, juices, deli-meat, sandwiches, bread rolls, mixed salads, bakery products, and ice. Of note is that contamination may occur at the start of the food chain, e.g., by use of contaminated irrigation water or by handling of berries during harvesting.

Bivalve molluscan shellfish

Gastroenteritis has been recognised as a clinical consequence of the consumption of contaminated shellfish for many years (Richards, 1985; Rippey, 1994; Lees, 2000). In industrialised countries, after the 1950s, when shellfish-associated cases of typhoid fever became rare, the majority of cases of shellfish-vectored gastroenteritis have appeared to be nonbacterial in nature. 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 that caused by NLVs. It is now widely recognised that enteric viruses, principally NLVs and hepatitis A virus, are the major causes of shellfish-vectored gastroenteritis (Lees, 2000).

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 affecting nearly 800 people (Appleton and Pereira, 1977). Small round virus-like particles, like those seen in outbreaks of "winter vomiting disease", were observed by electron microscopy in a high proportion of patients faeces. Subsequently, the same author examined clinical samples associated with nine separate shellfish-vectored gastroenteritis outbreaks and showed similar small round virus like particles in about 90% of samples. This indicated for the first time the probable broad significance of viruses in shellfish transmitted infections in the UK (Appleton *et al.,* 1981). Since these early observations, enteric viruses causing gastroenteritis have been epidemiologically linked to outbreaks of shellfish-vectored illness on numerous occasions and in many countries (Lees, 2000). The United States Food and Drug Administration (US FDA) risk assessments estimate cases of NLV gastroenteritis related to seafood consumption at some 100,000 per year (Williams and Zorn, 1997). Similar estimates for other countries have either not been performed or are not readily available in the scientific literature. Thus, although it is clear that shellfish-associated NLV infections represent a significant foodborne disease problem, the proportion of foodborne NLV infections that are associated with shellfish is still unknown.

An illustration of the problem can be given: In the winter season 2000/2001, in an European strain surveillance, a newly recognised strain of NLV that

initially emerged in a large waterborne outbreak in France in August 2000, was associated with several oyster-related outbreaks in December and January in France, and then with imported oysters in Denmark, the Netherlands, Finland, and Sweden. The variant subsequently spread efficiently in secondary and tertiary waves of person-to-person transmission, and in the Netherlands caused 25% of outbreaks in nursing homes of that winter. An investigation by French national authorities found no irregularities at the site of production based on bacteriological examination and concluded that no further action was required (Vennema *et al.*, manuscript in preparation).

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 (Sugieda *et al.*, 1996). In some shellfish associated outbreaks, patients may experience gastroenteritis followed by hepatitis (Richards, 1985; Halliday *et al.,* 1991) suggesting a mixed NLV/hepatitis A virus contamination. In a similar fashion, in an oyster-associated outbreak involving naval officers two episodes of gastroenteritis occurred, the first caused by NLV and the second seemingly by astrovirus. 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. These findings may have far-reaching epidemiological implications for the possible generation of new recombinant NLV strains. It also emphasises the potential significance of shellfish as a vehicle for spreading NLV strains, particularly given the increasingly global trade in such food commodities.

4.2.3. Infectious dose

Present knowledge indicates that the virus load required to cause disease is very low; <100 viral particles (Kapikian *et al*., 1996). This low infectious dose of NLVs facilitates the spread in the food production, distribution, and retail system of virus in amounts that can cause disease. Furthermore, the low infectious dose readily allows spread by droplets via person-to-person transmission, through fomites, or environmental contamination, as evidenced by secondary and tertiary spread in foodborne outbreaks.

4.2.4. Pathogenicity and virulence factors

Little is known about the mechanisms by which NLVs cause diarrhoea. In duodenal biopsies taken from infected volunteers, lesions were seen in the intestinal epithelium at 1-day post infection with the Norwalk virus or Hawaii virus as inoculum. The changes were villous broadening, abnormal epithelial cells, and an inflammatory response in the *lamina propria* with infiltration of polymorphonuclear leucocytes and lymphocytes. At 5-6 days after ingestion villous shortening and crypt hypertrophy were observed. D-xylose absorption was significantly reduced throughout this period (Schreiber *et al*., 1973, 1974). The observed inflammatory response in the *lamina propria* was similar to the damage that has been observed following rotavirus infections, where pro-inflammatory cytokines and chemokines are thought to trigger this process (Rollo *et al*., 1999). There are no known systemic effects.

4.2.5. Immunological response

Little is known about immunity to NLV infections. Antibody ELISA assays have been developed by using recombinant capsids as antigen, and preliminary studies in outbreaks and in volunteer studies suggest that people develop antibodies mostly restricted to the infecting genotype with some cross-reactivity. From experimental infections in volunteers it is known that infected persons may become protected from reinfection, but only for a short period, and again only when the challenge virus is closely related to the genotype of the strain that was used for the infection (Hale *et al.*, 1999; Noel *et al.*, 1997). Seroprevalence studies with the recombinant antigens have shown that antibodies to NLVs are very common in the population, even when the recombinant NV capsids are used in populations where viruses from the Norwalk cluster have not been identified for a long time. Volunteers with antibodies to the infecting genotype reportedly may have a higher risk of illness and a steeper dose-response curve. It is unclear what this means. It has been hypothesised that certain persons might be genetically more susceptible to NLV infections and disease.

Hinkula *et al.* (1995) have shown that the seroprevalence may differ markedly for different antibody isotypes, suggesting that the lack of protection in part may be explained by the fact that a different type of antibody was the better correlate of protection.

The lack of broadly reactive, long-lived immunity to natural infection suggests that development of a protective vaccine may be problematic.

4.3. Exposure assessment

4.3.1. Survival data

There is little precise information on the stability of NLVs because no *in vitro* culture systems exist to assess viability. Direct information about NLVs is available from epidemiological observations in foodborne outbreaks and from small number of experiments with Norwalk virus in volunteers.

NLVs infect via the gastrointestinal tract and therefore are acid-stable. People have developed viral gastroenteritis after eating shellfish pickled in brine and vinegar. In one volunteer study, Norwalk virus was shown to retain its infectivity after exposure to pH 2.7 for 3 hours at room temperature. Thus, even though pH can vary in environmental waters, pH is unlikely to be an important factor for survival of NLVs in the environment.

NLVs remain infectious after refrigeration and freezing. Frozen foods that have not received further cooking have been implicated in a number of NLV outbreaks. NLV's appear to be inactivated by normal cooking processes but are not always inactivated in shellfish given only minimal heat treatment.

NLVs appear to survive well on inanimate surfaces and in the environment. Epidemiological evidence from the lingering outbreaks that have occurred in hospitals, in residential homes and on cruise ships supports this. Virus has been detected by PCR in environmental swabs from one hospital outbreak

and in shellfish (Le Guyader, 1994; Lees, 1995b) and environmental samples (Cheesbrough *et al.,* 1997). Assessing the significance of these findings, which are based on the detection of viral genome by RT-PCR detection, is difficult because it is not possible to state that a positive RT-PCR indicates the presence of viable virus.

Since NLVs cannot be cultivated, it may be useful to consider survival data available for other enteric viruses, which may well have similar survival characteristics and can be cultivated and for which more extensive information is available. Human enteric viruses will potentially be present in any type of water contaminated by human faecal material and sewage.

Enteroviruses have been the most widely studied virus group. Monitoring fresh waters for enterovirus have reported very common detection with 75% of samples positive over a four years period in one study (Morris, 1984). The level of contamination varied widely from 0-720,000 pfu per 10 litres.

A similar picture has been described for marine waters. In a review of monitoring data for 1988-1992 in the UK, 31% of samples examined for viruses gave positive results with levels between 1 and >1,400 pfu per 10 litres (Wyer and Kay, 1993). Enteroviruses have also been detected in marine sediment.

The length of survival of enteroviruses in the environment is affected by a number of issues including temperature. One measure of the rate of survival is the time taken for 90% of the viable virus to be inactivated (T_{90}). Table 1 summarises the T_{90} for different enterovirus strains in a range of environmental circumstances.

These data show that enteroviruses will survive in the environment for long periods of time and that increased temperature is a critical factor in reducing environmental survival.

Heat inactivation data for feline calicivirus, a possible model for NLVs, showed this virus to be more readily inactivated than hepatitis A virus (Slomka and Appleton, 1998).

Available evidence suggests that salinity is of little significance to survival of NLVs (Dolin *et al.,* 1972).

The ability of NLVs to survive relatively high levels of chlorine (see ch. 6) and varying temperatures and other environmental factors facilitates spread through the recreational and drinking water, as well as food, including shellfish (CDC, 2001).

Table 1 T_{90} of different Enterovirus strains

Virus type	Conditions	T_{90} (h)	Ref.
Poliovirus 1	Estuarine water *in situ*, 4 - °C16	280	Lo *et al.*, 1976
Poliovirus 1	Estuarine water *in situ*, 21-26°C	170	Lo *et al.*, 1976
Poliovirus 1	Marine water *in vitro*, 20-25°C	17-56	Akin *et al.*, 1976
Poliovirus 2	Atlantic Ocean water, 4°C	>288	Bitton, 1978
Poliovirus 2	Atlantic Ocean water, 12°C	96	Bitton, 1978
Poliovirus 2	Atlantic Ocean water, 22°C	72	Bitton, 1978
Rotavirus SA – 11	Estuarine water *in vitro*, 20°C	17-38	Hurst and Gerba, 1980
Rotavirus SA – 11	Estuarine sediment *in vitro*, 20°C	70-100	Rao *et al.*, 1984
Echovirus 6	Marine water *in vitro*, 3-5°C	640-720	Bitton, 1978
Echovirus 7	Estuarine water *in situ*, 20°C	14-38	Hurst and Gerba, 1980
Echovirus 1	Marine water/sediment *in vitro*, temp ns*	60-144	Smith *et al.*, 1978
Coxsackie-virus B3	Estuarine water *in situ*, 4-16°C	29-240	Hurst and Gerba, 1980
Coxsackie-virus B3	Marine water/sediment *in vitro*, temp ns*	67-215	Smith *et al.*, 1978

* ns= not specified

4.3.2. Prevalence data

Detection of NLVs in naturally contaminated shellfish from polluted harvesting areas, or in shellfish associated with disease outbreaks, has been documented in only a few reports. In England and Ireland a two year study of commercial shellfish harvesting areas found that whilst NLVs were not detected by RT-PCR in an European Union (EU) class A site or an A/B site (see chapter 8.1.2 for explanation of sanitation categories for shellfish), they were detected in 6%, 23% and 33%, respectively, of samples taken from three commercial class B areas, and in 47% of samples taken from a prohibited site. In this study all NLV RT-PCR positive results were confirmed by sequence analysis. Occurence of NLV was found to correlate very strongly with the degree of faecal contamination as judged by either *E. coli* or FRNA bacteriophage levels in shellfish (Henshilwood and Lees, in preparation). Similarly in France, over a three year period, NLVs were detected in 23% of oyster samples and 35% of mussel samples collected in non-commercially producing areas (EU class B and D area) (Le Guyader *et al*., 2000). However in this case, no correlation was found with *E. coli*, especially in class B areas.

For hepatitis A virus, the levels of contamination in shellfish collected in contaminated areas (not commercial shellfish) reported in different studies are quite similar; 12% in China (Lee *et al*., 1999), 13% in France (Le Guyader *et al*., 2000), 15% in US (Chung *et al*., 1996). In Italy, about 36%

of samples collected from the Adriatic Sea were found to be contaminated (Croci *et al*., 2000).

4.3.3. Human consumption data

Only limited information is available on the consumption of various food categories, such as mollusc shellfish and specific fresh produce, in the EU. However, Eurostat gives production and trade information figures, which give an impression of the total consumption in the EU of food products of particular interest in relation to NLVs (Annex). Less information is available in regard to consumption patterns in various regions and among various age groups. Also, little crude information is available regarding the seasonal variation in consumption patterns.

4.4. Risk characterisation

Figure 1 illustrates the epidemiology of NLV infections and the various routes by which humans can be infected.

Due to the limited data that are available in regard to exposure to NLVs via various food categories, the lack of quantitative data, and the many uncertainties regarding how much foods contaminated with NLVs contribute to the burden of illness in humans, it is not possible for the time being to perform a quantitative risk assessment and to present a risk estimate regarding NLVs and various food categories. However, the data presented in the preceding chapters do show that NLV infections represent a significant proportion of gastroenteritis cases in humans, and that NLVs are often spread via food, causing outbreaks or sporadic cases of illness. Available data show that bivalve molluscs present a well-documented source of NLV infection. Thus, it can be concluded that bivalve molluscs represent a relatively high risk to the consumer in regard to NLVs unless preventive measures are applied. Also, other food categories, such as ready-to-eat foods and fresh produce that are handled by humans (or by other means may be exposed to human faecal material) and are eaten without further heat treatment, present a risk to the consumer in regard to NLVs, which can increase considerably if hygienic practices are sub-optimal. The hygienic practices exerted by food-handlers are a key issue. Furthermore, the hygienic production of fresh produce is an issue of the utmost importance.

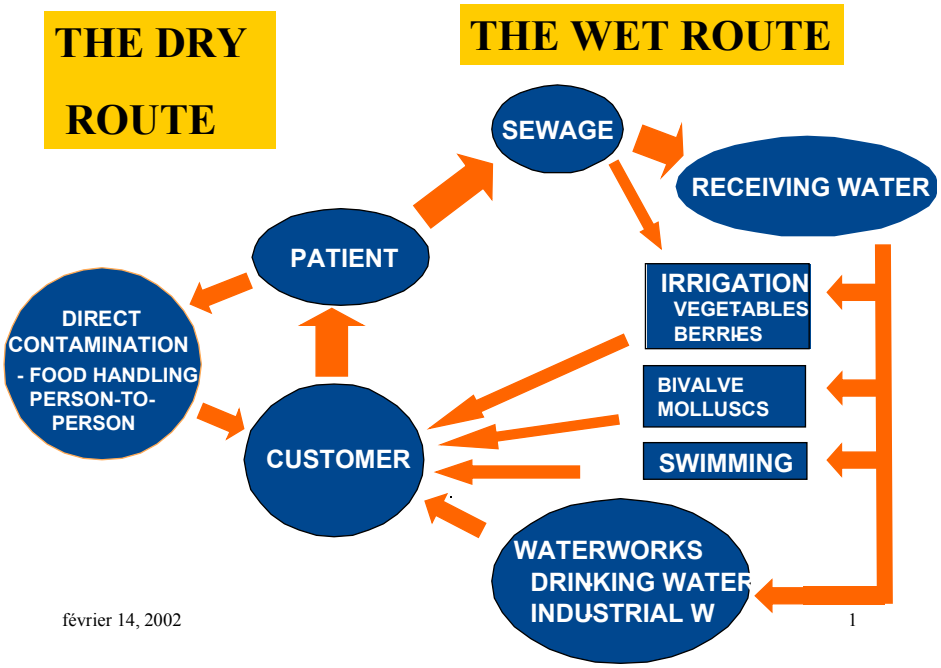


Fig. 1 The epidemiology of NLV and the various routes by which humans can become infected

5. METHODS FOR ANALYSES

5.1. Detection of NLVs

5.1.1. Detection of NLVs in clinical samples

Despite numerous attempts by several groups of investigators, NLVs have never been isolated using cell or tissue culture, and diagnosis has been made historically by visualisation of virus particles by electron microscopy (EM) (Atmar and Estes, 2001, Kapikian *et al.*, 1996). However, EM is a relatively insensitive technique, requiring the presence of a minimum of around 10^6 and probably more particles per ml of stool sample and, unlike some other enteric viruses, NLVs are not shed to very high maximum titres. This may not be a problem in outbreak investigations, when similar results may be obtained when stool samples have been collected promptly (Otsu *et al.*, 2000). For community-based studies however, this has been an impediment until the complete genome of the NLV prototype, the Norwalk virus, was sequenced from cDNA clones derived from RNA that had been extracted from a bulk stool specimen (Jiang *et al.*, 1990, 1993). From early work with immune electron microscopy (IEM), and later sequence analysis of genomes of different NLV strains, it became evident that the NLVs are in fact an antigenically and genetically diverse group of viruses (Dingle *et al.*, 1995; Lambden *et al.*, 1993; Lew *et al.*, 1991, 1994; Lewis 1990). Typically, variant viruses would be characterised by neutralisation assays using hyperimmune sera or panels of monoclonal antibodies in a tissue culture infectivity assay. However, because no one has succeeded in culturing these viruses *in vitro*, their antigenic relationships have been evaluated primarily

by cross-challenge studies and IEM or solid phase IEM (SPIEM) with viruses purified from stool samples (Dolin *et al.*, 1972; Kapikian *et al.*, 1972; Kaplan *et al.*, 1982; Lewis, 1990; Lewis *et al.*, 1995; Wyatt *et al.*, 1974).

At present, genome-based detection methods are available in which fragments of the viral RNA are amplified directly from stool samples by reverse-transcriptase polymerase chain reaction (RT-PCR) (Jiang *et al.*, 1992; De Leon *et al.*, 1992; reviewed in Atmar and Estes, 2001). Initial studies using these methods to detect viral RNA in outbreak specimens confirmed the unusual level of divergence, even when a highly conserved region of the viral genome was selected as a target for the RT-PCR (Ando *et al.*, 1994; Green *et al.*, 1993; Lew *et al.*, 1994). Since then, second generation assays have been developed which have been optimised for the detection of a broad range of NLVs by targeting conserved motifs in the non-structural protein genes (Fankhauser *et al.*, 1998; Green *et al.*, 1993; Noel *et al.*, 1997; Vinjé *et al.*, 1996).

Although NLVs cannot be grown in cell culture, efforts have been made at the development of antigen-based detection methods. For this purpose recombinant NLV capsids have been developed for use as control antigens (Green *et al.*, 1993; Hale *et al.*, 1999; Jiang *et al.*, 1992; 1996; Kobayashi *et al.*, 2000; Noel *et al.*, 1997; Vipond *et al.*, 2000). However, the current problem is that hyperimmune responses are predominantly type-specific, and that assays based on these reagents as a result are narrow in their applicability. Recently, a NV-specific monoclonal antibody was characterised with reactivity to strains from 4 out of 5 other genotypes within genogroup I NLVs that were tested (Hale *et al.*, 2000). This monoclonal antibody offers the first hope for the development of more broadly reactive detection assays. For genogroup II NLVs no common epitopes have been identified yet, although the low-level cross-reactivity observed in some studies suggests that such a group-specific epitope exists. For a complete overview of NLV diagnostics see Atmar and Estes (2001).

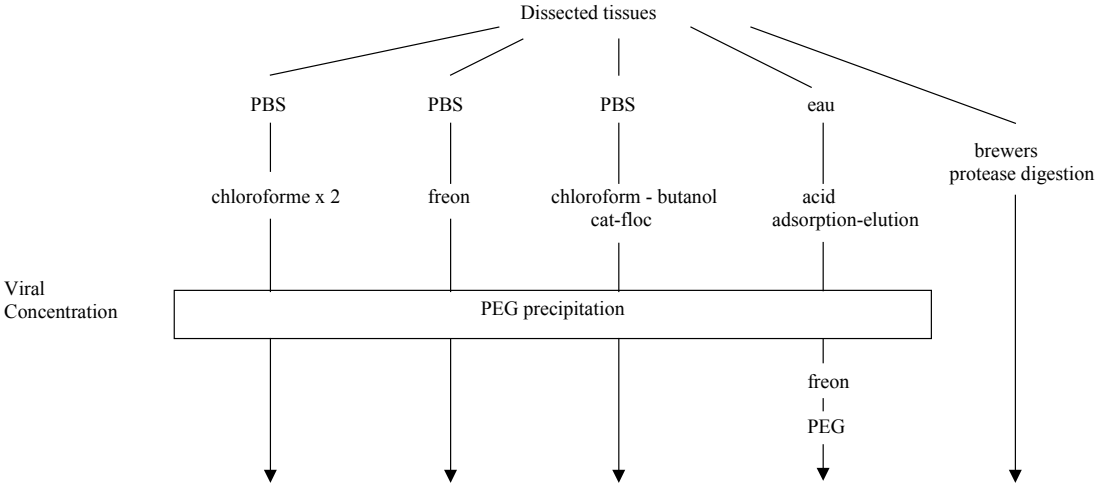
5.1.2. Detection of NLVs in shellfish

Only relevant methods for NLVs detection described during the last five years are reported here. However, two alternative approaches, the extraction-concentration methods and the adsorption-elution-concentration methods described for culturable enteric viruses still constitute the general draft to prepare virus concentrate (Lees, 2000). The main difference now is that nucleic acid is purified and then amplified by 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.

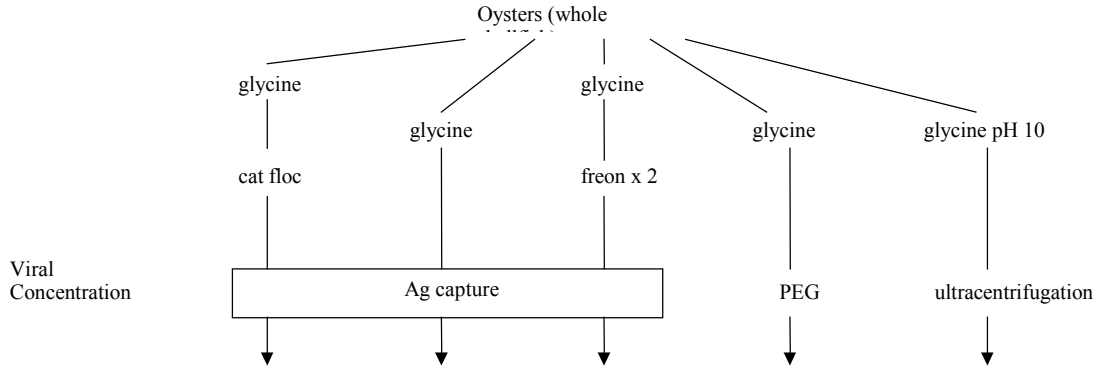
Progress with clinical PCR assays for NLV and other enteric viruses prompted the exploration of this technology to detection of viruses in food and more specifically in seafood. Several initial studies utilised culturable viruses, such as poliovirus, simian rotavirus or laboratory adapted strains of hepatitis A virus, in laboratory seeding studies to develop methods and assess the feasibility of PCR based assays (Lees *et al.*, 1994; Atmar *et al.*, 1995; Cromeans *et al.*, 1997; Lees *et al.*, 1995b; Jaykus *et al.*, 1996). 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. In figure 2 methods that have been validated on naturally contaminated samples are specified to be sure that the sensitivity threshold is compatible with viral concentration in natural samples. A later modification targeted extraction of the shellfish digestive organs (Atmar *et al.*, 1995, Sugieda *et al.*, 1996; Green et Lewis, 1999; Shieh *et al.*, 1999; Legeay *et al.*, 2000), since these are known to harbour most of the contaminating virus (Romalde *et al.*, 1994, Schwab *et al.*, 1998), thus minimising contamination 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 µl) 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 (Jaykus *et al.*, 1996; Atmar *et al.*, 1995). Recently, commercial kits have been applied for nucleic acid purification (Hafliger *et al.*, 1997; Shieh *et al.*, 1999; Lee *et al.*, 1999). A multi-centre collaborative study also demonstrated that NLV detection techniques could be reliably applied in a number of laboratories (Atmar *et al.*, 1996).

These methods are still under development and a monitoring method has not been designed yet.



NA extraction		PK phenol-chloroform CTAB ethanol precipitation	PK phenol -chloroform CTAB, chloroform ethanol precipitation	PK phenol-chloroform CTAB ethanol precipitation	Qiagen	Boom method
Sensitivity	NLV	/	/	5 RT-PCR units	/	/
	HAV	/	/	100 PFU	/	100 PFU
Validation on Natural samples		yes	yes	yes	yes	no
Reference		Green et al. 1999	Sugieda et al. 1996	Atmar et al. 1995	Shieh et al. 1999	Legeay et al. 2000



NA extraction	Qiagen Viral RNA kit	5 min/ 95°C	RNAzol	guanidium CsCl cushion	Boom
Sensitivity HAV	/	10 PFU	40 PFU	10^5 $TCID_{50}$	/
Validation on Natural samples	yes	no	no	yes	yes
Reference	Lee et al. 1999	Lopez -Sabater et al. 1997	Cromeans et al. 1997	Croci et al. 2000	Munian-Mujika 2000

Figure 2: Overview of methods for detection of viruses in food

5.1.3. Enumeration of NLVs

Since removal inhibitors of PCR amplification is a major factor affecting successful application of PCR to shellfish, most authors agree on 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 quantification, through the development of a competitive PCR approach. Real-time PCR is under development for environmental samples and at the present time only quantification for enterovirus in sludge has been published (Monpoheo *et al.*, 2001). In a recent study, about 10^6 enterovirus RNA copies/ml were detected in sewage treatment plant lagoons (Schvoerer *et al.,* 2001). Quantitative analyses of shellfish implicated in an outbreak using MPN-RT-PCR demonstrated the presence of approximately 10 000 genomic copies of NLV per oyster (Le Guyader *et al.,* Submitted). At present, the main obstacle for the development of NLV real time PCR is the lack of consensus sequence to design primers and probes. Good quality control procedures for amplification inhibition, and other aspects, remain an important area to be addressed.

5.1.4. Viability

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 affecting 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 (RNAases). It is debatable whether free virus genome, or virus genome unprotected by a complete protein coat, would remain intact for a long time in the RNAase rich environment of sewage or in the hostile environment of the shellfish digestive tract. Very little data are available although a laboratory seeding study on cooked shellfish showed that in some samples feline calicivirus, a possible model for NLVs, was detectable by PCR but not by culture (Slomka and Appleton, 1998). Clearly 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 NLVs. 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 to ensure that the PCR is detecting viable virus. This approach may prove useful for other enteric viruses, although 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.

5.1.5. PCR applications in routine laboratory

Despite these uncertainties PCR has proven to be a major step forward in the development of methods for detection of enteric viruses in shellfish. However, most data have 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 (8.1.2.5), 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.

5.2. Potential indicators for NLVs

The current regulatory controls in most countries rely heavily on the concept of faecal pollution indicator organisms to assess microbiological hazards (see chapter 8). 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 well illustrated in many outbreak reports by the failure of bacterial indicators to identify shellfish contaminated with NLVs (Bosch *et al.*, 2001; Chalmers and McMillan, 1995; Christensen et al. 1998; Gill *et al.*, 1993; Lees 2000). The problem arises probably because viruses are hardier than bacteria and therefore survive better in the marine environment and are more resilient to inactivation or removal during depuration or relaying of shellfish. It is likely that other potential bacterial indicators of faecal pollution, such as the faecal streptococci, would share similar disadvantages to the faecal coliforms for identification of viral contamination.

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 bacteriophages 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, 1991). Male-specific RNA (FRNA) bacteriophages 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). FRNA bacteriophages 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 FRNA bacteriophage in order to ascribe contamination to animal or human sources (Hsu *et al*., 1995; Beekwilder *et al*., 1995). 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).

During recent years, studies relating to bivalve shellfish have been performed. In studies in the UK, FRNA bacteriophage content in commercially depurated oysters was shown to be 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 *E. coli* (Dore *et al*., 2000). FRNA bacteriophage may have particular advantages as a 'viral' indicator for depurated shellfish as several studies have shown its elimination kinetics during the depuration process appear to reflect those of enteric viruses (Dore and Lees, 1995; Power and Collins, 1989). Other workers have also concluded that FRNA 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. However, it should be noted that these studies were performed in more polluted areas with oysters either requiring depuration or the area closed to commercial fishing. In the UK, analysis of shellfish associated with gastroenteritis have shown that 10 of 14 outbreak samples were negative for *E. coli* and only two were above the required bacteriological standard (<230 *E. coli* per 100g), whereas all samples were positive for FRNA bacteriophage with average levels of 2500 pfu per 100 g (range 60 to 17500) (Lees, 2000). Studies in France have shown that in cleaner (class A) harvesting areas with <230 *E. coli* per 100g, 13 out of 101 shellfish samples were positive for NLVs but only six were also contaminated by FRNA (Le Guyader, unpublished data). Although FRNA bacteriophage shows promise as a potential alternative indicator, results to date suggest it may perform better in more consistently polluted sites than in 'clean' sites. Where NLV contamination occurs without significant inputs of sewage effluent, such as in overboard dumping of faeces from boats or overflowing septic tanks from single dwellings, FRNA bacteriophage populations may not be detectable. In such situations, direct monitoring of NLVs by PCR may be the only option.

5.3. Molecular epidemiology

The early studies demonstrating the great variability of NLVs soon led to the notion that it was important to be able to distinguish between strains in order to better understand the epidemiology of NLVs. Typically, variant viruses would be characterised by neutralisation assays using hyperimmune sera or

panels of monoclonal antibodies in a tissue culture infectivity assay. However, because no one has succeeded in culturing these viruses *in vitro*, their antigenic relationships have been evaluated primarily by cross-challenge studies and immune electron microscopy (IEM) or solid phase IEM (SPIEM) with viruses purified from stool samples (Dolin *et al.*, 1972;Kapikian *et al.*, 1972; Kaplan *et al.*, 1982; Lewis, 1990; Lewis *et al.*, 1995; Wyatt *et al.*, 1974). Since this could not be done with every new variant that is identified, genome characterisation by sequence analysis has been used to provide an interim system of genotyping. As the genotypes ideally would correlate with serotypes, the sequence of the major structural protein gene was used as the basis for phylogenetic analysis. (Ando *et al.*, 2000; Koopmans *et al.*, 2000).

It is well established that many different types of NLVs co-circulate in the general population, causing sporadic cases and outbreaks (Fankhauser *et al.*, 1998; Farkas *et al.*, 2000; Greenberg *et al.*, 1979; Koopmans *et al.*, 2000; Pang *et al.*, 1999; Parks *et al.*, 1999; Schnagl *et al.*, 2000; Schreier *et al.*, 2000; Traore *et al.*, 2000). Typically strain sequences are almost identical within outbreaks, and different when specimens from different outbreaks are analysed. Thus, when identical sequences are found in different patients or different clusters of illness, a common source for the infection should be suspected (Beller *et al.*, 1997; Berg *et al.*, 2000; Brugha *et al.*, 1999; Cheesbrough *et al.*, 2000; Daniels *et al.*, 2000; Gaulin *et al.*, 1999; Green *et al.*, 1998; Hafliger *et al.*, 2000; Iversen *et al.*, 1987; Kohn *et al.*, 1995; Kukkula *et al.*, 1997; 1999; Marks *et al.*, 2000; Marshall *et al.*, 2001; Parashar *et al.*, 1998; Ponka *et al.*, 1999; Sawyer *et al.*, 1988; Sugieda *et al.*, 1996). Conversely, finding different sequences in people with a supposedly common source infection suggests independent contamination, unless there is an association with sewage-contaminated water: in epidemics due to sewage contamination often more than one strain is encountered (Gray *et al.*, 1997; Maunula *et al.*, 1999; Sugieda *et al.*, 1996). Molecular epidemiological methods have been used on many occasions to confirm (e.g., Beller *et al.*, 1997; Berg *et al.*, 2000, Brugha *et al.*, 1999; Kohn *et al.*, 1995; Kukkula *et al.*, 1997, 1999) or disprove links between outbreaks (e.g., Marshall *et al.*, 2001). Occasionally epidemics occur in which the majority of outbreaks are caused by a single genetic type (Hale *et al.*, 2000; Iritani *et al.*, 2000; Noel *et al.*, 1999, Vinjé *et al.*, 1997). These epidemics may be widespread and even global. The mechanisms behind emergence of epidemic types or fluctuations in the prevalent genotypes of NLVs are unknown. Hypotheses include large-scale foodborne transmission of a single strain and spill-over from a possibly non-human reservoir.

5.4. Use of molecular epidemiology for virus tracing

Based on the above principle a network was developed in which 12 laboratories collaborate in nine European countries to share their data on outbreaks of viral gastroenteritis in which food may be implicated (QLK1-CT-1999-00594). Starting in the winter season of 2001/2002, the groups enter epidemiological and virological information via web-based standardised forms into a central database at the co-ordinating institute (National Institute for Public Health, the Netherlands). The database can be

accessed and queried by all participants to facilitate rapid identification of potential international outbreaks.

6. METHODS FOR INACTIVATION

There is no precise information on the resistance of the NLVs to different chemical and physical methods of inactivation.

Two pieces of direct evidence are available on NLV heat stability. Norwalk virus heated to 60°C for 30 minutes remained infectious for volunteers. In the 1970s, many NLV outbreaks were linked to cockle consumption. Following laboratory based heat inactivation studies of Hepatitis A (Millard, *et al.*, 1987), the introduction of heat treatment at 90°C for 1.50 minutes of cockles harvested in the Thames estuary has prevented further NLV outbreaks. It is uncertain that Norwalk virus would be inactivated completely in many pasteurisation processes.

Chlorine-based disinfectants are thought to be effective against this group of viruses. NLVs are considered resistant to inactivation in the presence of 3.75-6.25mg chlorine/L, equivalent to free residual chlorine of 0.5-1.0 mg/L and consistent with that present in the drinking water system (Keswick *et al.,* 1985). However, the data to definitively prove this is lacking due to the lack of a simple culture system, such as tissue culture. Data from waterborne outbreaks suggest that drinking water chlorination does have an effect, since flaws in water processing are commonly detected in these incidents.

Norwalk virus is inactivated by 10 mg chlorine/L, which is the concentration used to treat a water supply after a contamination incident. It appears to be more resistant to chlorine than poliovirus or human rotavirus. It was also stable in 20% ether at 4°C for 18 hours (Dolin *et al.,* 1972).

Since NLVs cannot be cultivated, it is useful to consider inactivation data on other enteric viruses, which can be cultivated and for which a more extensive set of information is available. This is summarised in table 2.

Table 2: Food processes, virus inactivation factors and resulting risk of the product if viruses are present pre-processing.

Viruses, of which data were used to assemble this table are the (common) foodborne Hepatitis A virus (HAV), Norwalk like viruses (NLV) and Feline calicivirus (FeCV)), Human Rotavirus (HRV) or Rhesus Rotavirus (RV), and Poliovirus (PV).

Process	Example of food product	Virus inactivation (log10)	Risk to public health if viruses are present pre-processing *	Likelihood of presence Post-processing	Remarks
Thermal Treatments					
Boiling at 100 ^{o}C	Any liquid food (eg milk) or solid food boiled in water.	HAV and PV > 4	Negligible	Unlikely#	Kinetic data lacking.
60 ^{o}C, 30 min (liquids or solid foods)		HAV< 2 or HAV > 4 PV < 2 NLV: incomplete	Medium		Inactivation in solid foods lower than in liquids; dependent on fat and protein content.
Pasteurisation of solid foods (70^{o}C or equivalent, 2 min)	Pate and other cooked meats.	HAV < 2 FeCV > 3	Medium	Unlikely	Dependent on fat and protein content
Pasteurisation of liquids and immediate packing (e.g. HTST 71.7^{o}C for 15 sec)	Milk, Ice cream. .	HAV < 2	Medium	Possible	Dependent on fat and protein content
UHT & aseptic filling (> 120 ^{o}C)	Long life milk, other dairy products.		Negligible	Unlikely	
Other physical/chemical/biological processes					
Commercial drying (spray & freeze drying)	Dried milk, instant dried soups, dessert mixes, chocolate	HAV, FeCV<1	High	Unlikely	Insufficient data
Fermentation	Cheese, yoghurt	No information		Unlikely; is unlikely	Microbial inactivation of viruses is found for sludge

Acidification	Fruit juices, still fruit drinks.	NLV: pH 2.7, 3h incomplete HAV: pH 1, 5h incomplete	Medium	Possible	No quantitative data
Homogenization		Incomplete	High		
Rinsing of oysters and mussels		NLV Incomplete	High	Likely	
High hydrostatic pressure (600 MPa, 1h)		PV < 1	High		

Process	**Example of food product**	**Virus inactivation (log10)**	**Risk to public health if viruses are present pre-processing** *	**Likelihood of presence Preprocessing**	**Remarks**
Virus inactivation in water				Possible (drinking water) Likely (surface water	
Chlorination (0.5 mg free chlorine/litre, 1 min)		HAV > 3, HAV < 2 HRV < 2, PV > 3	Variable		Risk is low for PV but medium for HRV and HAV.
UV radiation (20 mJ/cm^2)		PV ≤ 3 HRV < 3	Low		
Ozone treatment (0.2 mg/l, 10 min)		HAV >3 PV ≤ 2 HRV < 1	Variable		Risk is low for HAV but medium/high for PV and HRV
Cleaning of equipment and surfaces					
Rinsing with (lots of) water		HAV < 2	Medium/low		
Ethanol (70%, 10 min)		HAV < 2 HRV < 3	Medium		
Chlorhexidine digluconate (0.05%, 10 min)		HAV < 1 HRV < 1	High		
Sodium hypochlorite (0.125%, 10 min)		HAV < 3 HRV < 3	Low		
Sodium chlorite (30%, 10 min)		HAV > 3 HRV > 5	Negligible		

Process	Example of food product	Virus inactivation (log10)	Risk to public health if viruses are present pre-processing *	Likelihood of presence Preprocessing	Remarks
Virus inactivation in water				Possible (drinking water) Likely (surface water	
Catering					
Washing, rinsing (where water > 1% of food) and the food is eaten without further cooking	Washed salads	No inactivation	High	Possible	Any removal of viruses will be by mechanical action only. It is very difficult to remove any microorganisms from foods by washing alone
Freezing of drinking water to prepare ice	Ice for drinks or for cold foods.	No inactivation	High	Possible	Freezing is an excellent way to preserve viruses. It is therefore best to assume there will be no inactivation after one freeze/thaw cycle.
Chilling of drinking water or use of mains water from tap without any treatment		No inactivation	High	Possible	Chilling will slow down the inactivation rate of viruses.

\# ***unlikely*****:** no reports are known in which NLV, HAV, RV or PV were found on the food items mentioned**.**
Possible**:** Sporadic contamination with NLV, HAV, RV or PV of the food items mentioned is reported**.**
Likely**:** Contamination with NLV, HAV, RV or PV of the food items mentioned is reported frequently.

****Negligible risk:*** product highly unlikely to contain viable viruses; treatment results in at least 4 $\log_{10}$ inactivation of common foodborne viruses.
Low risk: product unlikely to contain viable viruses in numbers likely to cause disease in healthy individuals: treatment results in approximately 3 $\log_{10}$ inactivation of common foodborne viruses.
Medium risk: product may contain viable viruses in numbers which may cause disease; treatment results in approximately 2 $\log_{10}$ inactivation of common foodborne viruses.
High risk: products where the level of viruses is likely to be high enough to cause disease in healthy individuals: treatment results in less than 1 $\log_{10}$ inactivation of common foodborne viruses.
Variable risk: treatment results in significant differences in inactivation of several common foodborne viruses

7. NLVs IN RELATION TO SPECIFIC PRODUCTION PRACTICES

7.1. Seafood

7.1.1. Introduction

Viruses can contaminate seafood either through contamination at source, principally through sewage pollution of the sea, or during seafood processing through inadequate hygiene practices of operatives or systems. This section deals with seafood contamination through pollution of the marine environment. Contamination during food processing or handling shares common features with contamination of other foodstuffs and is covered in section 7.2.

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 discharges constantly release human viruses into the marine environment. Following shedding into the environment, viruses can survive for weeks to months (Callahan *et al.*, 1995; Gantzer *et al.*, 1998; Nasser, 1994) 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 seafood species, only the filter-feeding bivalve molluscan shellfish have repeatedly proven to be an effective vehicle for the transmission of pathogenic viruses. Finfish and non-filter feeding shellfish (e.g., crustaceae) have not been associated with viral food poisoning following contamination at source.

7.1.2. 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.). Indigenous species such as cockles, mussels and the native oyster continue to be harvested from natural populations, although the characteristics of bivalve molluscs also make them suitable for cultivation. Nowadays, the cultivation of indigenous

species is supplemented by breeding and farming introduced species such as Pacific oysters and Manila clams.

Bivalve mollusc production makes a significant contribution to the European economy. Estimated production values for aquaculture species within the EU during 1997 were 270 million Euro for mussels, 161 million Euro for oysters and 149 million Euro for clams (MacAlister *et al.*, 1999).

Bivalve molluscs feed by filtering small particles such as algae from the surrounding water. Many commercial species are common in in-shore 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 sometimes contaminated with human sewage. In the process of filter-feeding, bivalve molluscs may concentrate and retain human pathogens derived from such sewage contamination. Bivalve molluscs can also accumulate naturally occurring toxic algae and pathogenic bacteria through filter-feeding activity. The hazards posed by bioaccumulation of harmful microorganisms are compounded by the traditional consumption of certain shellfish species (such as oysters) raw or only lightly cooked (mussels and clams), 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. The predominant association of oysters, mussels, cockles and clams with infectious disease incidents (Lees 2000, Jaykus *et al.*, 1994) 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 for viruses as do oysters, mussels, cockles and clams.

Viral food poisoning (gastroenteritis and hepatitis principally caused by NLVs and hepatitis A virus respectively) following consumption of sewage-polluted bivalve molluscs is very extensively documented in the scientific literature (see section 4.2.3 and Lees, 2000). In the majority of historical reports, the shellfish have been both produced and consumed within the same country. Investigation of disease outbreaks and, where appropriate, remedial control measures for production practices, have therefore been within the responsibility of the authorities of that state. However, during recent years the globalisation of food trade has altered this picture. Shellfish, like other food commodities, are now widely traded across the globe raising the potential for trans-national disease outbreaks. In addition to the direct effects on consumers, such outbreaks may be particularly significant with regard to providing an efficient vehicle for the wide scale dissemination of new emergent viral strains and for the importation of non-indigenous viruses (such as hepatitis E virus) into susceptible populations.

During recent years a number of examples of trans-national outbreaks have been reported following trade both between EU Member States (Christensen et al., 1998) and following importation of shellfish into the EU (Bosch *et al.*, 2001; Sánchez *et al.*, 2001). Such outbreaks raise new difficulties for authorities relating to both investigation of outbreaks and application of appropriate control measures.

7.2. Other food categories

7.2.1. Introduction

Epidemiological data have linked many types of food to outbreaks of NLV infections, including salad, sandwiches, cake icing, cold meats and hamburgers (Riordan, 1991; CDC, 2000). However, the burden of illness such outbreaks constitute is unknown. NLVs are heat sensitive and therefore the foods that have been implicated have largely been those that are consumed without heat treatment (e.g. fresh produce) or are not cooked after handling (e.g. deli foods). In published reports, foodborne outbreaks of NLV infections are most frequently associated with contamination by an infected food-handler (Dedman, 1996). The key role of food-handlers in these outbreaks is demonstrated by the fact that in all of the published outbreaks food-handlers either admitted to suffering an illness with typical symptoms prior to the outbreak or had laboratory evidence of NLV infection.

Foods become contaminated either directly by an infected person (food-handlers, cooks, produce pickers/harvesters) or though sewage pollution (runoff, use of contaminated irrigation or washing water, use of unclean water in food preparation or for cleaning dishes). Alternative methods of contaminating food have been suggested including gross environmental contamination following vomiting (Reid *et al.,* 1988). Although not covered in this report, drinking water contaminated with NLVs is an important source for foodborne NLV infections, both as sporadic disease and as outbreaks.

Surveillance, investigation and control of outbreaks is the responsibility of the national competent authorities and there is no overall EU responsibility. Several countries have published guidelines for managing food related outbreaks (CDC, 2000; CDR, 2000) and the key features are reviewed in section 8.3 of this report.

7.2.2. Fresh produce

Occurrence of viruses on fresh produce causing gastrointestinal disorders is well-documented (Beuchat, 1998; ICMSF, 1998), and fresh produce therefore represent a potential source for transmission of NLVs to the consumers. Transmission is mainly caused through surface contamination and no data confirm a possible route through damaged plant tissues. However, Oron *et al.* (1995) have reported the infiltration of polioviruses through the roots of plants. The low infectious dose of NLVs, less than 100 particles (Kapikian *et al.*, 1996), increases the risk associated with consumption of produce subjected to viral contamination. Even low contamination levels can result in substantial outbreaks (CDC, 2001). Epidemiological data linking viral gastroenteritis to fresh produce are, however, limited. In a recent paper by Seymour and Appleton (2001) they refer to data from Wales and England (Public Health Laboratory Service [PHLS]) showing that fruits and vegetables accounted for 4.3% of all foodborne outbreaks between 1992 and 1999. A total of 20% of the outbreaks were caused by NLVs and for 42% of the outbreaks the etiological agent was unknown. However, a majority (64%) of these outbreaks caused

by an unknown agent was suggested to be viral. Products like lettuce, tomatoes, raspberries and strawberries have been involved as carriers of viruses (ICMSF, 1998).

NLVs share with hepatitis A viruses a faecal-oral route of transmission. Fresh produce-associated viral gastroenteritis appears to have increased (Bean and Griffin, 1990). This observation may be a result of the recent development in molecular biological methods for detection of these viruses in clinical specimens (Svensson, 2000). In Finland, frozen raspberries imported from East European countries were epidemiologically associated with an outbreak of NLV infection (Pönkä *et al.*, 1999). The hypothesis was that the raspberries were irrigated with polluted water and the virus survived in the ice surface covering the berries. The occurrence of caliciviruses in European river water has been documented (Gilgen *et al.*, 1997) and viruses survive well in ice (Kapikian *et al.*, 1996).

Infected food-handlers are often expected to be the source of infection for fresh produce (Griffin *et al.*, 1982; Gross, 1989 and Beuchat, 1998). However, contamination can also be the result of pre-harvest and post-harvest practices. Production practices lay the basis for contamination of fresh produce with viruses from the human reservoir (Beuchat, 1998; ICMSF, 1998; Warner, 1991). The major sources of pre-harvest contamination are faecal polluted irrigation water and improperly composed manure and municipal sewage sludge and other bio-solids containing human faecal material used for fertilisation. It is known, that river water may contain a multitude of human enteric viruses (Gilgen *et al.*, 1997). Post-harvest practices that can represent a risk for contamination of products with NLVs include the use of polluted water for washing or ice made from polluted water for cooling. The hygienic practices by the workers throughout the production chain also represent an important risk factor.

Irrigation, washing, and use of polluted water as a solvent for plant protection products may contaminate the produce with pathogenic viruses. Different viruses may persist in vegetables for weeks or months (Larkin *et al.*, 1978). The risk related to introduction of hydroponic farming has not been assessed. By this technology sewage water can be used as a nutrient flowing around the plants in a solid layer.

The use of manure as a source of plant nutrition has a long tradition in farming. In the modern society, municipal wastes have been included in material used as an organic fertiliser. Recycling of municipal sewage and other forms of faecal material is important for the future sustainable society. The requested increase in organic farming will also depend on this source of plant nutrition. The risk associated with this trend is not well-documented. The potential contamination of domestic wastes with human faecal viruses reduces the safe use of this material as a fertiliser. Large number of virus particles can be excreted from infected persons, 106- 1011 units per gram faeces have been estimated (Feachem *et al.*, 1983). This high concentration has to be related to the low infectious dose of 10-100 particles (Kapikian *et al.*, 1996)

The ACMSF report (1998) also mentions the risk exerted by imported fresh produce. Today, various fresh produce from around the world has become available year round throughout the EU, which underlines the need for good hygienic standards for food in the global international trade.

8. PREVENTIVE MEASURES

8.1. Seafood

8.1.1. Seafood other than bivalve molluscan shellfish

Within the European Union, legislative requirements for the hygienic production and marketing of seafood (other than live bivalve molluscs) is contained in Council Directive 91/493/EEC. These measures principally relate to hygiene and quality standards for fishing vehicles and for landing and processing premises. The Directive sets bacteriological standards. However, these relate to food spoilage, food processing and food handler hygiene issues rather than to sewage contamination at source in the harvesting area. This is appropriate given that seafood species other than bivalve molluscs have not been associated with foodborne disease traceable to sewage contamination of the primary product. Council Directive 91/493/EEC does, however, also cover processed (dead) bivalve molluscs termed 'fishery products'. The processing of such bivalves is a critical control point and subject to specific requirements set out in Directive 91/493/EEC and in subsequent Council Decisions (e.g., 93/25/EEC). It is important to note that for such processed bivalves, all of the requirements of Council Directive 91/492/EEC (live bivalve molluscs – see below) apply up until the point of processing.

8.1.2. Bivalve molluscan shellfish

8.1.2.1.Background

The infectious disease hazards associated with consumption of bivalve molluscs have been recognised for many years. Consequently, most countries have enacted sanitary controls on the production of live bivalve molluscan shellfish. In the EU, these are covered by Council Directive 91/492/EEC (Anon, 1991a) and 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 harvesting area classification, bivalve transport, wet storage, depuration, relaying, analytical methods, movement and marketing documentation, and provisions for suspension of harvesting from classified areas following a pollution or public health emergency. The legislation requires that third country imports into the EU and US have to be produced to the same standard as domestic products. 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 100g of shellfish flesh, can be placed on the market for human consumption. This standard, together with standards for specific pathogens (such as *Salmonella* spp.), chemicals and algal biotoxins, has been adopted as an 'end-product' standard in Council Directive 9l/492/EEC. Shellfish meeting this standard, either directly from the harvesting area or following some form of processing (see below), can go for direct human consumption. It should be noted that viral standards are not currently set in either EU or US legislation. However, Council Directive 91/492/EEC refers directly to the problem of viral contamination in shellfish and the need to introduce standards when such techniques become available.

Sections 8.1.2.2 to 8.1.2.6 describe in detail the operation of the various production controls across the EU, their effectiveness with regard to viral contamination, and the level of consumer protection afforded. Section 8.1.2.7 considers issues relating to more effective control of sewage pollution of shellfish harvesting areas.

8.1.2.2. Microbiological monitoring and classification of shellfish harvesting areas

Both EU and US 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 US legislative standards for bivalve shellfish is set out in table 3. In the EU, faecal indicators are measured in shellfish flesh, whereas in the US, indicators are measured in the shellfish growing waters. Both the EU and the US systems base standards on a 5-tube 3-dilution most probable number (MPN) test.

Table 3 Synopsis of legislative standards for bivalve molluscan shellfish in the EU and the US.

Shellfish treatment required	US FDA classification	Microbiological standard per 100ml water (US FDA)	EU Classification	Microbiological standard per 100g shellfish (EU)
non required	Approved	GM < 14 FCs and 90% < 43 FCs	Category A	all samples <230 *E. coli* or all samples <300 FCs
purification 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

Abbreviations:

FCs = faecal coliforms, GM = geometric mean, 90% =90%-ile compliance

US 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. Shellfish exceeding these levels of contamination (EU ‘category B’ and US FDA ‘restricted’ classifications) may only be placed on the market following commercial depuration or relaying. 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 may also be treated by heat processing using an approved method (see below). In EU legislation, shellfish from harvesting areas exceeding the limits of category B (category C classification) may only be placed on the market following protracted relaying (a minimum period of two months is specified) or following commercial heat treatment by an approved method. Shellfish growing areas exceeding these prescribed levels of contamination, or areas for which harvesting area survey and classification has not been conducted, cannot be commercially harvested for human consumption in either US or EU legislation.

There is no European wide source of data relating to Member State classification of shellfish harvesting areas according to Council Directive 91/492/EEC. However, informal sources suggest quite large differences in percentage compliance with the different classification categories. In the UK (England and Wales) in the annual review of classification released in July 2001, 4% of areas were class A, 72% class B, 21% class C, and 3% prohibited. In France, 37.2% were class A, 48.4% class B, and 14.4% class C in 2001. By contrast, in the Netherlands all harvesting areas are designated as class A, but may occasionally be downgraded for short periods following poor monitoring results.

8.1.2.3. Monitoring of shellfish harvesting areas in relation to protection against enteric virus contamination

Given the inability of faecal coliforms in processed shellfish (currently the only available quality assurance measure) to predict viral hazard (see sections 8.1.2.4 – 8.1.2.6), the classification of shellfish harvesting areas provides the first, and in some cases (class A waters) the only, level of protection against virus contamination. It is therefore a fundamentally important measure for consumer protection. This section considers the degree of consumer protection against viral contamination afforded by faecal indicator monitoring and classification of shellfish harvesting areas (as required by Council Directive 91/492/EEC) - and how it could be improved.

It is firstly important to consider whether faecal indicator monitoring correlates at all with enteric viral contamination. During recent years, methods have become available for detection of NLV contamination of shellfish (see section 5.1) and a few studies have compared NLV occurrence with faecal coliform / *E. coli* levels. A three year study in France showed that in a category B site, NLVs were detected in 25 samples (23%), among which three were found contaminated by faecal coliforms. In a prohibited area, NLVs were detected in 20 samples (32%), among these 12 were also found contaminated with faecal coliforms (Le Guyader *et al.,* 2000). A comparable two year study was performed on six harvesting areas in the UK with pollution levels ranging from category A to prohibited. Like the French study, the results showed that NLVs could be frequently detected in harvesting areas judged suitable for commercialisation by faecal coliform criteria (category B). The study also demonstrated that the likelihood of NLV contamination correlated well with the extent of harvesting area contamination as judged by average levels of either *E. coli* (Pearson correlation coefficient 0.85) or FRNA bacteriophage (Pearson correlation coefficient 0.96) (Henshilwood and Lees, in preparation). These studies demonstrate that NLVs can be detected in commercially exploitable categories of shellfish harvesting area, and that the higher the average levels of *E. coli* at a site, the more likely it was to be so contaminated. It follows therefore that the classification of harvesting areas, whilst not preventing virally contaminated shellfish reaching the consumer, is providing some protection against the more frequent episodes of viral contamination found in more polluted areas. Given these findings, it is important that harvesting areas are classified using an approach that maximises consumer protection, in terms of protection from enteric virus contamination, such as envisaged by Council Directive 91/492/EEC.

Faecal indicator methods

Methods for analysis of *E. coli* and faecal coliforms in foods are quite diverse and include MPN assays of various types, various direct plating methods, spiral plater, and conductance/impedance methods. Some of these are available in a commercial format. For shellfish analysis it is important that the chosen test method is validated for the shellfish matrix since it is known that this may interfere with some assays. It is also important to consider the level of sensitivity required by Council Directive 91/492/EEC standards since many methods for foods are not capable of delivering sufficient sensitivity. An additional important consideration is that, unlike other foods, *E. coli*/faecal coliforms in shellfish have been exposed to the marine environment and may well be damaged or stressed. Direct

inoculation onto a relatively harsh selective media (such as MacConkey agar) is likely to 'kill off' such damaged cells and considerably reduce assay sensitivity. For these reasons, Council Directive 91/492/EEC requires *E. coli* or faecal coliforms in shellfish harvesting areas to be assayed by a 5-tube 3-dilution MPN test. It stipulates that the MPN assay, or one of equivalent accuracy, be used for monitoring processed shellfish ready for consumption. The MPN format requires initial inoculation into a liquid broth, which is likely to allow better recovery of stressed organisms than a solid agar. These are important requirements since some alternatives to the MPN assay are known to perform poorly for determination of *E. coli* or faecal coliforms in shellfish (Ogden *et al.,* 1998). Where laboratories have adopted other than the MPN approach, it is generally because of a reluctance to adopt the rather more laborious and time consuming MPN format. Examples include the use of direct plating onto prepared solid agar containing bile salts (e.g. MacConkey agar) or various brands of chromogenic media (e.g. Chromocult, TBGA, Petrifilm). Such methods do not comply with the requirements of Council Directive 91/492 and are unlikely to be suitable for shellfish testing. Unfortunately, even within the MPN format, some test methodologies are not optimised for the shellfish situation and may be unsuitable for sensitive detection of *E. coli* / faecal coliforms from shellfish (Araujo *et al.,* 1995).

It is clear that the method used for determination of *E. coli* / faecal coliforms in shellfish may influence the result and hence potentially the classification of the harvesting area. Given the importance of this for consumer protection, it is worth improving the nature and quality assurance of such faecal indicator tests. Critical to the accuracy of the results is the compliance of testing laboratories with the method requirements set out in Council Directive 91/492/EEC. Given modern developments in *E. coli* detection (such as the use of ß-glucuronidase) it is no longer technically necessary to set standards for both faecal coliforms and *E. coli*. Since the use of both indicators causes significant complications with regard to standardisation and harmonisation of methods, it seems advisable that such testing should be limited to *E. coli* using an internationally accepted method in accredited laboratory. This would also be in line with international developments in other foodstuffs.

Approaches to monitoring harvesting areas

The US legislation supplements the specified faecal indicator standards (see 8.1.2.2.) with various other detailed requirements including: growing area sanitary survey (including identification of pollution sources) prior to

classification and updated periodically thereafter; establishment of a prohibited zone adjacent to each sewage treatment plant outfall, the requirement to draw up maps showing the boundaries and classifications of each growing area; the stipulation that a minimum number of samples from each station shall be used to assess compliance (15 for approved areas), and the requirement to site sampling stations next to actual or potential sources of pollution and to time sampling to reflect adverse pollution conditions. EU Council Directive 91/492/EEC does not contain equivalent details relating to harvesting area classification. Consequently, a variety of different implementation protocols and approaches for interpretation of faecal coliform/*E. coli* monitoring data have been introduced. Because faecal coliform levels in shellfish harvesting areas are subject to wide variability (Beliaeff and Cochard, 1995), some classification approaches evaluate data trends over time whereas others base the classification on the results currently in hand. With regard to viral contamination, long virus survival times as compared to faecal coliforms/*E. coli* make an approach based on historical data trends likely to provide more consumer protection than an approach giving undue emphasis to the faecal coliform results currently in hand.

The implementation of Council Directive requirements by Member States and importing third countries are inspected by the European Food and Veterinary Office (FVO) (http://europa.eu.int/comm/food/fs/inspections/vi/reports).

Current approaches rely almost exclusively on faecal indicator monitoring against set microbiological standards. However, this may not provide the most optimum level of protection. In 1998, the World Health Organisation (WHO) in collaboration with the US Environment Protection Agency sponsored an expert consultation on possible new approaches to health based monitoring of recreational (bathing) waters. The result has been termed the 'Annapolis Protocol' and is available on the WHO website. This new approach proposes a more risk based holistic approach to assessment and management of risk in bathing and other recreational waters. The practical implications of this approach are currently being assessed by the European Commission for possible use in the bathing beach context in Europe. This approach might also be beneficial in the context of shellfish harvesting area monitoring and management and deserves further evaluation.

Classification standards in Directive 91/492/EEC

Observations that NLV occurrence in shellfish harvested from more contaminated areas correlates well with faecal indicator titre raises the issue of whether the *E. coli*/faecal coliform standards set out in Council Directive 91/492/EEC are stringent enough to protect against viral contamination. It can be argued that strengthening the permitted standards and a harmonised and comparable approach would provide further protection for the shellfish consumer.

An alternative is to apply risk based standards with different standards for shellfish eaten raw (such as oysters) and those normally cooked (such as mussels and cockles).

Potential for introduction of viral monitoring

Methods to detect NLVs and other enteric viruses in shellfish are now available. Also, workers have proposed various alternative 'viral' faecal pollution indicators such as FRNA bacteriophage. Could these methods now be used to improve monitoring of shellfish harvesting areas for enhanced consumer protection against enteric virus contamination? It is important to note that developments thus far have been restricted to the research field. Few, if any, countries have yet adopted viral monitoring in a formal statutory context although some countries have begun to use these techniques for investigation of outbreaks and for investigating viral contamination of harvesting areas associated with outbreaks. Experiences have been mixed, but some issues are immediately apparent. PCR based procedures for detection of NLVs in shellfish are technically complex and currently ill-suited to routine food control laboratories. Currently, no standard internationally accepted methods (such as ISO) exist for shellfish extraction or NLV PCR. Before non-specialist laboratories can apply these methods, further work is required on method standardisation, the generation of more widely available NLV control reagents, internal and external quality assurance procedures, and method interpretation and confirmation. It will also be important to reduce test costs. These issues currently limit the application of these methods for routine monitoring purposes such as end-product testing. However, the methods have proven useful in specialist laboratories for monitoring problematical or 'at risk' harvesting areas and outbreaks investigations.

The application of possible alternative indicators is also currently mainly restricted to the research field. However here the technical difficulties are less significant. ISO methods exist for alternative indicators, such as FRNA bacteriophage, and the methods are well proven and not expensive. Issues to

be considered are correlation between alternative indicators and pathogen, the merits of the various candidate indicator organisms, the selection of appropriate methods, performance in practice, quality assurance approaches, and numerical standards that might be applied. Currently, little has been published on this topic.

The broader uptake of either alternative indicators and/or PCR based approaches for shellfish monitoring within the EU would benefit from a wider technical discussion and further trials in official control laboratories. However, this will require research and development activities as well.

8.1.2.4.Heat treatment (cooking)

Shellfish from EU class B and C areas may be heat processed (cooked) by an approved method prior to sale. 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 were undercooking shellfish 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 log10 infectious units by raising the internal temperature of shellfish (cockle) meats to 85-90°C for 1 min (Millard *et al*., 1987). A 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 that these improvements were effective. Council Directive 91/493/EEC requires that commercial heat treatment methods be approved. Several commercial heat treatment processes 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). Since NLVs cannot be

cultivated, heat inactivation data for these viruses are not available. However, similar studies carried out on feline calicivirus, a possible model for NLVs, 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 based on the heat cook parameters of raising the internal temperature of shellfish meats to 90°C for 1.5 min seems effective for inactivating NLVs.

Although modern carefully regulated commercial heat treatment processes appear to have proven effective in controlling enteric virus contamination 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 most EU countries, 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 disease statistics as a cause of outbreaks of gastroenteritis (Anon, 1993c; Anon, 1998). However, the relatively low incidence of such reporting in relation to species commonly eaten raw, such as oysters, suggests that home or restaurant cooking may be having at least some protective effect. It seems likely therefore that viral problems associated with home and restaurant cooked mussels are a consequence of under or inconsistent cooking. Investigation into an epidemic of hepatitis A in China associated with clam consumption showed that attack rates were highest in those who ate raw clams (18%), but also higher among those who ate cooked clams (7%) than among those who did not eat clams (2%) (Wang and *et al*., 1990). Similarly, a recent study in the US following a multi-state outbreak of NLV infection 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 NLVs 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. The inability of home or restaurant cooking to provide adequate guarantees of consumer protection against viral contamination for bivalve shellfish emphasises the reliance on harvesting area controls and, for category B areas, depuration.

8.1.2.5.Purification (depuration)

Shellfish harvested from class B areas, which are intended for sale live, must be purified. 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 US (Otwell *et al.*, 1991). Depuration periods may vary from 1 to 7 days, with around 2 days being probably the most widely used period. Minimum time periods for depuration are not stipulated in Council Directive 91/492/EEC. Depuration systems also vary and include processes where water is static or changed in batches, through to systems where seawater is flushed through continuously or recycled through a sterilizer. Water sterilisation processes include ozone, chlorination, UV irradiation, and iodophores (Roderick and Schneider, 1994; Otwell *et al.*, 1991; Poggi and Le Gall, 1995). Depuration has been applied to most bivalve molluscan shellfish species sold live, including oysters, clams, mussels, and cockles.

Council Directive 91/492/EEC, 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. 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 fail to 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; Chalmers and McMillan, 1995; Ang, 1998; Perrett and Kudesia, 1995; Heller *et al.*, 1986) and the US (Richards, 1985). This strongly suggests 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 bacterial infections following shellfish consumption (Lees, 2000).

These epidemiological observations are 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; Sobsey and Jaykus, 1991; Jaykus *et al.*, 1994; Power and Collins, 1989; Dore and Lees, 1995; Power and Collins, 1990). Although elimination rates in individual studies vary significantly, the overwhelming finding from these studies is that viruses are eliminated from bivalve molluscan shellfish at a slower rate than faecal coliforms. These findings are confirmed by recent laboratory seeding studies using NLVs detected by PCR. NLVs were found to efficiently accumulate in shellfish (oysters and clams). However, they were only poorly removed by depuration compared to *E. coli* (Schwab *et al.*, 1998). Other studies have evaluated virus elimination in commercially depurated shellfish (oysters) as judged by both NLVs and male-specific RNA (FRNA) bacteriophage (a potential viral indicator) content (Dore *et al.*, 2000). Processed shellfish were found to be routinely compliant with faecal coliform end-product standards. However, significant numbers were contaminated with both NLVs and FRNA 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. A further finding was that virus contamination, as judged by both NLVs and FRNA 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 temperature (Dore *et al.*, 1998; Power and Collins, 1990; Jaykus *et al.*, 1994) 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.

It is probably the case that many aspects of depuration plant design and operation, optimised to ensure faecal coliform removal, require re-evaluation in the light of viral contamination. These included minimum depuration times, minimum depuration temperatures, the definition of ‘clean seawater’, and the over emphasis on compliance with faecal bacteria standards as a measure of success. It is important to note that the available evidence strongly suggests that reliance on faecal coliform end-product standard criteria alone for judging the acceptance of depuration systems and operating criteria, or depurated shellfish products, does not adequately protect the

consumer from virus contamination. Unfortunately, in some cases undue reliance seems to have been placed on compliance with faecal coliform criteria for both design and operation of shellfish depuration systems.

8.1.2.6.Relaying

Relaying involves the transfer of harvested animals to cleaner estuaries or inlets for self-purification in the natural environment. Shellfish harvested from class C areas, which are intended for sale live, can only be placed on the market following extended two months relaying. This process can also be used as an alternative to depuration for class B shellfish.

Relatively little information exists on the removal of viruses during shellfish relaying although factors such as seawater temperature and initial contamination levels appear critical (Cook and Ellender, 1986; Jaykus *et al*., 1994). Studies on NLVs and FRNA bacteriophage (Dore *et al*., 1998 and unpublished data) suggest that removal of viruses from heavily contaminated shellfish by a combination of relaying for 4-6 weeks followed by depuration can be effective, but is critically dependent 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). Although Council Directive 91/492 requires that minimum relaying seawater temperatures be set, it is not clear that this is always followed in practice. Probably this requirement would be more effective if guidance was available on appropriate minimum seawater temperatures for removal of virus during relaying.

8.1.2.7.Control of sewage pollution in shellfish harvesting areas

It is widely accepted that the most effective way to tackle shellfish transmitted viral disease is to prevent or reduce sewage pollution of shellfish harvesting areas. However, within Europe growing coastal populations and the high investment cost of sewage treatment processes have, in some cases, proven difficult obstacles to overcome. In some countries, such as in the UK, France, and Ireland, the shellfish industry is widely dispersed, whereas in others, such as the Netherlands, it is concentrated into a few geographical areas. 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 in some cases hampered investment. However, in recent years EU environmental quality legislation has become a major factor effecting expenditure on sewerage 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, Council 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 of Council Directive 91/492/EEC. In many EU countries, this has become an important driver for maintenance or improvement of water quality in molluscan shellfisheries. Council Directive 79/923/EEC remains the only piece of European legislation offering direct protection to the quality of shellfish harvesting areas. Its importance therefore in contributing to the sanitary quality of shellfish consumed by man should not be underestimated.

A particular issue is that Council Directive 79/923/EEC relies 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. For example Council Directive 79/923/EEC requires rather infrequent monitoring programs, which provide little protection 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 been reported on many occasions demonstrating the importance of rainfall-associated contamination events (Murphy, 1979, Grohmann *et al.*, 1981, Morse *et al.*, 1986, Truman *et al.*, 1987, Bird and Kraa, 1995). To maximise public health gains from expenditure on sewage infrastructure, it is therefore important 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 (such as set out in Council Directive 79/923/EEC) at a set monitoring point is unlikely to yield the optimum protection from viral contamination for vulnerable bivalve shellfisheries.

8.2. Other food categories

8.2.1. Food other than fresh produce

Food can become contaminated with NLVs either directly by an infected person or through sewage pollution. In order to prevent direct contamination of foods, it is important that everybody involved in food handling throughout the food chain from farm to table has knowledge about the importance of good hygienic practices and also behave accordingly. Especially people handling foods that will be consumed without further heat treatment must acknowledge the risk that they may represent to other people if good personal or kitchen hygienic practices are compromised. Commercial food establishments including restaurant, as well as kitchens in institutions, should have quality systems that also address hygienic practices. Education and information is a key issue in this regard.

The importance of good hygienic practices as a preventive measures for NLV infections as well as other infectious diseases, also apply to private homes. Information strategies addressing this could be promoted, and hygienic issues could be taught in public schools and other education systems.

In order to prevent indirect contamination of foods, it is of the utmost importance that the water being used in food production, either as irrigation water or as ingredient in a recipe, is of a quality such that it does not contain NLVs and in this way may jeopardise food safety.

8.2.2. Fresh produce

Fresh produce involved in foodborne viral diseases has either been contaminated by infected food-handlers or due to failing production practices. Strategies for preventing fruits and vegetables being contaminated during production have to rely on control measures taken during pre-harvest and post-harvest conditions (Cliver, 1997).

Pre-harvest measures

The most efficient way to improve the safety of fresh produce is to prevent contamination from the human faecal reservoir. This can be done by establishing effective Good Agricultural Practices (GAP), Good Hygienic Practices (GHP) or Hazard Analysis Critical Control Points (HACCP) based systems focusing especially on water quality and quality of organic fertilisers. Such practices are also of importance for preventing the

contamination of the fresh produce with bacterial pathogens from animal faeces.

The occurrence of human faecal viruses in water is well established, although the extent of such contamination is not well defined (Seymour and Appleton, 2001). The survival of viruses in water is sufficiently long to create a problem during irrigation and other purposes (Bosch, 1995). This means that water used for irrigation, washing, and for mixing plant protection products has to be of a hygienic quality that does not harm the consumer. In the draft Code on Hygienic Practice for Fresh Fruits and Vegetables on Step 8 within the Codex system, two qualities of water have been identified: potable water and clean water (Codex, 2001). WHO guidelines for Drinking Water define the hygienic quality of potable water. Clean water was defined as water that does not compromise food safety in the circumstances of its use.

Viruses in manure and sludge used as plant nutrition have to be inactivated before the material is added to growing edible plants. Composting at temperatures above 60-70°C has been shown to inactivate at least some viruses. However, the effect on NLV viruses of this treatment has not been estimated. Bagdasargan (1964) reported that different viruses could survive in soil for 150 to 170 days depending on pH, moisture content and temperature. ICMSF (1998) summarise published research that concludes that even the best treatment techniques will only reduce the virus load by 2-4 logs.

Regulations exist in many EU Member States on measures to be taken before manure and municipal sludge are used as plant nutrition. This area is, however, not harmonised within EU. The existing regulations identify the time to elapse before the material is supposed to be spread on the fields or the time in advance of harvesting that the material has to be applied. The UK regulation (ACMSF, 1998) is such an example prohibiting the application of sludge for fruit and vegetables 10 months before harvest. The Danish Legislation on the utilisation of sludge, solid waste and compost (Announcement from the Ministry of Environment and Energy, no 823, 16th September, 1996) divide the products into different categories and define the degrees of treatment of the offal and its potential use for different crops.

Post harvest reduction or inactivation of viruses

Methods for reduction of microbes on food are based on heating, drying, freezing, salting, acidifying, fermentation, washing and treatment with

various disinfectant agents. For fresh produce most of these methods are not applicable. Even though viruses are not growing on fruit and vegetables, their survival is affected by intrinsic and extrinsic conditions. Low temperatures have no retarding effect and in fact a better survival is observed compared to room temperature (Keswick *et al.*, 1985, Badaway *et al.*, 1985). Water is used in order to remove soil and other pollutants from vegetables and fruits. The use of washing may reduce the contamination of micro-organisms by 1-2 logs. However, this is not applicable for berries like strawberries and raspberries and the use of traditional decontaminants has a very limited influence on the reduction (Keswick *et al.*, 1985; Beuchat, 1998). Packaging lettuce in high content of CO_2 improves the survival of viruses. The survival was explained by the reduction in enzymatic activities on the vegetables preventing the production of toxic by-products (Bidawin *et al.*, 2001). Cliver and Kosten (1979) reported an interesting observation that substances present in fruit could cause an inactivation of viruses.

There is relatively little scientific literature available describing disinfection techniques for elimination of viruses on fresh produce, and Cliver (1997b) summarises that methods to be used for inactivating viruses on foods are relatively unreliable. However, viruses in and on exposed surfaces can be inactivated by ultraviolet light and with strong oxidising agents like chlorine or ozone. In water, 10 ppm chlorine for 30 min is requested for disinfection. Some times this level is insufficient (Keswick *et al.,* 1985). In this report, Norwalk viruses were found to be more resistant to chlorine compared to rotavirus and poliovirus.

Beuchat (1998) in his review on surface decontamination gives an overall opinion that, in addition to washing with water, it is not clearly known to what extent chemical agents are effective. He does not recommend the use of irradiation as a method for inactivation of viruses in fruits and vegetables due to the high doses required.

Seymour and Appleton in a recent review (2001) on foodborne viruses and fresh produce deal with survival of viruses in water, in soil, on surfaces and on fruits and vegetables and a part on washing and disinfectants. They conclude that fresh produce contributes to transmission of viral infections. There is a lack of information on the survival of viruses on fresh produce related to shelf life and types of packaging. Information is also lacking on the efficiency of current washing and decontamination processes for the removal of viruses.

Table 2 summarises some virus inactivation factors for food processes.

8.3. Food handling

In individual countries the management of foodborne NLV infections are the responsibility of the national competent authorities, and there is no uniformity of approach across the EU. However, there are some published guidelines (Lo *et al.,* 1976; Akin *et al.,* 1976), which employ similar approaches and the key measures are described below.

8.3.1. Criteria for suspecting an outbreak of NLV infection

There is no doubt that outbreaks associated with NLVs have a pattern, which, before any virological results are available, can lead to strong suspicions that NLVs are involved. Kaplan *et al.,* in 1982 defined the criteria listed in Table 4, which can give a strong positive prediction.

Table 4: Criteria for suspecting an outbreak is due to NLVs

1. Stool cultures negative for bacterial pathogens
2. Mean duration of illness 12-60 hours
3. Vomiting in > 50% of cases
4. Incubation period (if known) of 15-48 hours

The problems in controlling outbreaks of this type should not be under-estimated. The use of casual labour in the catering industry and the financial penalties incurred if staff are absent due to illness, make control measures difficult to enforce. This is frequently exacerbated by the fact that person-to-person transmission among the food-handlers prolongs the period of risk.

The main preventive and control measures that are employed are summarised in Table 5.

Table 5: Principles of control measures for food-borne NLV outbreaks

a) Elimination of potential reservoirs of NLVs
i) Exclusion of symptomatic food-handlers for 48 hours post-recovery ii) Destruction of potentially contaminated foods iii) Decontamination of kitchen surfaces and toilets using hot water and general purpose detergent followed by 500ppm hypochlorite iv) Segregation of shellfish to avoid cross-contamination
b) Prevention of contamination
i) Agree cleaning schedules for the kitchen and toilets ii) Ensure toilets are provided with toilet paper, soap and paper towels or functioning driers iii) Educate staff in personal hygiene and safe food-handling practices iv) Consider use of gloves

A key component of all control measures is that all food-handling staff with symptoms of gastroenteritis should be excluded from the site, irrespective of whether they have produced a specimen that is positive. Based on published incidents, it is desirable to extend this exclusion until 48 hours after recovery.

Particular care must be taken with high-risk food such as salads, cold meats and sandwiches. Following an outbreak, existing foods of this type may be destroyed since one cannot determine whether or not contamination may have occurred. In order to prevent a recurrence of the problem, an ongoing programme could be instituted. This will include cleaning schedules for the kitchen and toilets. The system must ensure that staff toilets are always supplied with soap and paper towels or functioning hand driers.

Staff education is needed to explain the chain of transmission, the importance of good personal hygiene, and the dangers of working when ill. Similarly, modification of food-handling practices to minimise hands-on contact with high-risk foods such as salads and cold meats may help to interrupt the chain of transmission.

Food examination

There are at present only research methods for detecting NLVs in food. However, early in an outbreak when the cause is not established it is desirable to submit any suspect foods for bacteriological examination.

Environmental investigations

Following a foodborne outbreak, standard procedures are adopted to try to identify any defects in food preparation or storage, which may have led to the outbreak. With NLV outbreaks factors such as cooking times, food storage temperatures etc., are of little direct relevance other than as an index of general standards in the kitchen.

The important areas to investigate are:

- evidence of illness among staff;
- instances of vomiting in kitchen environment;
- standard of toilet and hand-washing facilities available for food-handlers;
- standard of surface cleaning arrangements in the kitchen;
- presence of oysters or other bivalve molluscs;
- storage conditions for such shellfish, particularly with regard to cross-contamination.

Epidemiological investigations

Because there is no routine method currently available for testing food for NLVs, identification of the vehicle of infection depends totally on epidemiological methods, which should, where possible, include case control or cohort studies. Data are collected by questionnaires, which enquire about any illness suffered, the time and date of onset, the symptoms suffered and the food items consumed. In drawing up the questionnaire, a comprehensive list of the food and drink items served must be compiled, including such things as ice in drinks. In a cohort study all those who attended the function should be questioned whether or not they have been ill. The data provided by the questionnaires can then be used to derive food-

specific attack rates. Using statistical tests, such as the Chi square test, associations between illness and consumption of food items can be identified. In large outbreaks, very strong associations with a particular food may be established.

9. CONCLUSIONS

Epidemiology

- NLVs are a diverse group of highly infectious, poorly immunogenic, stable viruses that, although underreported, are one of the most commonly recognised causes of gastroenteritis in humans in the EU affecting people of all age groups.

- NLV infections occur both as sporadic cases and as outbreaks, including common-source international outbreaks.

- NLVs typically spread by a faecal-oral route. While person-to-person transmission is the most common mode of spreading NLVs, food- and water-borne outbreaks frequently occur. Sources of food-related illness are grouped into three categories:

  - Sewage-contaminated bivalve molluscs

  - Fresh produce for which contamination can occur at several stages throughout the food chain

  - Infected food-handlers

- There is a lack of data in Europe on the actual exposure to NLVs through foods as prevalence data are scarce and consumption data very limited.

- The potential for zoonotic transmission of NLVs is currently under investigation.

Methods

- Progress has been achieved in regard to methodology for detecting NLVs in bivalve molluscs, water and environmental samples, although improvement and standardisation of methods are needed. Further work is needed to make such new techniques applicable in a routine food control context. For foodstuffs other than water and bivalve molluscs, there are no generally applicable methods. Each foodstuff requires an individual approach.

- Progress has been achieved in regard to clinical diagnostics, which may aid epidemiological investigations.

- There is a need for test methodologies for NLVs as conventional faecal indicators are unreliable for demonstrating presence / absence of NLVs e.g., NLVs may be detected in shellfish in the absence of *E. coli* / faecal coliform.

- Progress has also been made in the development of alternative indicators (e.g., bacteriophages) that might more reliably indicate the presence of enteric viruses than do *E. coli* and faecal coliforms.

Preventive measures

- Surveillance of foodborne disease in humans is essential for the early detection of foodborne outbreaks, including those which may not immediately present as a common-source outbreak. Internationally harmonised methods for detection and typing of viral outbreaks and better exchange of data would improve this.

- As foodborne NLV infections are a result of human faecal contamination at some point in the food chain, controlling this contamination should be an important strategy for improving food safety. Contamination by food handling represents a specific problem relating to various food categories and various stages of the farm-to-table continuum, and needs to be addressed.

- Specific measures to be taken for fresh produce include the use of irrigation water and organic fertilisers not contaminated with human faecal material, as well as the use of uncontaminated potable water for washing and cooling (ice). Washing of fresh produce is not applicable for all products and seems to have a limited reducing effect on the number of virus particles. The potential of chemical decontaminants like chlorine, ozone and organic acids for reduction of NLVs on fresh produce have yet to be established.

- Shellfish purification cannot be completely relied upon to remove enteric viruses from shellfish.

- The reliance on faecal indicator removal for determining shellfish purification times is an unsafe practice.

- Commercial cooking of shellfish by an approved method appears to be an effective control measures for NLVs.

- Few applied studies have been performed with regard to behaviour of NLVs in foods and the effect of different control strategies.

- Health protection for consumers of bivalve molluscs is dependent on the protection of the shellfish harvesting areas from sewage pollution.

10. RECOMMENDATIONS

- To include NLVs under the EU communicable diseases surveillance network.

- To develop comparable methods for virus detection and outbreak investigations.

- To develop a European database of NLV strains to serve as a reference facility and to facilitate the identification of emergent strains including novel recombinant viruses.

- To support further work on NLV detection methods and NLV indicators.

- To assure the implementation of safe food handling practices throughout the food chain (GHP, GAP, HACCP).

- To assure the implementation of safe production practices for fresh produce including the exclusion of contaminated water for irrigation, washing and cooling (ice) and the exclusion of organic fertiliser contaminated with NLVs.

- To establish best practice guidelines relating to the microbiological monitoring and classification of shellfish harvesting areas.

- To consider the introduction of more risk based standards for shellfish harvesting areas applying, for example, more stringent standards to bivalves eaten raw than for shellfish that are to be cooked.

- To define actions to be taken to assure that NLVs are not further spread from a shellfish harvesting area after it has been linked to an outbreak.

- To introduce proactive quality management arrangements for shellfisheries based on environmental factors such as rainfall or pollution events.

- When applying bacterial indicators, to use *E. coli* rather than faecal coliforms of faecal contamination in shellfish harvesting areas.

- To establish optimal purification times for bivalve molluscs to ensure viral removal.

- To improve access to information on the hygienic quality of bivalve shellfish production throughout the food chain.

- To develop a harmonized policy on sewage effluents discharged in the vicinity of designated shellfish harvesting areas to provide better protection of vulnerable shellfish areas.

- To ensure that cooking processes that are to be approved are effective with regard to NLV inactivation.

- To promote studies aimed at better characterising the behaviour of viruses contaminating shellfish in order to formulate appropriate control strategies.

- To investigate the potential zoonotic transmission of NLVs.

11. ANNEX

Source of information : Eurostat
Unit of measurement : tonnes

Table A: Bivalve molluscs production in EU

Table B: Soft fruit production in EU

Table C: Bivalve Molluscs Trade Information for 1998, 1999 and 2000

Table D: Raspberries (uncooked or cooked by steaming or boiling in water, frozen, unsweetened) - Trade Information for 1998, 1999 and 2000

Table E: Raspberries, blackberries, mulberries, loganberries, black-, white- or red-currants and gooseberries (uncooked or cooked by steaming or boiling in water, sweetened, with sugar content of > 13 %, frozen) - Trade Information for 1998, 1999 and 2000

Table F: Raspberries, blackberries, mulberries, loganberries, black-, white- or red-currants and gooseberries (uncooked or cooked by steaming or boiling in water, sweetened, with sugar content of =< 13 %, frozen) - Trade Information for 1998, 1999 and 2000

Table G: Raspberries, blackberries, mulberries, loganberries, black-, white- or red-currants and gooseberries, (uncooked or cooked by steaming or boiling in water whether or not sweetened, frozen) – Imports into EU from EU candidate countries for 1997, 1998, 1999 and 2000.

Table A: Bivalve molluscs production in EU in tonnes live weight*
(Source: Eurostat)

	Wild caught		Farmed		Total production	
	1998	**1999**	**1998**	**1999**	**1998**	**1999**
Belgium	224	247	-	-	224	247
Denmark	110328	96469	-	-	110328	96469
France	20001	21892	203100	202700	223101	224592
Germany	-	-	31288	38039	31288	38039
Greece	6484	15907	14602	16930	21086	32837
Ireland	2013	2617	25239	23516	27252	26133
Italy	67793	74338	178000	180000	245793	254338
Luxembourg	-	-	-	-	-	-
Netherlands	68541	51194	115887	104014	184428	155208
Portugal	1761	1542	4327	3876	6088	5418
Spain	10992	11367	273895	276068	284817	287435
Sweden	38	4	455	954	493	958
United Kingdom	52889	47618	9941	10901	62830	58519
EU - 15	**390994**	**323195**	**856734**	**856998**	**1197728**	**1180193**

*includes the weight of the shells. The meat (product) weight is between 15-20% of the live weight

Table B: Production on soft fruits in EU in tonnes (Source: Eurostat)

	Currants		Black currants		Red currants		Raspberries		Gooseberries		Other soft fruit n.o.s.		Total soft fruit	
	1998	**1999**	**1998**	**1999**	**1998**	**1999**	**1998**	**1999**	**1998**	**1999**	**1998**	**1999**	**1998**	**1999**
Austria	19665	19537	5587	415	733	788	-	-	1673	1668	-	-	27658	22408
Belgium	1650	2200	-	-	-	-	200	220	280	200	500	650	2630	3270
Denmark	4992	4992	4087	4071	921	921	75	75	-	-	-	-	10075	10059
Finland	1913	1512	1541	1032	293	367	265	307	41	35	37	43	4090	3296
France	9699	10333	-	-	-	-	7116	7020	-	-	-	-	16815	17533
Germany	136200	-	-	-	-	-	75700	-	29900	-	-	-	241800	-
Greece	-	-	-	-	-	-	-	-	-	-	2220	2100	2220	2100
Ireland	1850	2442	1300	77	-	-	497	2365	120	685	68	-	3855	5569
Italy	551	585	28	36	523	549	1063	1167	43	29	135251	48106	137459	50472
Luxembourg	100	115	-	-	-	-	-	-	-	-	-	-	100	115
Netherlands	2250	3000	1000	1000	1250	2000	410	390	-	-	1270	1450	6180	7840
Portugal	-	-	-	-	-	-	-	-	-	-	1217	-	1217	-
Spain	-	-	-	-	-	-	-	-	-	-	93256	84061	93256	84061
Sweden	702	498	702	498	-	-	121	118	77	-	-	-	1602	1114
United Kingdom	7941	8863	7500	8400	441	463	12600	11100	1482	1708	2277	1038	32241	31572
EU - 15	**187513**	**54077**	**21745**	**15529**	**4161**	**5088**	**98047**	**22762**	**33616**	**4325**	**236096**	**137448**	**581178**	**239229**

Table C: Bivalve Molluscs Trade Information for 1998, 1999 and 2000 in tonnes (Source: Eurostat)

	Intra EU Imports			Extra EU Imports			Intra EU exports			Extra EU exports		
	1998	**1999**	**2000**	**1998**	**1999**	**2000**	**1998**	**1999**	**2000**	**1998**	**1999**	**2000**
Austria	389	372.3	426	31.6	43.6	41.7	10.1	15.8	13.2	0.8	0.8	0.3
Belgium	30563.6	32477.6	30385.1	808.6	804.3	218.6	1223.1	1411.9	1159.6	11.2	23.3	29
Denmark	221.1	438.1	391.7	1628.1	1526.9	1386.3	28066.7	25520.9	28946.7	73.6	123.5	237.7
Finland	133.2	75.7	98	0.6	1.3	0.9	-	-	-	5.8	4.4	10.2
France	63816.4	57586.8	49051.1	8413.9	8562.9	7953.7	12417.1	12992.6	13017.3	536.7	537	665.1
Germany	23124.5	25250.5	28401.1	624.7	473	450.4	24117.2	15534.1	16902.8	59.9	27.3	28.3
Greece	334.4	366.6	644.6	220	243	269.8	15640.5	18178.5	19288.9	8.3	6.7	2.4
Ireland	181.7	153.9	180.5	0.1	0.1	0.2	8754	7458.9	9949.5	83.9	432.4	649.9
Italy	38545.8	43259.5	34601.7	827.7	837.5	1228.8	9049.1	8555.9	9341.6	123.8	154.9	218.3
Luxembourg	-	821.2	852.9	-	13.8	18.5	-	32.9	63.2	-	-	-
Netherlands	20461.5	19959.2	10731.9	990.7	1047.4	1056.9	50059.9	49264.8	44310.4	138.9	277.8	311.2
Portugal	1091.4	1209.6	1171.3	148.7	342.2	411.6	47.7	321.9	194	9.6	3.7	5.9
Spain	13098.3	13560.6	15970.7	3124.8	2661.8	1906.7	24321.5	27023.8	25391.1	368	260.5	263.6
Sweden	85.9	96.1	118.8	139.4	201.6	288.8	125.1	311.1	578.2	91.4	113.1	111.1
United Kingdom	2467.1	3298.9	3226.7	1497.5	1906.7	1438.3	17381.3	15766.8	17236.5	739.4	1138.9	1824.7
EU – 15	**194513.9**	**198926.6**	**176252.1**	**18456.4**	**18666.1**	**16671.2**	**191213.3**	**182389.9**	**186393**	**2251.3**	**3104.3**	**4357.7**

Table D: Raspberries (uncooked or cooked by steaming or boiling in water, frozen, unsweetened)
Trade Information for 1998, 1999 and 2000 in tonnes (Source: Eurostat)

	Intra EU Imports			Extra EU Imports			Intra EU exports			Extra EU exports		
	1998	**1999**	**2000**	**1998**	**1999**	**2000**	**1998**	**1999**	**2000**	**1998**	**1999**	**2000**
Austria	822.8	1458.8	1834.3	5245.5	5761.9	7371.5	-	3134.2	5407.7	27.1	63.1	8.9
Belgium	1428.4	1314	2654.6	3434.3	4372.3	4429.6	6752.7	6592.6	8210.2	76.6	124	78.5
Denmark	1148.7	544	1445.1	2395.4	2604.8	2150.2	367.6	383.8	121.4	137.8	70.3	66.4
Finland	97	222.9	45	2351.4	2092.3	2140.7	-	0.5	0.9	22.4	62.6	2.4
France	6240.1	8297.5	8374	13275.1	13745.6	15368.4	754.9	961.6	741.3	577.5	315.7	328.3
Germany	5865.7	4534.6	4428.5	30241.6	33502.8	32763.1	5114.2	6113.7	6805.6	224.2	237.7	162.5
Greece	8.8	9.5	13.1	9	18	46.9	-	18	26.9	-	-	-
Ireland	213.9	190.3	107	22.7	-	-	6.5	3.4	2.4	-	-	-
Italy	1779.6	2127	2761.8	919.5	1083.1	1060.2	82.4	80.9	317	9.3	4.8	-
Luxembourg	-	30.2	27	-	-	-	-	2.9	1.2	-	-	-
Netherlands	3144.6	2064.8	1706.5	6663.3	6536.2	6680.4	2926.9	-	-	-	-	-
Portugal	24.2	56.8	39.9	-	-	-	-	-	-	-	-	-
Spain	281.3	365	586.7	-	22	1	138.4	263.9	371.1	-	-	-
Sweden	321.1	277.2	354.9	3173.4	2873	2705	56.9	55.1	25	34.8	26	6.1
United Kingdom	3399.9	2451.8	3618.9	3708.5	3766.7	3888.5	761.7	1675.5	1587.2	4.6	63.7	3.9
EU 15	**24776.1**	**23944.4**	**27997.3**	**71439.7**	**76378.7**	**78605.5**	**16962.2**	**19286.1**	**23617.9**	**1114.3**	**967.9**	**657**

Table E: Raspberries, blackberries, mulberries, loganberries, black, white or red currants and gooseberries (uncooked or cooked by steaming or boiling in water, sweetened, with sugar content of > 13 %, frozen)
Trade Information for 1998, 1999 and 2000 in tonnes (Source: Eurostat)

	Intra EU Imports			Extra EU Imports			Intra EU exports			Extra EU exports		
	1998	**1999**	**2000**	**1998**	**1999**	**2000**	**1998**	**1999**	**2000**	**1998**	**1999**	**2000**
Austria	46.7	128.8	76.4	-	-	-	46.7	60.6	127.4	-	-	-
Belgium	25.8	53.6	50.9	-	-	-	5.4	2.9	9.4	1.2	-	1.5
Denmark	327.3	618.6	436	-	-	-	2149.2	2118.4	2350.3	5.1	4.2	1.5
Finland	70.1	50.6	3.9	-	-	-	2	-	-	-	-	-
France	-	51.5	130.5	0.4	3.6	5.8	82.4	48.9	20.8	8.3	252.1	290.8
Germany	83.8	141.5	49.4	38	20	40	44.9	1.4	11.5	40	-	-
Greece	0.3	6.1	0.9	-	-	-	-	-	-	-	-	-
Ireland	4.4	70.6	32.7	-	-	-	-	-	-	-	-	-
Italy	66.3	154.6	156.7	-	-	1.8	77.4	15	21.1	4.8	8.6	49.1
Luxembourg	-	-	0.7	-	-	-	-	-	-	-	-	-
Netherlands	67.3	62.8	8.1	-	-	-	3.4	9.8	0.3	10.6	0.3	-
Portugal	-	-	0.6	-	-	-	-	-	-	-	-	-
Spain	9.4	5.6	12	-	-	-	-	1	0.6	-	-	-
Sweden	-	9.8	21	-	-	-	-	-	-	29.1	1.3	2.4
United Kingdom	195.7	174.4	146.6	18	20	0.3	-	-	17	-	7.4	17
EU 15	**897.1**	**1528.5**	**1126.4**	**56.4**	**43.6**	**47.9**	**2411.4**	**2258**	**2541.4**	**59.1**	**273.9**	**362.3**

Table F: Raspberries, blackberries, mulberries, loganberries, Black, white or red currants and gooseberries (uncooked or cooked by steaming or boiling in water, sweetened, with sugar content of =< 13 %,frozen)
Trade Information for 1998, 1999 and 2000 in tonnes (Source: Eurostat)

	Intra EU Imports			**Extra EU Imports**			**Intra EU exports**			**Extra EU exports**		
	1998	**1999**	**2000**	**1998**	**1999**	**2000**	**1998**	**1999**	**2000**	**1998**	**1999**	**2000**
Austria	177.6	194.6	129.1	8	-	20	-	-	1.3	19.5	0.1	0.1
Belgium	5	0.3	0.5	-	-	23	6.7	20.8	13.1	0.5	-	-
Denmark	256.9	205	197	49.2	20.2	30.8	-	-	-	-	0.8	2.2
Finland	15.2	43	-	-	2.5	18	-	0.8	-	0.3	0.1	0.3
France	32.9	138.8	169.9	-	30.1	-	506.9	147.4	200.7	14.4	38.2	35.9
Germany	210.5	128.3	75.3	22.2	-	65.7	23.5	13	23.8	6.3	0.5	7.2
Greece	0.4	0.2	0.2	-	-	-	-	20	-	-	2	-
Ireland	-	-	11	-	-	-	-	-	-	-	-	-
Italy	4.7	21.4	9	-	20.3	-	0.4	0.1	14.3	-	-	0.8
Luxembourg	-	1.6	0.3	-	-	-	-	-	-	-	-	-
Netherlands	4.3	451.1	120.1	90.2	18	-	22.6	1	-	78	16.1	13.8
Portugal	-	0.1	-	-	-	-	-	-	-	-	-	-
Spain	9	14.7	17.5	-	-	0.2	1	128.2	20.6	-	-	-
Sweden	6.8	31.4	3	1.9	-	-	12.4	4.4	-	6.1	2.2	14.7
United Kingdom	18.8	95.7	170.3	-	9	-	7.3	7.4	3.1	-	0.2	-
EU 15	**742.1**	**1326.2**	**903.2**	**171.5**	**100.1**	**157.7**	**580.8**	**343.1**	**276.9**	**125.1**	**60.2**	**75**

Table G: Imports of raspberries, blackberries, mulberries, loganberries, black-, white- or red- currants and gooseberries, (uncooked or cooked by steaming or boiling in water whether or not sweetened, frozen) into the EU from EU candidate countries for 1997, 1998, 1999, 2000 in tonnes (Source: Eurostat)

EU Candidate Country	1997	1998	1999	2000
Turkey	565.6	327.5	312.1	301.1
Estonia	270.9	221.7	109.0	338.4
Latvia	0.2	11.5	2806	1.2
Lithuania	168.7	182.3	153.0	324.5
Poland	46624.5	40170.1	56406.1	50804.0
Czech Republic	2451.5	3964.0	2738.2	3345.3
Slovakia	561.2	250.8	151.8	272.1
Hungary	11657.9	10023.4	11668.6	8166.0
Romania	37.2	15.7	237.3	343.1
Bulgaria	1360.8	1643.8	1311.3	1359.2
Total Imports	**63806.7**	**56895.6**	**73197.9**	**65650.2**

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