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Human Enteric Viruses in Bivalve Molluscs: Contamination and Detection

Bou m'handi Naïma
Seafood Processing Technology Center (CSVTPM), National Research Institute of Marine Fisheries (INRH), BP: 1050, Agadir- Morocco.

ABSTRACT

Human and animal fecal wastes and urine contain a large number of different viruses that can enter the environment through the discharge of waste materials from infected individuals. Despite the high diversity of viruses that are introduced into the environment by human fecal pollution, only a few have been recognized to cause disease in association with consumption of contaminated shellfish. Viruses are present in shellfish in very low numbers. Nevertheless, they are present in sufficient quantities to pose a health risk as presented above. This low level of contamination has made it necessary to develop highly sensitive viral extraction methods to ensure virus recovery from shellfish tissues. The aim of this paper is to outline the importance of human enteric viruses in bivalve molluscs, describes of shellfish contamination and describes processing methods to reduce or eliminate enteric viruses in shellfish and summarize the advantages and limitations of current detection methodologies. Recommendations are also provided on the need for new research directions to address shellfish-borne viruses.

Keywords: *Human Enteric Viruses, Shellfish, Contamination, Virus Inactivation, Detection Methods.*

1. INTRODUCTION

Filter-feeding bivalve molluscan shellfish (oysters, clams, mussels and cockles) have a history of association with viral foodborne disease (FAO/WHO, 2008). The presence of several human enteric viruses (norovirus [NoV], Aichi [AiV], rotavirus [RV], enterovirus [EV], adenovirus [AdV], astrovirus [AV], sapovirus [SaV], Hepatitis A [HAV] and Hepatitis E [HEV]) have been identified in shellfish, although not all have been clearly linked with documented disease outbreaks (Le Guyader *et al.*, 2000; Nakagawa-Okamoto *et al.*, 2009; FAO/WHO, 2008). Based on the symptoms of infection, these viruses can be grouped into those which cause gastroenteritis (NoV, RV, AV, AiV, AdV and SaV), enterically transmitted hepatitis (HAV and HEV) and those which replicate in the human intestine, but only cause illness after they migrate to other organs such as the central nervous system (EV) (FAO/WHO, 2008).

NoV and HAV infection have been shown to be widely associated with the ingestion of contaminated shellfish (Le Guyader *et al.*, 2000), although HEV (Meng, 2011) and AiV infection (Le Guyader *et al.*, 2008; Ambert-Balay *et al.*, 2008) have also been implicated in the consumption of infected oysters. NoV is the most common cause of foodborne viral gastroenteritis worldwide, while HAV continues to pose an international health threat. In the US the majority of all foodborne illness (58%) are caused by NoV (Scallan *et al.*, 2011). Between 1980 and 2012 there were 368 reported shellfish associated viral outbreaks in the scientific literature (Bellou *et al.*, 2013). The most common viral pathogens involved were NoV (83.7 %) and HAV (12.8 %) with the most frequent shellfish implicated in outbreaks being oysters (58.4 %). NoV outbreaks following shellfish consumption are attributed to growing waters contamination by human faeces and consumption of raw or lightly cooked product (Bellou *et al.*, 2013). Recent evidence has shown that NoV bind specifically to shellfish tissue receptor sites and can persist in contaminated shellfish digestive tissue for 8-10 weeks, which would explain why these viruses persist after depuration (Le Guyader *et al.*, 2006; FAO/WHO, 2008).

To protect the consumer, it is important to have sensitive and rapid methods for directly detecting the viral pathogen of concern in shellfish. A number of methods to do this have been described over the past 15 years, demonstrating that detection of viruses in shellfish is possible. The detection of enteric viruses relies mainly on the use of reverse transcription-PCR (RT-PCR) assays (Duizer *et al.*, 2004), but the low quantity of virus in environmental samples usually requires a time-consuming hybridization step, which enhances both the sensitivity and the specificity of the assays, or sequencing of the obtained amplicons. Recently, real-time reverse transcription-PCR (rRT-PCR) has been applied to the detection of the main enteric viruses, including NoV (Kageyama *et al.*, 2003 ; Le Guyader *et al.*, 2009 ; Loisy *et al.*, 2005), HAV (Costafreda *et al.*, 2006), AsV (Le Cann *et al.*, 2004) and EV (Donia *et al.*, 2005). The aim of this paper is to outline the importance of human enteric viruses in bivalve molluscs, describes of shellfish contamination and describes processing methods to reduce or eliminate enteric viruses in shellfish and summarize the advantages and limitations of current detection methodologies. Recommendations are also provided on the need for new research directions to address shellfish-borne viruses.

2. NOROVIRAL GASTROENTERITIS AND HEPATITIS A

Norovirus belong to the *Caliciviridae* family, a group of nonenveloped, icosahedral viruses with a single-stranded, positive sense RNA genome (Atmar, 2010). These viruses are highly diverse and are currently divided into 5 genogroups (Zheng *et al.*, 2006). Genogroups I, II and IV contain human

strains. Each genogroup is further subdivided into genotypes based upon analyses of the amino acid sequence of the major capsid protein, VP1. Other genotyping systems based upon shorter sequences (Kageyama *et al*., 2004) or analysis of the polymerase gene (Kroneman *et al*., 2011) have also been described. New strains and genogroups infecting animals also have been described (Mesquita *et al.*, 2010). NoVs infection causes gastroenteritis that is characterized by vomiting and diarrhoea (Glass *et al.*, 2009). The prevalence of vomiting along with the short incubation period (1-2 days) and short clinical illness (1-3 days) have been used epidemiologically to identify probable outbreaks of NoV-associated gastroenteritis (Turcios *et al.*, 2006; Kaplan *et al.*, 1982). The infectious dose 50% has been estimated to be as low as fewer than 20 virions (Teunis *et al.*, 2008). NoVs bind to Histo-blood group antigens (HBGAs), phylogenetically highly conserved complex glycans present on many different cell types and proposed as an attachment factor necessary to initiate infection in people (Tan and Jiang, 2011; Donaldson *et al.*, 2010). NoVs are the major cause of epidemic non bacterial gastroenteritis worldwide and have been identified as the cause of 73% to more than 95% of outbreaks (Atmar, 2010). These outbreaks involve all age groups in a wide variety of settings, with a large dominance of GII strains (Table 1) ((*in* Le Guyader et al., 2012). Over the past 10 years, NoV sequence analyses of outbreak strains collected from around the world show that GII.4 viruses have accounted for ~70% of all human cases (Siebenga, 2010). The hepatitis A virus belongs to the genus *Hepatovirus* of the family *Picornaviridae*, and is very stable in the environment, remaining viable for up to several weeks in water or on fomites (Abad *et al.*, 1994; Arnal *et al.*, 1998; Hollinger and Emerson 2007). Hepatitis A virus infection has a long incubation period and is generally asymptomatic or associated with a mild illness in young children, while in older children and adults the illness is characterized by jaundice in more than 70% of individuals (CDC, 2006). There is only a single serotype, and an effective vaccine is available for prevention of infection (CDC, 2006).

Table 1: Norovirus genotypes reported from shellfish-related outbreaks (*in* Le Guyader et al., 2012).

Date	Country	Stool			Shellfish			
		# pos/ # analyzed	NoV GI genotype	NoV GII genotype	species	# pos/ # analyzed	NoV GI genotype	NoV GII genotype
May 1998	US	1/2	nd	4	oyster	2/3	nd	4
March 2000	France	4/4	1, 2, 3	nd	oyster	2/2	1	nd
February 2001	Netherlands	8/9	1, 4	b, 7	oysters (France)	5/5	4	7
Dec 1998- Feb 2002	Japan	84/108a	1-5, 7-9, 11-14	1, 3-12, 14, 16	oyster - no sample			
March- Apr 2002	Italy	24	4	8, b	mussels	5/11	4	II.b
December 2002	France	29/53	4, 6	4, 8, b	oysters	3/3	4	4, 8
Nov 2003-Jan 2004	Australia	8/?	2, 4	5, 6, 7, 9, 12	oysters (Japan)	1/1	nd	4
January 2004	UK	10/11	1, 2	3, 4	oyster - no sample			
Jan /March 2004	Canada	26/50	1, 2	3, 4, 5,	oysters	12/19	1	12
October 2005	Japan	18/37	nd	1, 4, 5, 6	oyster - no sample			
June 2006	New	4/4	nd	3, 6, 12	oysters (Korea)	4/6	3	3, 6, 8, 12
February 2006	Zealand	12/12	1, 2, 4	2, 4, 7, 17, b	oysters	9	1, 2, 4	4, 17
Jan 2002- March	France	71[b]	1-5, 8, 10, 13-15	3-6, 8, 12	oyster - no sample			
2007	Japan	1/1	1	nd	oysters	1/1	1	3
January 2007	Sweden	4/5	nd	4	oysters	4/4	nd	4
February 2008	France	11/24	1	4, 8	clams	3	1	8
June 2008	Japan	3/6	nd	12	oysters- no sample			
December 2009	US							

Nd: not detected,Two manuscripts report data from 21 (a) and 11 (b) individual outbreaks.

3. VIRUS CONTAMINATION OF SHELLFISH

3.1 Localization within bivalve molluscs.

Virus entry into molluscan shellfish is achieved by normal shellfish feeding activities, where the viruses are filtered from the water by the gills, and the filtered materials (viruses and associated solids) enter the digestive tract starting from the mouth. Once in the stomach and digestive diverticula, some viruses may pass through the shellfish and exit in the feces; however, some of the viruses are transported through the walls of the digestive tract into interior portions of the shellfish (Le Guyader *et al*., 2006; McLeod *et al*., 2009). It has been suggested that the phagocytic process in hemocytes, which involves lysosomal enzymes, toxic oxygen intermediates, and antimicrobial peptides, may be responsible for killing bacteria in bivalves (Canesi *et al*., 2002), but this has not been demonstrated for human enteric viruses to date. Viruses are known to be retained by bivalves for significantly longer periods of time than bacterial indicators such as *E. coli* and fecal coliforms

(Cook and Ellender, 1986; Power and Collins, 1989, Power and Collins, 1990).
Several studies have examined the localisation of norovirus in oyster tissues. Noroviruses were detected in the gills, stomach, digestive diverticula and cilia of the mantle (Wang *et al.*, 2008a; Wang *et al.*, 2008b). Recent research has shown that noroviruses can bind specifically to antigens in the oyster gut which are similar to human blood group antigens (HBGA) (Le Guyader *et al.*, 2006; Tian *et al.*, 2006), and can be internalised within cells of both digestive and non-digestive tissues (McLeod *et al.*, 2009) which could explain why viruses persist after depuration.

3.2 Harvesting.

Common sense dictates the need to harvest shellfish from clean waters, but the determination of what constitutes clean waters is often difficult to discern. As in Europe, Moroccan sanitary controls subscribes to *Escherichia coli* standards under Regulations (EC) 852/2004, 853/2004, 854/2004 and Decision N°1246/01 of Ministry of Maritime Fishing, Moroccan Government, which classifies shellfish based on tissue levels of bacteria under four classifications: class A, where shellfish may be directly sold without processing; class B, where shellfish must be depurated or relayed before marketing; class C, where shellfish must be subjected to prolonged relay or cooking; and class D, where shellfish harvesting and distribution are prohibited (Anonymous 2004a, b and c). In the United States, sanitary surveys are performed on seawater from shellfish growing areas under the National Shellfish Sanitation Program Model Ordinance (Anonymous 1999), which classifies waters as approved, conditionally approved, restricted, or prohibited. Shellfish from approved beds may be marketed directly, those from conditionally approved and restricted areas must be processed according to an approved plan, and shellfish from prohibited areas may not be harvested or distributed. Fecal coliforms or *E. coli* limits serve as indicators of recent contamination events; however, shellfish, which are free from perceived bacterial contaminants may contain viruses, which can persist for longer periods than the indicator bacteria. Nevertheless, the first precaution in reducing shellfish-borne viral illnesses is to restrict shellfish harvesting in accordance with the applicable standards.

3.3 Handling.

Improper post-harvest handling of shellfish can lead to product contamination and illness. Shellfish contamination may also come from the use of contaminated ice or water used in storing or rinsing products. Only potable water should be used in processing and for the preparation of ice, since ice has been epidemiologically linked to hepatitis A and NoV illnesses (Beller 1992; Khan *et al.* 1994). The shucking of shellfish offers another means of potential product contamination from the hands or gloves of shuckers or from the shucking knives, tabletops, or containers in which the shellfish are stored. Good sanitary practices are essential in reducing product contamination (Mokhtari and Jaykus, 2009). Some shellfish dealers may subject shellfish to a process known as “freshening up” where previously harvested and stored shellfish are returned to the sea for a short period so that they may feed and be revived after a period out of water. This can lead to product contamination if the seawater that they are transplanted into is not clean. Freshening up is not an approved practice in the US or the EU, but has been known to occur. Shellfish handling and processing procedures should follow regulatory guidelines and good manufacturing practices, but strict adherence to these procedures may not be sufficient to eliminate all traces of virus contamination. There is no guarantee that shellfish or any other food item is absolutely safe, so risks must be minimized to the extent possible.

4. VIRUS INACTIVATION IN SHELLFISH

Washing and surface disinfection of shellfish may be effective in reducing post-harvest contamination, but most of the virus outbreaks associated with shellfish are from pre-harvest sources of contamination where the viruses are bioaccumulated within the tissues, as mentioned above. Since these viruses are internalized within the shellfish, they cannot be disinfected by traditional surface treatment, such as washing, exposure to UV light, or chemical disinfectants. The following section covers processing interventions, which likely reduce or in some cases eliminate infectious viruses within molluscan shellfish tissues.

4.1 Shellfish Depuration.

The practice of shellfish depuration originated over a century ago (Herdman and Scott 1896; Herdman and Boyce 1899; Belding and Lane 1909) and has significantly reduced the levels of shellfish-borne illnesses since its inception. Depuration is a commercial processing strategy where shellfish are placed in tanks of clean seawater and allowed to purge the contaminants over a period of several days (reviewed in Richards 1988, 1991). Water may be purified by replacement in what are termed flow-through systems, or using chlorine, ozone, or UV light (reviewed in Richards 1988, 1991). Studies have repeatedly shown that shellfish purge enteric viruses more slowly than most bacteria. Depuration is intended to reduce relatively low levels of contamination from shellfish and was never intended for highly contaminated products. In some cases, depurated shellfish have been associated with outbreaks of norovirus, hepatitis A, and other viral diseases (Grohmann *et al*. 1981; Ang 1998; Conaty *et al*. 2000; Le Guyader *et al*. 2008). Some outbreaks have been associated with contamination of product during the depuration process or from inadequate depuration controls (Guillois-Bécel *et al*. 2009; Richards 1988).
Depuration is a beneficial treatment to reduce bacterial contaminants from shellfish in a simulated natural environment, and can reduce virus levels in the process; however, depuration should not be relied on to reduce virus levels sufficiently for virus contaminated shellfish to be considered safe. This is because viruses, unlike bacteria, are generally infectious at very low levels, perhaps as low as 5 or 10 virus particles per meal. Thus, there is a need to reduce

viruses to near negligible levels to improve the safety of shellfish.

4.2 Relaying.

Another method to disinfect bivalve shellfish involves a longer-term purification process known as relaying (reviewed by Richards 1988). In relaying, shellfish are harvested from a contaminated area and transplanted to clean areas where they are broadcast on the ocean floor, or placed into containers, which are laid on the bottom or are suspended in racks or other devices for quick retrieval after the process is complete (Richards 1988). Where commercial depuration may be performed for only 2-3 days to meet regulatory requirements, relaying often requires 10 days or longer. Both depuration and relaying are generally acceptable for the depletion of bacterial contaminants, but appear insufficient for total enteric virus elimination. Very long-term relaying is a possible solution to the virus problem, if clean waters are available and if the cleanliness of the waters containing the shellfish can be maintained. This is a challenge, since: a) boats may illegally discharge waste in areas that were previously clean; b) changing winds, currents, and tides may carry polluted waters into harvesting areas; c) floods and storm water runoff may contaminate coastal waters; d) malfunctioning sewage treatment plants may allow the release of insufficiently treated effluent; and e) private septic systems may leach or overflow into coastal areas. Long-term relaying may be impractical from a commercial standpoint because of increased production costs associated with the additional handling and because it reduces product availability.

4.3 Cooking and Heat Pasteurization.

The most effective method to reduce viruses from any food product is to cook the food thoroughly. In the case of shellfish, thorough cooking changes organoleptic characteristics and can toughen shellfish to an unpalatable state. Light cooking may be acceptable to some consumers, but is generally inadequate for the elimination of enteric viruses, since most of the viruses are inside the shellfish and would not be subjected to sufficient heat for their total inactivation. Shellfish affected by likely surface contamination, as might occur through handling or processing, could benefit from a quick cooking or blanching process. Local customs and preferences often dictate whether the product will be consumed raw or cooked. Heat pasteurization may be performed on in-shell or shucked products (Brown 1982). In some countries, oysters are shucked, pasteurized, and refrigerated until sale and may be readily accepted in this form by some consumers. There remains a substantial portion of shellfish consumers who demand raw shellfish and for them alternate processing strategies, like high pressure processing may be desirable (see below).

Studies to evaluate the thermal resistance of viruses in foods, including molluscan shellfish, have given variable results, in part because of the use of different testing methods, different virus strains or surrogates, and different food compositions and shellfish species. Early studies using poliovirus (PV) showed that virus inactivation rates in oysters (*C. gigas* and *O. lurida*) depended on the manner in which the oysters were cooked (fried, baked, stewed, or steamed) (DiGirolamo et al. 1970). Only a 2-log10 decrease in viable viruses was seen after steaming for 30 min to an internal temperature of 93.7°C. Virus survivals ranging from 7-13% were observed using different cooking methods (DiGirolamo et al. 1970). Hewitt and Greening (2006) showed differences in HAV and NoV inactivation in New Zealand greenshell mussels (*Perna canaliculus*) depending on the method of cooking, where boiling for 3 min was more effective than steaming for 3 min to inactivate HAV.

Common sense would suggest that the manner and duration of cooking, as well as the final temperature achieved may be important to the inactivation of HAV, NoV, and other viruses. HAV and PV present in cockles were both shown to be reduced by > 4 logs when an internal temperature of 90°C was maintained for 1 min (Millard *et al*. 1987). Similarly, heat inactivation processes (90°C for 90 sec) undertaken on shellfish products in processing establishments in the UK have been shown to be effective in inactivating NoV (as evidenced by a decrease in human illness resulting from the consumption of these products) (Lees 2000). Canned oysters are likely to be safe from a virus standpoint, since the canning process provides sufficient heat to essentially sterilize the product. It is unclear to what extent pasteurization is effective in virus elimination, but there are reports that *C. botulinum* spores resist the pasteurization process (Chai *et al.* 1991).

4.4 High Pressure Processing (HPP).

In recent years, HPP has emerged as a processing intervention to inactivate vibrios and spoilage bacteria in shellfish and to facilitate in the shucking of oysters. Commercial processors use around 275−300 MPa of pressure for about 3 min to disinfect oysters. This non-thermal process produces shellfish, which have the taste and texture of raw product, remain plump and juicy, and have a slightly cooked appearance from partial denaturation of oyster proteins by the pressure treatment. In 2002, Kingsley and colleagues were the first to demonstrate the effectiveness of HPP to inactivate HAV and FCV (Kingsley *et al*. 2002). RNase protection assays showed that the pressure-treated viral capsids remained intact, since the virus RNA's were not degraded by RNase after pressure treatment (Kingsley *et al.* 2002). This led us to conclude that virus inactivation was likely from denaturation of virus capsid proteins, rather than from lysis of the virus capsid. Such denaturation would prevent the viruses from binding to host cells both *in vivo* and *in vitro*. Pressures of 250 and 450 MPa for 5 min were sufficient to inactivate 7-log10 of FCV and cell culture-adapted HAV, respectively, in culture media containing 10% fetal bovine serum (Kingsley *et al*. 2002). Different food matrices were expected to affect virus inactivation rates, as demonstrated for HAV where virus inactivation was affected by pH, salt content, and temperature (Kingsley and Chen 2009). High salt appeared to decrease the effectiveness of HPP in inactivating HAV and FCV (Kingsley and Chen 2008; Grove *et al*. 2009). Oysters subjected to treatments of 350, 375, and 400 MPa for 1 min showed

reductions in HAV greater than 1, 2, and 3 log10, respectively (Calci *et al.* 2005). Pressure oscillations did not significantly affect the inactivation of HAV in culture media; however, HAV was found to be more susceptible to inactivation when pressurization was performed at warmer temperatures (Kingsley *et al.* 2006). The advent of a quantitative assay for MNV-1, a closer relative of human NoV than FCV, led to a study showing a 4-log10 decrease in virus infectivity after pressure treatment of oysters for 5 min at 400 MPa (Kingsley *et al.* 2007), therefore, it was uncertain which surrogate was more representative of human NoV inactivation under high pressure. Studies showed that PV could not be inactivated by pressures as high as 600 MPa, but that its close relative, HAV, was inactivated at substantially lower pressures (Kingsley *et al.* 2002). In addition, Aichi virus and coxsackievirus B5 were found to be resistant to 600 MPa for 5 min, but human parechovirus-1 and coxsackievirus A9 were sensitive to 400 MPa, further demonstrating major differences in pressure response by closely related viruses (Kingsley *et al*. 2004).

4.5 Irradiation.

Enteric virus inactivation studies have included work on the effects of ultraviolet light and ionization radiation on virus levels. Ultraviolet irradiation is effective in reducing NoV surrogates and HAV on the surface of product, but does not have penetrating power to inactivate viruses deep within the shellfish. UV irradiation at 120 J/m2 and 200 J/m2 reduced the infectivity of FCV and a presumed canine calicivirus by 3-log10, respectively, while 650 J/m2 was required to reduce MS2 phage counts by the same amount (de Roda Husman *et al*. 2004). In PBS, MNV-1 was readily inactivated by UV; however, the amount of UV exposure was not reported (Wolf *et al*. 2009). Ionizing radiation was used to inactivate PV in shucked and in-shell oysters (*C. gigas* and *O. lurida*), but the levels required to inactivate 90% of the viruses imparted undesirable organoleptic qualities, rendering the shellfish unpalatable (Di Girolamo *et al*. 1972). Jung and colleagues (2009) showed the D10 value for PV at 2.94 kGy, and referred to PV as a surrogate for NoV (Jung *et al*. 2009). Gamma irradiation of clams (*M. mercenaria*) showed poor reduction of F-coliphage, where the mean D10 value was 13.5 kGy and where > 0.5 kGy was reportedly lethal to the shellfish (Harewood *et al.* 1994). HAV and rotavirus were eliminated from oysters (*C. virginica*) and clams (*M. mercenaria*) at D10 values of 2.0 and 2.4 kGy, respectively (Mallet *et al*. 1991). These authors reported that at these levels of irradiation, shellfish survival rates and organoleptic characteristics were relatively unaffected. Gamma irradiation at 0.5, 0.3, and 0.1 kGy produced 3-log10 decreases in FCV, canine norovirus, and MS2 titers, respectively, in low protein solutions, but high amounts of protein appreciably reduced the effectiveness of ionizing irradiation (de Roda Husman *et al*. 2004). Some consumers have expressed uncertainty about the safety of irradiated foods; however, education about the food benefits of irradiation is likely to improve consumer perceptions. The variability in the results among the studies may be attributed, in part, to the use of different viruses, shellfish species, exposure methods, and matrix compositions. Further studies on the effectiveness of irradiation to inactivate human enteric viruses are warranted.

4.6 Freezing.

Freezing of shellfish is a potential processing method of limited value. Raw shellfish meats are often frozen to await subsequent processing, such as breading or cooking either at the restaurant or at home. Although freezing tends to preserve viruses, there can be an initial loss in virus titer with each freeze-thaw cycle. That loss in titer may reduce virus levels in minimally contaminated product to enhance safety. Studies on PV survival in oysters under frozen conditions showed an approximately 1-log10 decrease in infectious viruses after storage for 4 to 12 weeks at -17.5°C (Di Girolamo *et al*. 1970). In 2010, Richards and colleagues see an approximately 10% decrease in NoV titer (based on RT-PCR assay) for each round of freezing and thawing, suggesting that freezing and thawing may cause lysis of the viral capsids (Richards *et al*., 2010). Freezing by itself seems inadequate to protect the consumer from even lightly contaminated shellfish, but when freezing is combined with cooking or HPP, the additive effect of both processes would further enhance shellfish safety.

4.7 Smoking.

Smoking is another commercial method of processing shellfish. There are various, non-standardized methods for smoking shellfish and the amount of heat applied in this process is likely to vary considerably from one facility to another and perhaps from one batch of shellfish to another. It is uncertain if smoking alone is effective in reducing virus levels in shellfish, but many smoked products are also canned, which provides sufficient heat to inactivate viruses. Outbreaks of *Listeria monocytogenes* have been associated with smoked mussels (Brett *et al*. 1998; Baek *et al*. 2000), so it seems likely that HAV and NoV would also survive some smoking processes unless the product was canned after smoking.

4.8 Other Techniques.

A report by Mormann *et al*. (2010) claimed that cooling, freezing, acidification, and pasteurization were ineffective in inactivating NoV. We would temper that claim somewhat, as some losses in viral infectivity might occur for cooling and freezing, but not at the levels desirable for a food processing intervention. In fact. cooling of viruses tends to preserve viruses and would not be expected to exert a major role in reducing virus titers. Likewise, enteric viruses are accustomed to the acidic environment of the human gut and are, therefore, acid resistant, except at very low pH's (e.g., < pH 3.0). Mussels subjected to the marinade process were reported to retain NoV and HAV after 4 weeks at pH's as low as 3.75, but FCV was readily inactivated by the low pH (Hewitt and Greening 2004). The determination of NoV and HAV infectivity in that study was based on RT-PCR, which as previously stated, is not a good indicator for virus infectivity. Finally, there is some evidence to suggest that virus titers are reduced with drying/desiccation, which may be applicable to viruses on the surface of processing

equipment. Although dehydrated shellfish are not a widely known commercial product, there are reports that oysters have been freeze dried, placed into capsules, and marketed as health foods in New Zealand.

5. DETECTION METHODS

Viruses are present in shellfish in very low numbers. Nevertheless, they are present in sufficient quantities to pose a health risk as presented above. This low level of contamination has made it necessary to develop highly sensitive viral extraction methods to ensure virus recovery from shellfish tissues. The observation that viruses are concentrated in digestive diverticulum tissues led to the development of a method that represented a major step in the improvement of extraction methodologies (Metcalf *et al*., 1980; Atmar *et al*., 1995). This observation was subsequently confirmed by detection of hepatitis A virus (HAV) (Romalde *et al.*, 1994) as well as through the tissue-specific quantification of infectious enteric adenoviruses and rotaviruses in mussels previously contaminated by bioaccumulation of such viruses and similarly of Norwalk virus in oysters and clams (Abad *et al*., 1997; Schwab *et al*., 1998). Analysis of digestive tissues provides several advantages, including increased sensitivity, decreased processing time and decreased interference with RT-PCR by inhibitory substances (Atmar *et al*., 1995). Focusing the analysis of shellfish on the digestive tissues enhances assay performance by eliminating tissues (i.e. adductor muscle) that are rich in inhibitors (Atmar *et al*. 1995). The digestive tissues represent about one tenth of the total animal weight for oysters and mussels. With the exception of small species, such as clams or cockles, in which dissection may be technically difficult, most of recent methods are based on dissected tissues and thus will be discussed here. Extraction of enteric viruses from shellfish is based on several steps: virus elution from shellfish tissues, recovery of viral particles, and then virus concentration (Table 2) (EFSA, 2012). The weight analyzed generally ranges from 1.5 to 2 g of digestive tissues. Some recent methods propose larger weights for the first step but thereafter analyze only a fraction of the extracts (Boxman *et al*., 2006). Viruses are eluted from shellfish digestive tissues using various buffers (i.e. chloroform-butanol or glycine) before being concentrated either by polyethylene glycol precipitation or ultracentrifugation (Atmar *et al*., 1995; Nishida *et al*., 2003; Myrmel *et al*. 2004; Milne *et al*. 2007). Direct lysis of virus particles has also been used, including methods utlizing proteinase K or Trizol to destroy shellfish tissues or Zirconia beads and a denaturing buffer for virus and/or nucleic acid elution (Lodder-Verschoor *et al*., 2005; Kittigul *et al*. 2008; Umesha *et al*. 2008; Lowther *et al*. 2008). In addition to the in-house protocols that have been used for nucleic acids extraction and purification (Le Guyader and Atmar, 2007), a number of commercial kits can also be successfully applied to accomplish this task. Advantages of the commercial kits used for nucleic acid purification include their reliability, reproducibility and ease of use. Most of these kits are based on guanidium lysis followed by capture of nucleic acids on columns, beads or silica (Nishida *et al.* 2003; Lodder-Verschoor *et al.* 2005; Costafreda *et al*., 2006; de Roda-Husmann *et al*., 2007; Kingsley 2007; Milne *et al*., 2007; Fukuda *et al*.,2008; Nenonen *et al*. 2008; Umesha *et al*., 2008). One of the goals of extraction methods is to remove inhibitors of the RT and PCR reactions sufficiently to allow detection of viral nucleic acids. Polysaccharides present in shellfish tissue are at least one substance that can inhibit the PCR reaction (Atmar *et al*., 1993). Elimination of inhibitors is difficult to evaluate and depending on the time of the year and shellfish life, different compounds may be present (Di Girolamo *et al*. 1977; Burkhardt and Calci, 2000). Internal amplification control standards are used to detect the presence of significant sample inhibition, and the amount and frequency of sample inhibition has varied depending upon the shellfish tissue being analyzed (Atmar *et al*.,1995; Schwab *et al*., 1998; Le Guyader *et al*., 2000). Recent advances in food virology re-enforce the need of harmonization of methods as well as addressing quality assurance and quality control (Pinto and Bosch, 2008). The addition of an external virus to a shellfish sample has been proposed as a control to evaluate the extraction efficiency of molecular virus detection methods (Costafreda *et al*., 2006; Nishida *et al*., 2007; Lowther *et al.*, 2008). An ideal candidate would have the following properties: (1) it would be an encapsidated RNA virus with properties similar to the enteric viruses contaminating shellfish, (2) it would normally not be present in field samples (thus RNA phages may be problematic), and (3) it would be non-pathogenic. Based on these considerations Costafreda *et al.* (2006), proposed to use a mengovirus strain MC0 as a control for extraction efficiency.

Table 2: Overview of methods used for virus elution, concentration and nucleic acid (NA) extraction based on RT-PCR detection in shellfish. (EFSA, 2012).

Shellfish mass	Virus elution	Virus concentration	Mass extracted	NA extraction	% analysed
Oysters					
25g	Glycine	PEG	0.5g	QIAamp (Qiagen)	3
50g	Water	PEG, precipitate	0.5g	Boiling	nc
18g	Glycine	Ultracentrifugation	1g	GuSCN	10
50g	Sonication	PEG	nc	GuSCN	nc
25g	Glycine	PEG	0.4g	Tri-reagent (Sigma)	10
1.5g DT	CHC13-but, Catfloc	PEG	1.5g	Prot. K, CTAB	20
1.5g DT	Gycine-threonine	PEG	0.12g	GuSCN+ QIAamp	16
1.5g DT	Chloroform-but, Catfloc	PEG	1.5g	Prot. K, CTAB	20
1.5g DT	PBS pH7.4, CHC13-but, Catfloc	Ultracentrifugation	1g	QIAamp	25
1.5g DT	Zirconia beads	nc	0.09g	RNEasy (Qiagen)	6
DT of 1 oyster	Stainless-steels beads	nc	nc	Silica & guanidium	17
2g DT	Proteinase K	nc	0.01g	GuSCN	4
10g DT	TRIzol (Gibco)	nc	0.08g	GuSCN	8
1.5g DT	Zirconia beads	nc	nc	RNeasy	5
nc	Glycine, pH 10	Ultracentrifugation	nc	Silica-method	10
DT of 3 oysters	Phosphate saline buffer	PEG	1g	QIAamp	5
5g DT	Buffer pH 8 + Prot. K	nc	0.05g	Silica & guanidium	10
25g flesh	Adsorption-alkaline elution	PEG (twice)	5g	flesh RNeasy	3
DT of 6 oysters	Buffer pH 8 + Prot. K	-	nc	Silica & guanidium	11
1.5g DT	Glycine pH 9	PEG	1.5g	Nuclisens (BioMerieux)	20
5g DT	Finely chopped + Prot K	-	0.15g	RNeasy	4
25g flesh	Adsorption/elution	PEG (twice)	25g flesh	RNeasy	6
25g flesh	Adsorption/elution	PEG (twice)	25g flesh	RNeasy	6
2g DT	TPB and CHC13-but	PEG	nc	Silica & guanidium	nc
Mussels					
20g	Glycine, Catfloc	Antigen capture	0.04g	QIAamp	21
50g	Glycine	PEG	nc	Guanidium, CsCl	100
100g	Glycine	Ultracentrifugation	1g	GuSCN	5
10g	Glycine	PEG	0.8g	RNeasy	50
25g	Threonine	PEG	nc	GuSCN	2.5
25g DT	Glycine	Ultracentrifugation	1.5g	TRIzol (Gibco) + Boom	6
2g DT	Glycine	Ultracentrifugation	0.1g	GuSCN	10
75xg total	Glycine buffer pH 9.2	PEG	8.3g	Guanidium	100
1.5g DT	Glycine buffer pH 9.5	PEG	1.5	Nuclisens	5
25g DT	Glycine buffer pH 9.2	PEG (twice)	0.75g	Nucleospin RNA (M-N)	4
Clams					
25g	Glycine, CHC13	Ultracentrifugation	1.25g	Nucleospin RNA	10
1.5g DT	CHC13-but, Catfloc	PEG	0.07g	RNeasy	6
1g DT	PBS	Ultracentrifugation	nc	QIAamp	1.5

Mengovirus, a *Picornaviridae* family member, was initially proposed as a control in validation studies of HAV removal in blood products manufacturing by several agencies such as the European Agency for the Evaluation of Medicinal products or the American Food and Drug Administration (Pinto and Bosch, 2008). Advantages of mengovirus are that it is unlikely to naturally contaminate shellfish, it is non-pathogenic for humans and it can be grown in cell culture.

The use of a single extraction control for different enteric viruses that may be detected in shellfish or other type of food is also considered to be important for method standardization (European working group CEN/Tag4) and for comparisons between different laboratories. Since the most important shellfish-borne viral pathogens (enteric hepatitis viruses A and E and noroviruses) are either non-culturable or grow only poorly in cell culture, RT-PCR and real-time RT-PCR have become the methods of choice for their detection (Straub *et al.*, 2011). In addition to the problems posed by the presence of inhibitory substances in samples, there are other difficulties encountered when molecular analyses are performed for the detection of viruses in shellfish samples. These include low virus concentrations in the sample and genomic diversity of the contaminating viruses. The extraction-concentration procedure is not virus specific, allowing the nucleic acid of several viruses to be extracted simultaneously. RT-PCR must be performed under stringent conditions and confirmed by hybridization. A number of reviews on RT-PCR methods are now available that address issues related to these methods, including assay specificity and sensitivity (Wyne-Jones, 2007; Le Guyader and Atmar, 2007). Real-time PCR assays allow the combination of RT, PCR and confirmatory hybridization assays in a single well, and these assays are now being used to detect enteric viruses in shellfish (Nishida *et al*., 2003; Loisy *et al*., 2005; Jothikumar *et al*., 2005; Costafreda *et al.*, 2006; Lowther *et al*. 2008; Stals *et al.*, 2012). This technology significantly shortens the time needed for virus detection by removing the need for gel electrophoresis and the additional hybridization step. When extraction, RT and PCR efficiencies are measured, virus quantification in the sample can be estimated (Costafreda *et al*., 2006). The efficiency of the virus nucleic acids extraction is evaluated through the use of a model virus (as described above) while the efficiency of the RT-PCR reaction must be tracked by amplification of a RNA standard using the same combination of primers and probe used for virus detection (Pinto and Bosch, 2008). Such internal RNA controls have also been used for the detection of amplification inhibitors in qualitative assays (Schwab *et al*., 1998; Le Guyader *et al*., 2003).

6. QUANTIFICATION

The development of quantitative molecular assays for the analysis of shellfish has allowed estimates of the level of virus in naturally contaminated shellfish. However, relatively few data are currently available in this area. One report estimated the amount of HAV in coquina clams implicated in an outbreak to be between 7.5x103 to 7.9x 105 genome copies per g of digestive tissues (Coastafreda *et al*., 2006). Another recent report used a semi-quantitative approach for norovirus detection to compare the levels of virus contamination between sites without describing the amount of shellfish analyzed (Lowther *et al*., 2008). Japanese investigators estimated the levels of norovirus contamination in oysters collected from two areas range from <100 copies to 7.9x 103 genome copies per g of digestive tissues in two consecutive studies (Nishida *et al*. 2003, 2007). These estimates did not include an adjustment for extraction efficiency, as proposed by Costafreda *et al.* (2006). Similar data have been obtained for the levels of norovirus contamination in oysters from France (Le Guyader *et al*., 2008). Quantitative estimates of norovirus contamination in shellfish implicated in outbreaks are even more uncommon. By using MPN PCR or real-time RT-PCR, about 100 copies of genome/g of digestive tissues were found in oysters in France (Le Guyader *et al*., 2003, 2006, 2008). This level of contamination is sufficient to cause human infection based upon the low infectious dose of the Norwalk virus, prototype for norovirus, in a volunteer study (Teunis *et al*., 2008). The availability of quantitative assays offers the potential to perform risk assessments associated with the consumption of virus-contaminated shellfish.

7. PERSPECTIVES

Although seafood can generally be regarded as a wholesome, safe, and nutritious food, it may occasionally pose consumer risks. Regulations are currently based on routinely monitoring shellfish for fecal bacteria to determine their sanitary quality. However, viral contamination of bivalve molluscs is currently recognised as one of the major causes of illness associated with seafood. Monitoring viral contamination is complex and must take into account different factors such as detection methods, technology, social demands and the sustainable development of aquaculture. Recent advances in technology, especially in developing molecular tools, make it possible to look for pathogens directly in shellfish implicated in outbreaks. Evaluation of seafood for the presence of norovirus and hepatitis A virus for regulatory purposes has been recommended by experts involved in EU, FAO and WHO working groups. Mandatory surveillance for virus may be implemented in the near future but additional information including level of exposure and virus infectious dose are still needed to assess risks. Different actions may be considered to lower the contamination of shellfish bed in coastal area:

Reduction of fecal input in coastal areas. Water quality studies have demonstrated the feasibility of determining the main sources of fecal contamination and to identify the critical points in the catchments. Hydrodynamical models when applied to these contaminants, even if they need further development and have to be validated by data bases, could lead to rational bases for the choice of treatment levels based on results from screening purposes to limit the contamination.
Implementation of warning systems: in developed countries, some data are already available on outbreaks occurring in the population, so, associated with forecast information, salinity, STP failure and other, a warning system could lead to real-time assessments of water quality in bathing or harvesting areas. The development of new tools for rapid pathogen detection (for example micro-array) may help to collect additional information on the presence of pathogens in sewage or waters.
To limit shellfish contamination, the most desirable and effective option is to reduce the viral input. Villages, small towns and dwellings must be equipped with small individual treatment tanks to comply with the regulations. New technologies may be needed to improve removal of viruses from sewage effluents (Shannon *et al.*, 2008). Another

solution could be the relocation of shellfish aquaculture away from the contamination sources.

Other factors will also need to be considered to protect the consumer and to provide safe shellfish on the market. An important aspect of monitoring concerns the sustainable development of aquaculture. This development is closely linked to environmental quality in shellfish breeding areas. The nested–regulations set up for water quality, bathing areas and shellfish growing areas, if well applied, should provide the guarantees and the management tools. There are promising examples of coastal management designed to reduce the fecal load which could make recovery of water quality feasible. Used in association with early warning systems, they could help ensure shellfish quality and increase consumer confidence and thus, would greatly contribute to sustainable development of aquaculture. Shellfish have long been recognized as being beneficial to human health and this benefit should also be taken into consideration in managing the coastal areas and preserving the water quality.

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