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Norovirus and other human enteric viruses in Moroccan shellfish

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Abstract:

The aim of this study was to evaluate the presence of human enteric viruses in shellfish collected along the Mediterranean Sea and Atlantic Coast of Morocco. A total of 77 samples were collected from areas potentially contaminated by human sewage. Noroviruses were detected in 30 % of samples, with an equal representation of GI and GII strains, but were much more frequently found in cockles or clams than in oysters. The method used, including extraction efficiency controls, allowed the quantification of virus concentration. As in previous reports, results showed levels of contamination between 100 and 1,000 copies/g of digestive tissues. Sapoviruses were detected in 13 % of samples mainly in oyster and clam samples. Hepatitis A virus was detected in two samples, with concentrations around 100 RNA copies/g of digestive tissues. Only two samples were contaminated with enterovirus and none with norovirus GIV or Aichi virus. This study highlights the interest of studying shellfish samples from different countries and different production areas. A better knowledge of shellfish contamination helps us to understand virus levels in shellfish and to improve shellfish safety, thus protecting consumers.

Keywords: Shellfish ; Norovirus ; Hepatitis A virus ; Environmental conditions ; Quantification ; Morocco ;

1. Introduction

Shellfish consumption is becoming increasingly popular in Morocco, where they are considered as a healthy food both by the local population and by tourists visiting coastal areas. Trading and exportation may also be considered as a good source of income for producers. As in many countries, sanitary controls are based on *Escherichia coli* counts in shellfish flesh and liquor (Ministerial Decision N° 1246/01 Ministry of Maritime Fishing, Moroccan Government). Although shellfish grown in good quality areas (such as class A areas in the EU or good quality areas in the US) are rarely contaminated by human enteric viruses, it has been known for more than 30 years that bacteria and viruses show differences in terms of concentration, accumulation and depuration in shellfish (Metcalf 1982). As a consequence, absence of virus contamination cannot be reliably deduced from failure to detect bacterial contamination.

Among human enteric viruses, noroviruses (NoVs) are recognized as being the leading cause of epidemics and sporadic cases of gastroenteritis in all age groups (Atmar 2010). They are discharged in large amounts in sewage and, being very resistant to inactivation, have been detected in wastewater treatment plant effluents, surface waters and environmental samples (Wobus and Nguyen 2012). The sanitary consequences are contamination of drinking water and foods, including shellfish, leading to disease outbreaks among consumers (Maalouf et al. 2010; Lopman et al. 2012). NoVs are members of the *Caliciviridae* family and are highly genetically and antigenically diverse. Based on relatedness of the VP1 capsid protein, they are classified into five genogroups (G), with GI, GII and GIV infecting humans. In humans, different strains bind to different histo-blood group antigens (HBGAs), complex glycans present on many types of cell (Tan and Xiang 2011). Such ligands are also important for their bioaccumulation in oysters and, presumably, in other shellfish species (Tian et al 2007; Zakhour et al. 2010; Maalouf et al. 2011).

Many other human enteric viruses may be detected in the environment and in shellfish, such as sapovirus (SaV), enterovirus (EV), Aichi virus (AiV) or hepatitis A virus (HAV) (Ueki et al. 2010; Woods et al. 2010). All these viruses have been implicated in some disease outbreaks, for example SaV and AiV in Japan and France (Yamashita et al. 2000; Le Guyader et al. 2008; Nakagawa-Okamoto et al. 2009), or HAV in Spain (Pinto et al. 2009). The aim of this study was to evaluate the presence of these human enteric viruses in shellfish collected along the coasts of the Mediterranean Sea and Atlantic Ocean in Morocco.

2. Material and methods

2.1. Shellfish sampling and preprocessing

Bivalve molluscan shellfish were collected from two areas over several years, from October 2006 to August 2010. Moroccan cockles (*Acanthocardia tuberculatum)* and smooth clams (*Callista chione)* were collected between 2006 and 2008 from the Martil coast (Mediterranean Sea), site 1 and 2 (Figure 1), over 14 months. Oysters (*Crassostrea gigas*) were collected from Oualidia, site 3, (Atlantic Ocean) over 13 months during 2009 and 2010. More than 61% of samples (47/77) were collected between October and April, with no difference for the two areas. Shellfish samples were shipped to the Moroccan laboratory (Pasteur Institute, Virology laboratory) in refrigerated boxes and dissected on arrival. Each sample consisted of at least of 12 individuals for oyster and 30 for the other species. On arrival, shellfish were washed, shucked and their stomachs and digestive diverticula (DT) were removed and frozen at -80°C in 1.5 g portions. All analyses were performed within a short period of time following sample collection in order to optimize subsequent comparisons between different samples.

2.2. Virus concentration and nucleic acid extraction

Briefly, DT were homogenized, extracted with chloroform-butanol and treated with Cat-floc (Calgon, Ellwood City, PA, USA). Viruses were then concentrated with polyethylene glycol 8000 (Sigma, St Quentin, France) precipitation (Atmar et al. 1995). Viral nucleic acids (NA) were extracted using Nuclisens kit (BioMerieux, Lyon, France), suspended in 100 µl elution buffer with 20 units of RNAse inhibitor (Invitrogen, Saint-Aubin, France) and analyzed immediately or kept frozen at -80°C (Le Guyader et al. 2009).

2.3. Virus detection and quantification

NA extracts were screened by real-time RT-PCR (*r*RT-PCR) using the previously published primers and probe for NoV (GI, GII and GIV) and for SaV, HAV, AiV and EV (Oka et al. 2006; Trujilo et al. 2006; Le Guyader et al. 2008). *r*RT-PCR was performed on a MX3000 (Stratagene, Massy, France), using the Ultrasens® one step Quantitative RT-PCR System (Invitrogen). All samples were analyzed in duplicate using 5 µl of undiluted or ten-fold diluted RNA extracts. Two negative amplification controls (water) were included in each amplification series and no more than six samples were analyzed in a *r*RT-PCR assay. Precautions, such as the carrying out of certain steps in isolated rooms and the use of filter tips, were taken to prevent false positive results.

The cycle threshold (C_T) was defined as the cycle at which a significant increase in fluorescence occurred (i.e. when fluorescence became distinguishable from background). To be considered as positive, all wells had to yield a C_T value $\leq$ 41. The number of RNA copies present in positive samples was estimated using standard curves. To be included in the quantitative analysis, all wells had to yield a C_T value < 39. The final concentration was then determined based on the NA volume analyzed (5 µl of 100 µl of NA extract), and the measured weight of DT (1.5 g analyzed) (Le Guyader et al. 2009).

2.4. Quality controls

The efficiency of virus extraction procedures was determined for each extraction by seeding 10^4 50% tissue culture-infective doses of mengovirus (MgV) prior to sample processing, and determining MgV recovery by *r*RT-PCR, as previously described (Pinto et al. 2009). The extraction efficiency was evaluated by comparing the C_T value for the mengovirus-positive amplification control with the C_T value for a sample and was classified as poor (<1%), acceptable (1 to 10%), or good (>10%). All extracts were also checked for the absence of inhibitors (Le Guyader et al. 2009).

2.5. Statistical analysis

As the groups of samples were independent, a Fisher exact test was performed to compare NoV and SaV contamination using StatGraphic (version 15.1.02) (Statpoint, Inc., Herndon, VA, USA). A *P* value of <0.05 was considered as significant.

3. Results

3.1. Extraction efficiency

A total of 77 shellfish samples were collected, displaying an average MgV extraction efficiency of 16.4%. More than half of the sample (53%) displayed a good extraction efficiency (above 10%), and 30 samples showed an acceptable extraction efficiency. However, despite repeated extraction, six samples still had a poor extraction efficiency (Table 1). Among the samples analyzed, 14 were cockles and 29 were clams, collected in the northern part of Morocco, and 34 samples were oysters, collected in the southern part of Morocco. Cockles and clams belong to the order *veneroida* and live in the sediment; oysters belong to the order *ostreoida* and live in the water above the sediment. If we consider these three groups, four of the six samples with poor extraction efficiencies were cockle samples. Among the other cockle samples, five had an acceptable extraction efficiency and five had a good extraction efficiency. Overall, the average extraction efficiency for these samples was 7.4 %. For the clam samples, only one had a poor extraction efficiency, and most had a good extraction efficiency. For the oyster samples, the average extraction efficiency was about 18.5%, with 19 of the 34 samples analysed presenting good extraction efficiencies and one a poor extraction efficiency. Considering the six samples with poor extraction efficiency, five were negative for all viruses assayed and the one cockle sample was positive but thus was not considered for quantification.

3.2. Viral contamination

Among the 77 samples analyzed, 23 were positive for NoV, ten for SaV, two for EV and two for HAV (Table 2). Among NoV positive samples, nine were found contaminated only by NoV GI, seven by NoV GII and seven by both GI and GII NoV. No sample was found contaminated by NoV GIV, or AiV.

Regarding NoV contamination, only one oyster sample was found to be contaminated (3%) compared with 41.4% of the clam samples and 71% of the cockle samples, making oyster samples significantly different from cockles and clams (p=0.0001 and p=0.0003, respectively). A significant difference was also observed for SaV contamination between oysters and clams (p=0.0237) but not between oysters and cockles (p= 0.657). No difference was observed for EV or HAV contamination between oysters and cockles (only one oyster and one cockle sample being contaminated).

3.3. Viral concentration

Among the ten cockle samples positive for NoV, one sample could not be quantified due to poor extraction and two were under the limit of quantification (i.e. less than 50 RNA copies/g of DT). For the seven samples for which quantification was possible, the geometric mean concentration was 278 RNA copies/g of DT. Among the 12 clam samples positive for NoV, quantification was possible on seven samples. The geometric mean concentration was 208 RNA copies/g of DT. The positive oyster sample was under the limit of quantification.

Sapovirus was detected without any other viruses in five oyster samples. Four of these samples were suitable for quantification and the geometric mean concentration was 141 RNA copies/g of DT. The three cockle samples contaminated with SaV were also contaminated with NoV. Among these samples, two were suitable for quantification and gave a geometric mean concentration of 167 RNA copies/g of DT. The concentration of SaV in the positive clam sample was 16056 RNA copies/g of DT (this sample was also contaminated by NoV). The concentration of HAV detected in the oyster-positive sample was 74 RNA copies/g of

DT. The cockle sample detected positive for HAV showed a concentration of 166 RNA copies/g of DT. This sample was also found to be contaminated with GII NoV (70 RNA copies/g of DT).

4. Discussion.

Viral contamination in shellfish is a serious problem and recent papers have demonstrated contamination of different bivalve molluscs worldwide (Myrmel et al. 2004; Nishida et al. 2007; Vilarino et al. 2009; Boxman et al. 2010; dePaola et al. 2010; Greening et al. 2010; Terio et al. 2010; Woods et al. 2010; Alfano-Sobsey et al. 2012; Lowther et al. 2012; Pepe et al. 2012). Meanwhile, food exchanges between countries are increasing and imported shellfish have been implicated in outbreaks (Simmons et al. 2007; Webby et al. 2007; Pinto et al. 2009). To protect local consumers and to prevent exportation of contaminated shellfish, data are needed in different production areas. To the best of our knowledge, this is the first report on NoV contamination in Moroccan shellfish. One study reported the detection of adenovirus in 20% of mussel samples collected in Casablanca area (Karamoto et al. 2005). In Tunisia, NoVs were detected in 35% of mussel samples and HAV in 26% (Elamri et al. 2006).

In our study, shellfish were collected from Mediterranean and Atlantic coasts, each site being sampled for 14 months, but in different years. As environmental events are known to impact shellfish quality, this difference in sampling times should be borne in mind when comparing the data with those of previous studies (Suffredini et al. 2008; Maalouf et al. 2010). Winter gastroenteritis outbreaks due to NoV are predictable and, although there are variations in intensity or duration from year to year, many clinical cases occur every winter (Rohayem 2009; Atmar 2010; Phillips et al. 2010). Based on French data, the two winter epidemics during the sampling periods were similar (www.sentiweb.org), allowing some qualitative comparisons. All precautions were taken to monitor extraction efficiencies and to test for inhibitors, making us confident of the results reported in this study. This method has been used for several years now in our laboratory (Le Guyader et al. 2009) and there is no indication that the freezing procedure used to facilitate analysis is responsible for any difference in results, as the least contaminated samples (oysters) were the last to be collected.

Both study sites are known to be fecally contaminated, based on the detection of high *E. coli* levels (data not shown). Finding human enteric viruses such as SaV, HAV or EV at the two sites confirmed that both are impacted by human sewage. The SaV concentrations detected were in the same range at both sites, and HAV and EV were detected once in each area at similar concentrations. Concerning NoV, GI was detected as frequently as GII, confirming observations made in other studies and outbreak reports (EFSA 2012; Le Guyader et al. 2012). Nevertheless, an unexpected and intriguing observation was made regarding NoV contamination: There was a large difference in NoV contamination in Moroccan cockle and clam samples compared with oyster samples, which had never been reported elsewhere. Such differences were not noticed for other human enteric viruses, so we can postulate that the difference observed between oysters and cockles or clams is not due to human contamination of water but rather to characteristics of these shellfish species or to specific environmental conditions. Both sites were sampled during winter months, reducing the impact that seasonality could have had on this observation.

For many years, oysters have been known as concentrators of virus particles. However, specific binding of Norwalk virus in *C. gigas* oysters *via* a carbohydrate structure very similar to human histo-blood group A antigen was demonstrated and subsequently confirmed to occur in another oyster species (*C. virginica*) (Tian et al. 2006). In humans, genetic diversity of

NoVs is reflected in their binding capacity to various HBGA structures (Tan and Jiang 2011), and such differences also occur in oyster tissues (Tian et al. 2007; Maalouf et al. 2010). The seasonal variations observed in ligand expression are also important to note. For example in Brittany, oysters were able to bind Norwalk virus-like particles much more efficiently from January to March compared with the rest of the year, presumably in relation to the water temperature (Maalouf et al. 2010). We further demonstrated that ligand expression had a clear impact on bioaccumulation both through a laboratory study and an environmental investigation (Zakhour et al. 2010; Maalouf et al. 2011). Some environmental conditions such as water temperature, salinity, chlorophyll *a* concentration and the presence of phytoplankton may differ between Morocco and previously studied shellfish production sites (Gentry et al. 2009; Grangere et al. 2009). The ability of oysters to bioaccumulate NoVs may be low in Morocco, where temperatures resemble those of summer in Western Europe and, inversely, the bioaccumulation in clams and cockles may be greater.

In Italy, clams were found to be more contaminated than oysters (Gabrieli et al. 2007) or mussels (Suffredini et al. 2008). In Galicia, all cockle or clam samples collected were found to be contaminated compared with only 50% of mussel samples (Vilarino et al. 2009). Similarly, a more frequent contamination was detected in clam samples compared with mussel samples collected in Tunisia (Elamri et al. 2006). These animals live in contact with the sediment, which is known to be potentially contaminated by different types of enteric virus and may protect viral particles (Rao et al. 1984; Bosch et al. 1988). A recent study demonstrated that there were about 100 RNA copies of EV/g of sediment, based on samples collected in Japan (Miura et al. 2011). Also, as observed for oysters, some difference in bioaccumulation may exist depending on the species of cockle or clam in question (Tian et al. 2007). No difference was observed between clam and cockle NoV or SaV concentrations. The observed concentrations for NoV are in the same range as concentrations previously reported in shellfish-related outbreaks or market studies (Le Guyader et al. 2009; Le Guyader et al. 2010; Woods et al. 2010; Lowther et al. 2012). In contrast, HAV concentrations detected in the two positive samples seem to be below the concentration estimated to pose a risk for human consumption, especially for cockles because they are usually consumed cooked (Pinto et al. 2009).

In conclusion, this study is informative for several reasons, (i) this is the first report on virus levels in Moroccan shellfish; (ii) introduction of quality controls are important to allow result comparisons and interpretation; (iii) NoV GI was detected as frequently as NoV GII; (iv) clams or cockles living in sediment or possibly warmer waters are likely to be more contaminated than other shellfish.

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Tables

Table 1 : Extraction efficiency

Shellfish	Poor[a] (<1%)	Acceptable[a] (1-10%)	Good[a] (>10%)	Average extraction efficiency	Total no. of samples
Oyster	1	14	19	18.5	34
Cockle	4	5	5	7.4	14
Clam	1	11	17	18.3	29
Total	6	30	41	16.4	77

a: number of samples with the following extraction efficiency

Table 2 : Viruses detected in shellfish samples.

Shellfish	# samples	NoV[a]	SaV[a]	EV[a]	HAV[a]	AiV[a]
Total	77	23	9	2	2	0
Oyster	34	1	5	1	1	0
Cockle	14	10	3	1	1	0
Clam	29	12	1	0	0	0
Total	77	23	9	2	2	0

[a] abbreviations: norovirus, NoV; sapovirus, SaV; enterovirus, EV; hepatitis A virus, HAV; Aichi virus, AiV.

Figures

Figure 1 : Localization of sampling areas. Two sites are located on the Mediterranean Sea (point 1: cockle samples, point 2: clam samples) in the same area, one site (point 3: oyster samples) is located on the Atlantic Ocean.