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Detection of human enteric viruses in shellfish collected in Tunisia

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Abstract:

Aims: The aim of this study was to detect the main pathogenic human RNA enteric viruses able to persist in the environment such as astrovirus, enterovirus, norovirus and hepatitis A virus (HAV) in shellfish collected from two locations in northern Tunisia.
Methods and Results: Viruses were eluted from digestive tissues and concentrated by polyethylene glycol precipitation before nucleic acid extraction and purification. After checking for inhibitors, all viruses were detected by reverse transcription-polymerase chain reaction (RT-PCR) and confirmed by hybridization. Overall, 83% of the samples were found positive for at least one virus. Astrovirus was detected in 61% of the samples, norovirus in 35% and HAV in 26%. Surprisingly, only one sample was found positive for enterovirus.
Conclusions: The mean number of positive samples found in this study is in accordance with the data found in the literature, indicating that no real difference exists in this respect among countries studied. A notable exception is HAV, which reflects the epidemiological status of the population.
Significance and Impact of the Study: This study highlights the interest to analyse shellfish samples from different production areas. These data will be helpful to understand virus circulation and to improve shellfish safety. The results, which confirm contamination, necessitate the development of appropriate studies and monitoring in all shellfish-producing countries.Résumé

Keywords: human-enteric-viruses; reverse-transcription-PCR; shellfish

INTRODUCTION

Coastal areas can be contaminated by human sewage, which can contain more than 100 types of viruses (Metcalf *et al.*1995). When sewage treatment plants are unable to eliminate all of these pathogens, shellfish, during filter feeding activity, may accumulate viral particles. Some examples of norovirus-gastroenteritis or hepatitis A outbreaks after contaminated shellfish consumption can be found in the literature (Bosch *et al.* 2001; Le Guyader *et al.* 2003; Kageyama *et al.* 2004, Ng et al. 2005).). In addition to documented outbreaks, viral pathogens have been detected in shellfish from countries with large amounts of shellfish production or consumption, including countries in Europe and Asia (Henshilwood et al. 1998; Le Guyader et al. 2000; Croci *et al*. 2000; Formiga-Cruz *et al.* 2002; Myrmel *et al.* 2004; Kageyama et al. 2004; Cheng *et al.* 2005; Ueki et al. 2005). However overall data are scarce, particularly from Africa, and, to our knowledge, only one study on Moroccan shellfish has been published (Karamoto et al. 2005).

. Technological advances in molecular detection methods have led to the development of sensitive and specific assays for the detection of viruses, that grow poorly or not at all in cell culture, such as norovirus, or hepatitis A virus (Bosch *et al.* 2003). In Tunisia, monitoring of shellfish beds relies on *E.coli* counts according to the current European Community regulation (Directive 91/492/EC). However, bacteria have a limited predictive value for viral pathogens, and shellfish meeting bacterial sanitary criteria have been involved in outbreaks (Le Guyader *et al.* 2003; Kageyama *et al.* 2004; Butt *et al.* 2004). The aim of this study was to detect RNA enteric viruses, as norovirus and HAV are the most important human pathogens linked to shellfish consumption, in shellfish samples harvested from natural sites in northern Tunisia.

MATERIALS AND METHODS

Shellfish sampling

From July 2000 to September 2001, six mussel samples (*Mytillus galloprovincialis*) and 17 clam samples (*Ruditapes decussatus*) were collected from different sampling points in two sites (4 points numbered P1 to P4 in the lagoon called site 1, and 2 points numbered P5 and P6 in the lagoon called site 2) located in northern Tunisia. Shellfish were kept at 4°C during shipment and arrived at the laboratory one day after collection. Upon arrival to the laboratory, shellfish were processed immediately or stored at – 20°C.

Shellfish processing for virus concentration

Shellfish samples were processed on ice as described previously (Le Guyader *et al.* 2000). Briefly shellfish samples were shucked and the stomach and digestive diverticula were removed by dissection, cut into small portions, mixed, divided into 1.5 g portions and kept frozen at – 20°C until use. For analysis, each 1.5 g portions were homogenized, extracted with chloroform-butanol (1:1 v/v) and Cat-Floc (Calgon corp, Ellwood City, PA) followed by polyethylene glycol 6000 (PEG, 24% w/v- sodium chloride (1.2M) (Sigma, St Quentin, France) precipitation. Viral nucleic acid was extracted and purified from the suspended polyethylene glycol pellet by digestion with 0.2 mg of proteinase K (Amresco, Solon, Ohio) per milliliter, phenol-chloroform (Applied Biosystems, Foster city, CA) extraction, ethanol precipitation, 1.4% (wt./vol.) cetyltrimethylamonium bromide (Sigma) precipitation, and a final ethanol precipitation. Viral nucleic acid was suspended in 100 µl of RNase-free water.

Primers and probes

All primer and probes are detailed in Table 1. For astrovirus, primers and probe located in the 3' conserved region were used (Mitchell et al., 1995). Enterovirus and hepatitis A virus were detected by amplification of a fragment located in the 5' non-coding region of the genome (Bosch et al. 2001). For norovirus detection, primer sets NVp110/Nvp36, Nvp110/NI, Nvp110/SR48-50-52 were used for amplification, and probes NVp116, Nvp117, Nvp118, SR47, and SR61 were used for confirmation (Le Guyader et al., 1996, Ando et al. 1995).

RT-PCR

RT-PCR was performed according to the instructions of the murine leukemia virus RT and *Taq* polymerase supplier (Perkin-Elmer Corp.). Two microliters of extracted nucleic acids were used for reverse transcription with 2.5 µM of the upstream primer, and incubated for 30 min. at 42°C and heat denaturated for 5 min. at 95°C. After addition of the PCR master mix to yield a mixture containing 1 µM each of the downstream and upstream primers, amplification was performed for 40 cycles (94°C for 30 s, 50°C for 30 s (55°C for HAV), 72°C for 30s) with final extension at 72°C for 7 min. A negative amplification control (water) was included in each amplification series. To prevent false positive results, precautions were taken such as separate rooms, filter tips and several negative controls for the different steps. The amplified products were detected by electrophoresis on a 9% polyacrylamide gel and stained with ethidium bromide.

Detection of inhibitory compounds

To monitor inhibitory compounds, a single-strand RNA internal control (ssRNA IC) constructed from the enterovirus genome was used as previously described (Le Guyader et al. 1997). The ssRNA IC (50 to 100 RT-PCR detectable units per reaction) was added to EV-specific amplification reactions with each nucleic acid extract (Le Guyader *et al.* 2000).

Hybridization

For hybridization, the PCR product was denaturated and blotted onto a positively charged nylon membrane, hybridized for 2 h at 50°C. Probes were labeled with digoxigenin using the 3' tailing kit (Roche, Meylan, France). The hybridized probes were detected by chemiluminescence (Roche) according to the manufacturer's protocol using a Bio-Rad multi-imager. A sample was considered positive if a positive signal was obtained after hybridization with or without previous observation of a band of the expected size on the gel (Le Guyader *et al.* 2000).

RESULTS

Between the two sites, 23 samples were analyzed, 18 for site 1 and 5 for site 2. After amplification and hybridization, 4 samples were negative and 19 samples (83%) positive for at least one virus (Table 2 and 3). Astrovirus were detected in 14 (61 %) samples, norovirus in 8 (35 %) samples, hepatitis A in 6 (26 %) samples, and enterovirus in 1 sample.

In site 1, the clams collected from the point 2 appeared to be the most contaminated, as multiple viruses were detected in all but the first sample (Table 2). Four of the 5 samples

positive for hepatitis A virus were taken from site 1, mainly during January and February. All of the samples collected in February were found to be contaminated.

Very few samples were collected from site 2 but all were contaminated with astrovirus (Table 3). In June, norovirus and hepatitis A virus were also detected in point 5 and 6 respectively.

DISCUSSION

This study is the first to analyze viral contamination of shellfish beds by RT-PCR in Tunisian shellfish samples, and to our knowledge in Africa, except one report on adenoviruses in shellfish in Morocco (Karamoto et al. 2005). Though the number of samples is limited, these results allow some conclusions and comments as numerous samples were found contaminated by human enteric virus particles. Although conclusions should be made with caution since nucleic acid amplification does not tell whether the detected viruses were infectious, free viral RNA was unlikely to have been detected inside the shellfish (Atmar et al. 1996; Bosch et al. 2003).

Comparing the occurrence of viral pathogens in shellfish is difficult since few data are available in the literature and conditions are always different (including site conditions, sampling, and detection methods). However this study can be compared with a previous study conducted in France, which employed the same method, primers, and probes (Le Guyader et al.). For sites occasionally impacted with sewage, astrovirus were detected in 47% of the French samples versus 61% in this study, and norovirus in 25% of the French samples versus 35% here.

In this study, astrovirus were frequently detected (up to 60% of the samples). The prevalence of astrovirus in the samples implies that these viruses are easily transmitted through the environment, though little data are available to confirm this. However, one study using real-time RT-PCR, showed that about 10^5 genomes /100ml were detected in sewage, with no seasonal effect (Le Cann *et al.* 2004). As these viruses are resistant in water environment, they may persist for long period of time, explaining their detection all year long (Bosch *et al.* 1997; Le Guyader *et al.* 2000). On the contrary, it was surprising to detect only one sample positive for enterovirus, as these viruses are usually more frequently detected (Le Guyader et al. 2000; Formiga-Cruz et al. 2002; Shieh et al. 2003). We believe that it is not a technical problem, as the primers and probe, located in a conserved area of the genome, were validated in previous studies (Shieh et al. 1997; Bosch et al. 2001) and the efficiency of our RT-PCR was controlled by running separate amplifications on positive controls.

The most notable result is the percentage of shellfish samples positive for hepatitis A virus. About 26% were found positive, mainly in February, and most positive samples were collected in point 2 from site 1, confirming human sewage impact at this location. The low water circulation in this part of the lagoon may contribute to the contamination (Harzallah *et al.* 2003; Pommepuy *et al.* 2004). In Tunisia, the impact of hepatitis A virus infection is significant and the virus is circulating actively in the population, as 58% of the population under the age of 15 years carry antibodies (unpublished data). Similar data were obtained in Italy, Greece or Spain where the virus is circulating (Formiga-Cruz et al., 2002; Croci et al. 2000; Lopalco *et al.* 2005). It is clear from these data that it will be important to set up an efficient monitoring system for this virus in shellfish. This also confirms that epidemiologic status of the population has a direct correlation with shellfish contamination.

Noroviruses, the most common human gastroenteritis agent in all age classes, were detected in 35% of the samples. Although data are not available at present on norovirus strains circulating in the Tunisian population, we postulate that it is the same as in other countries (Widdoson *et al.* 2005) and we used primer sets and probes that can amplify almost all strains identified to date. A number of different RT-PCR assay formats have been developed, and due to the genetic diversity of norovirus no one single assay stood out as the best (Vinje et al. 2003). This is particularly important when analyzing environmental samples, which tend to have low viral concentrations. To enhance norovirus detection, we selected different primer pairs targeting small portion of the polymerase region, increasing the assay sensitivity (Le Guyader et al. 1996, Atmar and Estes, 2001). The drawback is that the amplified area is of little interest for molecular epidemiology, though that was not the aim of this short study since no epidemiological data from the local population was obtainable. The other main concern when analyzing environmental samples is the specificity of the amplicons. All PCR products were confirmed by hybridization and these primers have been validated either in clinical or environmental studies (Bon et al. 2005; Maunula et al. 2005), demonstrating also that they are still efficient on new strains currently circulating though they were designed few years ago. About 35% of the samples were found positive for norovirus. This is comparable to data published in a collaborative study where 41%, 16%, 10% and 2% of shellfish samples collected in Sweden, United Kingdom, Spain and Greece, respectively were positive for norovirus (Formiga-Cruz et al. 2002), 7% in Norwegian samples (Myrmel et al. 2004), or 60% in Japanese samples (Ueki et al. 2005). Recently, oysters imported to Hong Kong have been found contaminated by noroviruses from 0 to 67% depending on the country of origin

(Cheng et al. 2005). The development of sensitive and simple methods for their detection in the environment would help to obtain more data (Loisy *et al.* 2000, 2005).

The mean number of positive samples in this study is in accordance with data found in the literature, indicating that viral contamination of shellfish is similar among countries investigated, with the exception of HAV, which reflects the epidemiological status of the population. Our results confirm that shellfish in Morocco were contaminated with several viral pathogens. To protect human health worldwide, research should be dedicated to better understand virus circulation and to develop appropriate monitoring in all shellfish-producing countries. This will be helpful to understand virus circulation and to improve shellfish safety;

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Table 1 Primers and probes used to detect human enteric viruses in shellfish nucleic acid extracts.

Virus	Primers	Probes	Locations*	reference
AV	Mon2-Mon1	Ap	6709-6797	Mitchell *et al*. 1995
EV	PV444-P1	P2	225-460	Bosch *et al*. 2001
HAV	HAV240-HAV69	HAV probe	68-240	Bosch *et al*. 2001
NoV	NVp110-NVP36	NVp116, 117	4487-4865	Le Guyader *et al*. 1996
	NVp110-NI	NVp118	4768-4865	Le Guyader *et al*. 1996
	NVp110-SR48-50-52	SR47,61	4766-4865	Ando *et al*. 1995

* nucleotide positions are in reference to AV serotype 2 (L13745), poliovirus type 1 (Mahoney strain)(V011148), HAV strain HM175 (M16632) and Norwalk virus (M87661).

Table 2 Detection of human enteric viruses in shellfish collected in site 1.

Date	P1 clams	P2 clams	P3 mussels	P4 mussels
28/07/2000	Negative*	negative	negative	EV, NoV
12/01/2001	AV	AV -HAV	NoV	no sample
09/02/2001	HAV	AV, HAV, NoV	no sample°	HAV, NoV
18/04/2001	NoV	AV, NoV	negative	no sample
31/05/2001	AV	AV, NoV	AV	no sample
11/09/2001	AV	AV, HAV	no sample	no sample

*: sample detected negative for all viruses after RT-PCR and hybridization,

: sample detected positive for astrovirus (AV), hepatitis A virus (HAV), enterovirus (EV), or norovirus (NoV) after RT-PCR and hybridization.

°: no sample was collected.

Table 3 Detection of human enteric viruses in shellfish collected in site 2

Date	P5 clams	P6 clams
31/07/2000	AV*	AV
12/06/2001	AV, NoV	AV, HAV
21/09/2001	AV	no sample°

*: sample detected positive for astrovirus (AV), hepatitis A virus (HAV), or norovirus (NoV) after RT-PCR and hybridization.

°: no sample was collected.