Enteric viruses in molluscan shellfish

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SUMMARY

One hundred and thirty-seven bivalves were collected for environmental monitoring and the market; all the samples were analysed by RT-PCR test.

Bacteriological counts meeting the European Union shellfish criteria were reached by 69.5% of all the samples, whereas the overall positive values for enteric virus presence were: 25.5%, 18.2%, 8.0% and 2.1% for Rotavirus, Astrovirus, Enteroviruses, Norovirus, respectively. Mussels appear to be the most contaminated bivalves, with 64.8% of positive samples, 55.7% and 22.7% respectively for clams and oysters, whereas in the bivalves collected for human consumption 50.7% were enteric virus positive, as compared to 56.4% of the samples collected for growing-area classification. The overall positive sample was 54.0%.

KEY WORDS: Bivalves, Rotavirus, Astrovirus, Enterovirus, Calicivirus, Molecular biology

Faecal bacteria are normally accepted under European Law (Anonymous, 2004, 2005) as the criterion for the hygienic quality of both shellfish and their growing waters, but their presence does not reflect the presence of human enteric viruses (Croci *et al.*, 2000). In recent years attempts have been made to use other indicators, such as phages, in particular the B40-8 phage of the *Bacteroides fragilis* (Havelaar, 1987*).* However, the use of these microorganisms as indicators of viral presence has been widely questioned (Xavier Abad *et al.*, 1997). Several viral outbreaks have been associated with the consumption of bivalves that met the bacteriological standards (Sugieda *et al.*, 1996).

In past years the method for detecting enteric

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viruses was based on virus infectivity on cell lines, but this method was expensive, time-consuming and difficult to perform because of the absence of a susceptible cell line for each enteric virus.

The nucleic acid based method has proven to be highly specific and can detect even very small amounts of viral particles in the mussel homogenates (LeGuyader *et al.,* 2001). However, the successful application of the molecular method for enteric virus identification has been limited by the presence of RT-PCR inhibitors in shellfish bodies and several methods for viral genomic extraction have been reported (Casas and Sunen, 2001). The dissection of the digestive tract and diverticula (hepatopancreas) appears to reduce the presence of inhibitors and to increase the sensitivity of the molecular method (LeGuyader *et al.,* 2006).

The aim of the present study was to evaluate the presence of enteric viruses, like Rotavirus (RV), Astrovirus (AV), Enterovirus (EV) and Norovirus (NV), in bivalves collected both for environmental monitoring and sale, as well as the possibility of using molecular methods to detect enteric

viruses and improve food quality given the inadequacy of standard bacteriological limits.

One hundred and thirty-seven molluscan shellfish were collected during the one-year study and monitored for the presence of RV, AV, EV and NV. Specifically, 62 samples were collected for environmental monitoring and 67 for sale, while, with regard to bivalves, 61 were clams (*Tapes decussates* and *semidecussatus)*, 54 mussels (*Mytilus galloprovincialis)* and 22 oysters (*Crassostrea gigas)*. As for the source, 15 samples were obtained from France, 11 from Greece and the remainder were domestic Italian produce. Some information was lost during sample collection.

All the bacteriological analyses, Faecal Coliforms, *Escherichia Coli* (*E. Coli*) and *Salmonella* sp, were performed in accordance with the method accepted by the International Organization for Standardization (Anonymous, 1996, 2002), Italian law (Anonymous, 1995), European Law (Anonymous, 2005) as UNI CEI EN ISO/IEC 17025; whereas for the virological analyses 15 grams of digestive apparatus (hepatopancreas) were removed from the bivalves by dissection and the virus eluted. Seventy-five ml (ratio 1:5) of 0.05M glycine pH 9.5 were added, and the sample was homogenized at the maximum speed for 5' in a Waring blender. The homogenate was stirred for 15-20 min at +4 °C and centrifuged at 10,000 x g for 15 min at 4°C. The supernatant fluid was collected, and the pH was adjusted to 7.2-7.4 and concentrated with final 10% (w/v) polyethylene glycol 6000 (Merck, Milan, Italy) and 0.3 mol l^{-1} NaCl (Merck, Milan, Italy). The viruses were precipitated by centrifugation at 10,000 x g for 45 min, and the pellet re-suspended in the smallest volume of sterile phosphate buffered salts (PBS, Gibco, Milan, Italy). After 1-2 standard chloroform extraction (20% v/v), viruses in the aqueous phase were purified and the aspecific inhibitors eliminated by ultracentrifugation at 60,000 x g for 1h at 4°C in a Beckman Optima TL-100 rotor TLA 100.4.

All the samples were extracted using the QIAmp viral RNA kit (Qiagen, Milan, Italy). One fifth of the final elution volume, corresponding to 1.5 g of hepatopancreas, was used for each RT-PCR test. A specific amplifier of 155 bp, after nested RT-PCR, was obtained for EV, 121 bp for AV, 150 bp for RV, 241 bp for NV type I and 203 bp for NV type II.

The RT-PCR conditions and primer sequences were previously reported for EV (Divizia *et al.*, 1999). For RV and AV. The molecular tests were performed using the methods proposed by Villena *et al.* (2003) and Noel *et al.* (1995) respectively, for NV by Beuret *et al.* (2000). All the enzymes for RT-PCR were obtained from Promega, Milan, Italy.

The bacteriological analysis respected the European limits in 57.4% of samples, not taking into consideration the types of molluscs and their source. When the origin of the samples was considered, 67 were collected for sale, with 26 samples showing normal bacteriological values (38.8%), and 62 were collected for environmental monitoring, with 47 falling within the limits (75.8%).

The microbiological values exceeding the limits, both for *E.coli* and Faecal Coliforms, ranged from 400 to more than 54,200 MPN/100g. The limits were exceeded just for Faecal Coliforms in only 3 samples. One sample was positive for the presence of Salmonella with bacteriological standard parameters exceeding the limits. The same sample was also positive for EV.

The overall percentage of positive samples for one or more viruses was 54.0% (74 samples out of 137) (Table 1), whereas double viral contamination was present in 6.1% of the samples, and 7 samples showed RV-AV and 2 EV-AV. Norovirus genotypes I and II were both found to be present in two samples.

Sixty-one samples of clams were analysed and 34 showed specific bands of amplification: 29.5% for RV, 14.7% for AV, 9.8% for EV and 1.6% for NV, with an overall prevalence of 55.7% (Table 1). Thirty-five out of 54 (64.80%) mussel samples showed the presence of enteric virus, 31.4% positive samples for RV, 24.0% for AV , 5.5% for EV and 3.7% for NV (Table 1). Only 5 samples out of 22 (22.7%) of the oysters were found to be positive, but they showed the presence of only AV (13.6%) and EV (9.0%).

The samples collected by the Zooprophylactic Institute of the Latium and Tuscany Regions (IZSLT) can be catalogued on the basis of the source. The samples can be collected for monitoring to classify the growing-beds, and for the market, in the case of direct human consumption. Sixty-two samples were collected for monitoring and 35 (56.4%) showed a specific band of amplification (Table 2), with respective percentages of

TABLE 1 - *RT-PCR positive sample distribution for the different bivalves.*

	Sample	RT-PCR Pos (%)	RV Pos (%)	AV Pos (%)	EV Pos (%)	NV Pos (%)
Clam	61	34 (55.7)	18 (29.5)	9 (14.7)	6 (9.8)	1 (1.6)
Mussel	54	35 (64.8)	17 (31.4)	13 (24.0)	3 (5.5)	2 (3.7)
Oyster	22	5 (22.7)	0	3 (13.6)	2 (9.0)	0
Total	137	74 (54.0)	35 (25.5)	25 (18.2)	11 (8.0)	3 (2.1)

TABLE 2 - *RT-PCR positive samples, based on the purpose.*

Purpose	Sample	RT-PCR Pos (%)	RV Pos (%)	AV Pos (%)	EV Pos (%)	NV Pos (%)
E. Monitoring	62	35 (56.4)	16 (25.8)	12 (19.3)	7 (14.2)	0
Market	67	34 (50.7)	19 (30.6)	11 (16.4)	4 (6.7)	3 (2.3)
Total	129	69 (53.5)	35 (31.8)	23 (17.8)	11 (10.0)	11 (10.0)

25.8%, 19.3% and 14.2% for RV, AV and EV. No samples were positive for NV. Among the samples collected for market (67 samples), the overall percentage of positive samples was 50.7% (Table 2), with 30.6% for RV, 16.4% for AV, 6.7% for EV and 4.4% for NV.

Bivalves collected in a growing-area classified as A can be used directly for human consumption. Twenty-two out of 41 clam samples (53.6%) scored positive under molecular methods, showing the presence of all the tested enteric viruses, whereas mussels had a higher contamination: 65.3%. Oysters collected in a limited number showed only the presence of AV (25.0%). The overall positive presence was 53.1%: 42 samples out of 79.

In Italy a large percentage of the bivalves marketed is imported from either European Countries or non-EU Countries. In particular, 15 samples were imported from France and 11 from Greece. All the samples imported from Greece were positive, but only for RV, whereas 46.6% of the samples imported from France showed a specific band of amplification: one sample was positive for RV, four for AV, and two for EV.

Shellfish are able to filter seawater while feeding and to accumulate enteric viruses in their filtration apparatus (Le Guyader *et al.,* 2006). Bivalves, eaten raw or partially cooked, are the most important risk factor for the transmission of these viruses to human beings.

Detection of viral pathogens can be performed using several methods, but cell culture, at the moment, represents the only method for isolating infectious viruses (LeGuyader *et al.,* 2000). However, cell culture is not a feasible method because of the absence of a common cell line; the method is time-consuming, in addition to which immunofluorescence, ELISA or molecular tests are needed to confirm the presence of viruses. The crucial point in the application of RT-PCR is the manipulation of large amount of shellfish bodies and the presence of RT-PCR inhibitors. The knowledge that virus particles can get trapped in the stomach apparatus has resolved both problems (Le Guyader *et al.,* 2006). The dissection of the apparatus has led to a reduction of the amount of homogenate to be treated, an increase in the number of bivalve samples per test and a reduction in the molecular test inhibitor (LeGuyader *et al.,* 2000a). In our hands, 15 g of hepatopancreas were equivalent to 15, 60 and 10 mussels, clams and oysters respectively.

The extraction of human pathogens from shellfish is a complex process and the molecular methods, because of the presence of inhibitors, can be,

to a limited extent, applied to shellfish. The inhibitors in shellfish homogenate are low molecular compounds not precipitating in the ultrapellet concentration procedure. This method can reduce false negative tests improving the virological analysis (data not shown). A similar protocol of ultrapelleting was adopted by Formiga-Cruz *et al.* (2002) in an intercalibration test among several laboratories.

Three different types of molluscs have been considered: clams, mussels and oysters (Table 1). Mussels appear to be the most contaminated, with 64.8% of the samples positive, as compared to 55.7% and 22.7% respectively for clams and oysters. Unlike the Hepatitis A cases recorded by SEIEVA, 50%-60% of which are associated with bivalve consumption, in Italy there is no surveillance system to correlate the human transmission of enteric viruses associated with seafood, as reported by Sanchez *et al.* (2002). Based on our results, we can only postulate the influence of bivalves on episodes of gastroenteritis.

To date, bivalves in Italy have been tested only for the presence of HAV or to identify enteric viruses involved in an outbreak (Chironna *et al.*, 2002). Our data, however, show a high circulation of RV and AV (25.5% and 18.2%) (Table 1) and a limited presence of EV (8.0%) and NV (2.1%). Similar virological results were obtained by LeGuyader *et al.* (2000a) in seawater with occasional faecal presence, whereas bivalves in polluted seawater could present two or more viruses. In our case, double contamination was present only in 11 samples out of the 137 collected, whereas Romalde *et al.* (2002) reported 8.0% of double contamination in Galicia (Spain).

Norovirus was identified in only three samples with a double contamination from genotypes I and II. Noroviruses have been identified, in low number of positive samples: 3 (2.1%) in bivalves linked to an outbreak (Prato *et al.*, 2004). The apparently low number of positive NV can be explained by the limited circulation of this virus, or by a lower standardization of the RT-PCR test. In effect, diverse sets of primers with different levels of sensitivity have been reported in the literature. We cannot exclude that the primer set used was not sensitive enough in our hands.

The only parameters considered in the European Community to guarantee safe consumption of bivalves and the quality of their growing-waters classified as A are the *E. Coli* (230 MPN/100g) and *Salmonella* sp (absence in 25g). However, the absence of bacteria does not guarantee an absence of viruses (Croci *et al.*, 2000). This study once again confirms the lack of correlation between bacteria and virus presence; in fact 42 samples out of 79 (53.1%) collected from a growing-area classified as A presented a specific band for one of the enteric virus and, according to the European Community (Anonymous 1995), the samples collected from growing-area A can be used for human consumption without any depuration or treatment.

Italy, like other European countries, is an importer of molluscan shellfish from several countries, some of them not European.

The possibility of introducing new strains of enteric viruses or new viruses not endemic in the country, like Hepatitis E virus, must be carefully monitored. In this study, 100% of the samples from Greece and 40% from France were positives for RV or AV.

In conclusion, in evaluating bivalves for safety, bacteriological parameters alone are not sufficient for predicting viral presence. Although the RT-PCR test does not yield the infectivity of the enteric viruses tested, only this fast method is able to evaluate the presence of enteric virus not usually investigated, such as RV, AV, EV and NV, and, according to other authors (Le Guyader *et al.*, 2000), our study confirms the feasibility of the molecular methods.

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