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Qualitative and quantitative assessment of viral contamination in bivalve molluscs harvested in Italy

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ABSTRACT

Bivalve molluscs are a well documented source of viral infection. Further data on shellfish viral contamination are needed to implement European Regulations with sanitary measures more effective against viral pathogens. To this aim, 336 samples of bivalve molluscs (185 mussels, 66 clams, 23 oysters and 62 samples from other species) collected in harvesting areas of class A and B of four Italian Regions were analyzed for qualitative and quantitative determination of hepatitis A virus (HAV) and Norovirus (NoV) GI and GII, using real time RT-PCR.

The results showed a wide diffusion of viral contamination in the shellfish production areas considered. HAV prevalence was low (0.9%) with contamination levels that varied from 5 to 7×10^2 copies/g. On the contrary, NoV showed a high prevalence (51.5%), with a large variability according to the group considered (e.g. 47.8% for *Crassostrea* in Veneto, 79.7% for *Mytilus* in Campania, 84.6% for *Tapes* in Sardinia). NoV contamination affected class A and class B production areas to a different extent, with a statistically significant difference in both contamination prevalence (22.1% vs. 66.3%; $p < 0.0001$) and quantity (average contamination level of 3.1×10^2 vs. 1.9×10^3 copies/g; $p < 0.05$). The different species analyzed from class B harvesting areas (*Mytilus*, *Tapes*/*Ruditapes* and *Crassostrea*) showed a NoV prevalence respectively of 70.3%, 66.0% and 47.8% but comparable NoV contamination levels (between 8.4×10^2 and 4.9×10^3 copies/g). Other two bivalve species considered in the study (*Donax* spp. and *Solen* spp.) showed a relevant NoV presence (40.0% and 34.4% of samples). Finally, samples analyzed before and after commercial purification treatment showed a decrease of contamination prevalence after the treatment, but inconsistent results were recorded on NoV levels.

The data obtained, together with other quantitative information to estimate consumer exposure, in association with studies on dose–response and on the effectiveness of post-harvest treatments, will provide a useful tool for the definition of microbiological criteria related to the different shellfish species.

1. Introduction

Bivalve molluscs are internationally recognized as a potential vehicle for human enteric virus transmission (Bellou et al., 2013; Lees, 2000; Potasman et al., 2002), especially when consumed raw or improperly cooked. These filter feeding animals may accumulate in their tissue particles present in the surrounding water, including viruses (Burkhardt and Calci, 2000). Current hygienic measures, reported in the EU Reg. 854/2004 (classification of harvest areas, monitoring of production areas for *E. coli*, post harvest treatments; European Parliament, 2004) and Reg. 2073/2005 (*E. coli* and Salmonella as food safety criteria for marketed products; European Commission, 2005) are oriented to the control of fecal contamination of live bivalve molluscs (LBM). However, although these measures have been effective against bacterial pathogens with oral–fecal transmission, they are less efficacious against viral pathogens, as shown by cases where shellfish as oysters, compliant with the legislative microbiological standards, were associated with Norovirus (NoV) and hepatitis A (HAV) outbreaks (Baker et al., 2011; Guillois-Becel et al., 2009; Le Guyader et al., 2010). Acquiring knowledge on virus prevalence and levels in shellfish has therefore become increasingly important in countries with relevant production, as Italy, which is the third main European producer of bivalve molluscs, with an average of 100,000 tons per year (Bronzi et al., 2011).

For many years, the main problem to this aim has been the lack of testing methods to directly detect the enteric viruses in shellfish. In

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the past decade, however, the molecular approach has led to the development of a large number of methods (Atmar et al., 1995; De Medici et al., 2004), especially for the detection of viruses such as HAV and NoV, which grow poorly or even do not grow in cell culture (Duizer et al., 2004). Therefore, despite the awareness that polymerase chain reaction (PCR) methods may overestimate health risk by detecting both infectious and non-infectious virus (De Medici et al., 2001), PCR has become the method of choice for monitoring virus contamination of bivalve molluscs. The availability of molecular methods allowed to gather knowledge on the incidence of enteric viruses associated with shellfish-related outbreaks (Gallimore et al., 2004; Prato et al., 2004; Sanchez et al., 2002) and their spread both in Italy (Croci et al., 2007; Pavoni et al., 2013; Pepe et al., 2012; Suffredini et al., 2012) and in other EU countries (Formiga-Cruz et al., 2002; Le Guyader et al., 2000; Myrmel et al., 2004).

More recently, a standardized method based on RT-qPCR detection of NoV and HAV in food, developed by the European Committee for Standardization (CEN) working group TC 275/WG6/TAG4, has been published as an ISO technical specification (ISO/TS 15216–1:2013; International Organization for Standardization (ISO), 2013a), providing a standardized and internationally recognized tool to quantify the viral contamination in shellfish and other foods. Clinical studies have demonstrated a dose response relationship between the amount of NoV genome and the onset of the disease (de Wit et al., 2007; Teunis et al., 2008; Visser et al., 2010). In addition, a correlation has been found between the number of viral genome copies in bivalve shellfish and the amount of reported illness for both HAV and NoV, suggesting that detection of high levels of virus RNA in shellfish is indicative of a high health risk (Lowther et al., 2010, 2012a; Pinto et al., 2009). Data available indicate that the NoV concentration in shellfish linked to human cases varied greatly from less than 100 to more than 10,000 copies/g (EFSA, 2012), however the lack of extensive suitable data hampered the performance of a quantitative risk analysis and the definition of microbiological criteria for viruses in shellfish.

The aim of this study was to collect both qualitative (contamination prevalence) and quantitative data (contamination levels) for NoV and HAV in bivalve molluscs collected from harvesting areas representative of the major Italian production zones, obtaining useful information to estimate consumer exposure, optimize sampling monitoring plans and improve the safety of the products.

2. Material and methods

In this study, the qualitative and quantitative data on viral contamination of different species of bivalve molluscs from harvesting areas classified either A or B according to EU Reg. 854/2004 (European Parliament, 2004) were reported. The areas were located in four Italian Regions (Veneto, Lazio, Campania and Sardinia) which host the most relevant national production areas. A total of 336 samples were analyzed in the period between 2008 and 2012 as a part of different regional monitoring programs conducted by the Istituto Superiore di Sanità (ISS), as National Reference Laboratory (NRL) for viral contamination of bivalve molluscs, in collaboration with other public health Italian laboratories (Istituti Zooprofilattici Sperimentali) and two Universities (University of Naples "Federico II" and University of Sassari).

2.1. Samples

The bivalve species analyzed included mussels (*Mytilus galloprovincialis*, n = 185), clams (*Tapes philippinarum* and *Ruditapes decussatus*, n = 66), oysters (*Crassostrea gigas*, n = 23) and other species (*Donax* spp. and *Solen* spp., n = 62, collected from areas under classification). For a number of samples collected from class B areas in Sardinia (n = 22), a test aliquot was taken before as well as after purification treatment to evaluate effects of commercial post-harvest treatments on viral contamination.

Samples collected were transported to the laboratory under refrigerated conditions, and were subjected to viral analysis for quantitative and qualitative determination of hepatitis A virus (HAV) and Norovirus (NoV) GI and GII, using an NRL's in-house validated procedure corresponding to ISO/TS 15216-1 and -2 final drafts (International Organization for Standardization, 2013a,b).

2.2. Samples processing

Depending on species' size, 15 to 60 individuals of each sample were randomly selected for the analysis and digestive tissue was dissected, cleaned and finely chopped with a sterile razor. Aliquots of 2.0 g, spiked with 10 μl of process control (titrated suspension of Mengovirus), were treated for digestion with 2 ml of proteinase K (0.1 mg/ml) at 37 °C for 60 min with shaking, and then placed at 60 °C for 15 min to produce inactivation of the enzyme. Finally, samples were centrifuged at 3000 ×*g* for 5 min, supernatant was collected, volume recorded (range from 2.3 to 3.0 ml) and the volume taken was normalized to 3.0 ml by addition of sterile PBS. Nucleic acid extraction and purification were performed using the Nuclisens extraction kit (BioMerieux, Paris, France) according to the manufacturer's instructions, and eluted RNA (100 μl) was stored at −80 °C until real time RT-PCR analysis.

2.3. Real time RT-PCR

The real time RT-PCR for HAV and NoV detection was carried out on a ABI Prism 7700 SDS system (Applied Biosystems, Foster City, California, US) using amplification conditions, primers, probes and reagents (RNA UltraSense™ One-Step Quantitative RT-PCR System, Life Technologies, Carlsbad, California, US) reported in ISO/TS 15216–2:2013 (International Organization for Standardization, 2013b) and previously published in separate papers (Costafreda et al., 2006; Kageyama et al., 2003; Le Guyader et al., 2009; Loisy et al., 2005; Pinto et al., 2009; Svraka et al., 2007). Undiluted and 1/10 diluted samples were tested. The presence of PCR inhibitors was evaluated testing samples in association with an external control RNA (EC-RNA, approximately 10^4 copies of target sequence) and amplification efficiency was calculated by comparison to the *C*q value of EC-RNA alone ($E = 2^{-\Delta Ct}$). Results from undiluted samples were considered acceptable if amplification efficiency was ≥50%, otherwise only results from dilution 1:10 were considered. In each run two negative controls (molecular grade water) and a positive control (the same EC-RNA as above) were added. The efficiency of the extraction procedure was evaluated through the recovery of the process control, comparing the *C*q values obtained for Mengovirus on shellfish samples extracts to the viral stock, taking into account the dilution factor due to the extraction procedure and the aliquot of sample subjected to analysis. Recovery was considered acceptable if ≥1%; samples failing to reach this criterion were re-extracted.

Samples that resulted positive for either HAV or NoV were analyzed for virus quantification. Each sample was tested in duplicate. Analyses of undiluted samples and 1/10 samples and EC-RNA were performed as above described. Calibration curve for quantification was generated using plasmids containing the appropriate target sequence, as reported in ISO/TS 125216–1 (International Organization for Standardization, 2013a). Quantitative results for NoV were expressed as a sum of the two detected genogroups (GI and GII) according to conclusions of EFSA opinion on NoV in oysters (EFSA, 2012).

2.4. Statistical analysis

Quantitative results for each sample group were elaborated by calculating the geometric mean, the standard deviation and the range of values (geometric mean ± 2 geometric standard deviations). Contamination prevalences were compared using the Fisher's exact test, while quantitative contamination levels were compared with the Student's