RESEARCH ARTICLE **(Open Access)**

Occurrence of norovirus and hepatitis A virus in shellfish

VALENTINA TERIO[1]*, ANGELA DI PINTO[1], MARILISA BOTTARO[1], FATMIRA SHEHU[2], CRISTIANA CATELLA [1], VITO MARTELLA[1].

[1]Department of Veterinary Public Health, Valenzano (Bari) Italy

[2] Department of Veterinary Public Health, Faculty of Veterinary Medicine (FVM), Kodër-Kamëz, Tirana, Albania

*Corresponding author: valentina.terio@uniba.it

Abstract:

Norovirus (NoV) and hepatitis A virus (HAV) are a common cause of gastroenteritis outbreaks associated with consumption of raw shellfish. The majority of NoV infections worldwide are due to geno group II NoVs. The predominant HAV strains belong to sub-genotype IB. A total of 369 bivalve molluscs (294 mussels, 42 clams and 33 oysters) from several retail points and harvesting class-A areas of the Adriatic basin in South Italy, North Italy and Albania (Butrinti Lagoon) were sampled between 2008-2013. All the samples were screened by a hemi-nested RT-PCR specific for NoV geno group II and by a nested RT-PCR for the VP1/2A region of HAV. NoV RNA was detected in 10,5% of samples and ranged from 3% in 2008 to 85% in 2013. HAV RNA was detected in 32,5% of samples and ranged from 90% in 2008 to 3,1% in 2013. The marked decrease in HAV prevalence may be the related to the vaccine-induced immunity, able to interrupt the ecological cycle of HAV. Monitoring the epidemiology of the virus strains circulating in the fieldis pivotal to develop and assess the efficacy of new control strategies to reduce the risks for public health.

Key words: Shellfish, HAV, Norovirus,.

1. Introduction

Bivalve shellfish are known to concentrate efficiently waterborne viruses, and are regarded as a major source of HAV and NoV outbreaks [5,24,26]. In some of the Mediterranean regions, including Southern Italy, HAV and NoV have been frequently detected in bivalve mussel *(Mytilus galloprovincialis*) and others shellfish that are sold at public markets [2,4,6,17,20,27,29]. Similar, in Albania different cases of gastroenteritis and hepatitis have been associated with NoV and HAV infection, respectively [8].

Importantly, mussels are often served and eaten raw, making the consumption of bivalves a significant risk factor for acquisition of gastroenteritis.

NoVs and HAV are non-enveloped viruses and are very stable in the environment. Thus they can often contaminate rivers or coastal waters that support shellfish growth [19]. Due to their filter-feeding activity of molluscs, these viruses may be detected in shellfish in a number of countries [7,21,28,29].

According to the current European Union standards, conventional monitoring of shellfish relies solely on bacteriological parameters. The European (EC) Regulations 852/2004, 853/2004 and 854/2004 setstandards based on *E. coli*, and classify shellfish-harvesting areas on the basis of the bacterial tissue levels into three areas: class A area, where shellfish may be directly sold without depuration processing; class B area, where shellfish must be depurated or relayed before marketing; class C, where shellfish must be subjected to prolonged relay or cooking; [22]. However, the presence of *E. coli* does not correlate with the presence of enteric viruses such as NoVs and HAVs, as these viruses are more persistent than fecal bacteria in brackish and sea water (Reg. EC No. 2073/2005). Therefore, the Council Regulation (EC, No. 2073/2005) recently proposed that sanitary controls of shellfish should include viral parameters to guarantee safety for human consumption. This document specifies that standardised methods should be developed before the establishment of virological criteria.

In this study, we evaluate the presence of viruses within the bivalve mussels (mussels, clams and oysters) collected from different harvesting areas of the Adriatic basin, located in Southern and Northern Italy, and Albania, between 2008-2013.

2. Materials And Methods

2.1 Shellfish sampling

A total 369 bivalve molluscs (294 mussels, 42 clams and 33 oysters) from several retail points and harvesting areas “A” in South Italy, North Italy and Albania (Butrinti Lagoon) were sampled between 2008-2013.

2.2 Bacteriological control

Salmonella spp. and *E.coli* were isolated using MPN standard methods as described in UNI EN ISO 6579:2004 and ISO TS 16649-3:2005.

2.3 Detection of HAV by RT-PCR

RNA extraction and concentration were performed following the glycine-PEG-Tri-reagent-poly (dT) extraction method (GPTT) procedure, as described by Kingsley and Richards (2001). Viral nucleic acid was analysed for HAV by a nested RT-PCR assay using gene-specific primers (dkA24-dkA25) targeting a VP1/2A junction region [13] in order to amplify a 200 bp fragment using a Hot Star Taq Master mix kit (Qiagen). First round RT-PCR was performed using Superscript II One step (Invitrogen, Paisly, UK) in order to amplify a 267bp fragment, [23].

2.4 Detection of NoV by RT-PCR

Viral nucleic acid, obtained using GPTT method, was analysed and typed for NoV by a hemi-nested RT-PCR assay. For NoV GII detection, oligonucleotide primers (NVp110 and NI) [15] targeting a highly conserved region in the RNA-dependent RNA-polymerase (RdRp) in the ORF1 were used. For NoV GI, oligonucleotide primers (GISKF and GISKR) [14] targeting a region of capsid N/S domain ORF2 were used. First-round RT-PCR was carried out using Superscript II One step (Invitrogen, Paisly, UK) in order to amplify a 273 bp (GII) and 368 bp (GI) fragments. A hemi-nested PCR, specific for genogroupII and I was carried out using AmpliTaq Gold (Applied Biosystems Foster City, CA) in order to amplify a 120 bpand (300) fragments, respectively. All the PCRs were performed in a GeneAmp PCR System 2700 thermocycler. The amplified products were analyzed by gel electrophoresis on ethidium bromide-stained 2% agarose. Several procedures were adopted during RNA extraction and PCR amplifications to avoid cross-contaminations.

3. Results

NoV: Of the 369 samples examined during the 5 years of monitoring, NoV RNA was detected in 39 samples (10,5%). The positivity rate during the course of the study ranged from a minimum of 3% (2008) to a maximum of 85% (2013). With regard to the different matrices tested, 21 of the 294 mussels samples tested positive (7,14%), while the percentage of positive samples was 36,4% for oysters and 14,3% for clams (Table 1). All the NoV strains were characterised as GII genogroup, as reported elsewhere [18].

HAV: Out of the 369 samples examined, 120 (32,5%) tested positive for HAV. In 2008, the positivity rate was 90%. Between 2009-2013 the prevalence markedly decrease, to as low as 1%. With regard to the different matrices tested, HAV RNA was detected in 66 of the 294 mussels samples (22,4%), 36/42 (85,7%) of the oysters 18/33 (54%) of the clams examined (Table 1)

Upon sequence analysis, the HAV strains were characterized as either subtype IA or IB, as described in other studies [3].

Table 1. Distribution of positive samples

YEAR	*mussels positive*		*Oysters positive*		*Clams positive*	
	NoV GII	HAV	NoV GII	HAV	NoV GII	HAV
2008	0	59	0	32	0	16
2009	0	5	0	3	0	1
2010	2	2	0	1	0	1
2011	5	2	1	0	0	0
2012	5	0	1	0	1	0
2013	17	0	10	0	5	0

4. Discussion

Filter-feeding bivalve shellfish can accumulate human pathogenic viruses, such as NoV and HAV when grown in environments polluted with human fecal material. The consumption of shellfish contaminated with human viruses has been associated with several outbreaks of gastroenteritis [16].The risks associated with bivalve shellfish are well documented [9].

HAV has been a serious public health problem in Europe and in particular in Puglia where between 1996-97 there was a large epidemic. After this period, the incidence of HAV in Puglia has steadily declined since 2008. One crucial point for HAV control in Puglia is due to the actual policy of universal vaccination of toddlers and adolescents [3]. It is safe to assume that the decreasing positivityrates of HAV in shellfish from class-A harvesting areas is due to the vaccination coverage. Currently, NoV represents the

main cause of outbreaks of gastroenteritis associated with shellfish consumption in several European countries [31].The development of vaccines for NoV is hampered by the large genetic/phenotypic variability of NoVs and by their uncultivable nature.

In our study, the virological investigations performed on shellfish collected in 2012 from Albania showed the presence of NoV GII. The first documented outbreak of NoV in Albania dates back to the period 2010-2012 [8]. Moreover, the presence of NoV GII was not unexpected as this genogroup is predominant worldwide [12]. In Albania fecal contamination of water has been responsible for different outbreaks in the past years and, since 2002, circulation of enteric viruses has been documented with NoV infection accounting for nearly 12% of the cases [1].

In Italy, viral contamination was detected throughout the whole monitoring period, demonstrating the constant circulation of enteric viruses in the environment, with marked fluctuations.The European guidelines in EC Regulation 2073/2005 base shellfish safety exclusively on specific bacteriological parameters, *Salmonella* spp. and *E. coli*, which do not correlate with the presence of viruses [11]. It is important to remark thatbacteria, unlike viruses, are easily cleared with the depuration systems used routinely [10,30]. Indeed, epidemiological and laboratory studies have shown that the depuration times and conditions currently used are inadequate to remove the viruses [22].

5. Conclusion

Our study provided a clearevidence of the potential risks associated with the presence of NoV in seafood matrices and it confirms the inadequacy of current microbiological criteria for commercialization of shellfish, demonstrating the need, as asserted in EC Regulation 853/2004, to ‘‘lay down additional health standards for live bivalve molluscs, including virus testing procedures and virological standards’’ (Reg EC N. 853/2004).

6. Reference

1. Arrivi. F, Donia. D, Gabrieli. R, Petrinca. AR, Cenko. F, Bebeci. D, Altan. AMD, Buonomo. E, Divizia. M: **Influence of EntericViruses on Gastroenteritis in Albania: Epidemiological and Molecular Analysis.** *Journal of Medical Virology*, 2007, 79, 1844–1849
2. Chironna. M, Germinario C, De Medici. D Fiore. A, Di Pasquale S, Quarto M, Barbuti S. **Detection of hepatitis A virus in mussels from different sources marketed in Puglia region (South Italy).** *International Journal of Food Microbiology*, 2002, 75, 1-2, 11-18
3. Chironna M, Prato R, Sallustio A, Martinelli D, Tafur. S, Quarto M, Germinario C. **Hepatitis A in Puglia (South Italy) after 10 years of universal vaccination: need for strict monitoring and catch-up vaccination.** *BMC Infectious Disease*, 2012,12, 271
4. Croci L, Ciccozzi M, De Medici D, Di Pasquale S, Fiore A, Mele A, Toti L. **Inactivation of hepatitis A virus in heat-treated mussels.** *Journal of Applied Microbiology*, 1999, 87, 884–888
5. Croci L, Losio MN, Suffredini E, Pavoni E, Di Pasquale S, Fallacara F, Arcangeli G. **Assessment of human enteric viruses in shellfish from the northern Adriatic sea.** *International Journal of Food Microbiology*, 2007, 114, 2, 252-257
6. Di Pinto A, Forte VT, Tantillo GM, Terio V, Buonavoglia C. **Detection of hepatitis A virus in shellfish (Mytilus galloprovincialis) with RT-PCR.** *Journal of Food Protection*, 2003, 66, 9, 1681-1685
7. Diez-Valcarce M, Kokkinos P, Söderberg K, Bouwknegt M, Willems K, de Roda-Husman AM, von Bonsdorff CH, Bellou M, Hernández M, Maunula L, Vantarakis A, Rodríguez-Lázaro D. **Occurrence of human entericviruses in commercial mussels atretaillevel in three European countries.** *Food and Enviromental Virology*, 2012, 4, 2, 73-80
8. Donia D, Kota M, Leno L, Ylli A, Cenko F, Divizia M. **First outbreak of norovirus in Albania.** *Letters in Applied Microbiol*, 2011, 53, 3, 283-287
9. EFSA Panel on Biological Hazards (BIOHAZ). **Scientific Opinion on Norovirus (NoV) in oysters: methods, limits and control options.** *EFSA Journal*, 2012, 10, 1, 2500
10. Franco E, Toti L, Gabrieli R, Croci L, De Medici D, Panà A. **Depuration of Mytilus galloprovincialis experimentally contaminated with hepatitis A virus.** *International Journal of Food Microbiology*, 1990, 11, 3-4, 321-327.
11. Goyal SM, Gerba CP, Melnick JL. **Human enteroviruses in oysters and their overlying waters.** *Applied Environmental Microbiology*, 1979, 37, 3, 572-581
12. Iritani N, Vennema H, Siebenga JJ, Siezen RJ, Renckens B, Seto Y, Kaida A, Koopmans M. **Genetica nalysis of the capsid gene of genotype**

GII.2 noroviruses. *Journal of Virology*, 2008, 82, 15, 7336-7345.

13. Kingsley DH and Richards GP. **Rapid and efficient extraction method for reverse transcription-PCR detection of hepatitis A and Norwalk-like viruses in shellfish.** *Applied and Environmenta lMicrobiology*, 2001, 67, 9, 4152-4157
14. Kojima S, Kageyama T, Fukushi S, Hoshino FB, Shinohara M, Uchida K, Natori K, Takeda N, Katayama K. **Genogroup-specific PCR primers for detection of Norwalk-like viruses.** *Journal of Virological Methods*, 2002, 100, 107–114.
15. Le Guyader F, Estes MK, Hardy ME, Neill FH, Green J, Brown DW, Atmar RL. **Evaluation of a degenerate primer for the PCR detection of human caliciviruses.** *Archives of Virology*, 1996, 141, 11, 2225-2235
16. Lees D. **Viruses and bivalve shellfish**. *International Journal of Food Microbiology*, 2000, 59, 1-2, 81-116
17. Macaluso A, Petrinca A, Lanni L, Saccares S, Amiti S, Gabrieli R, Divizia M. **Identification and sequence analysis of hepatitis A virus detected in market and environmental bivalve molluscs.** *Journal of Food Protection*, 2006, 69, 2, 449-452
18. Pavoni E, Consoli M, Suffredini E, Arcangeli G, Serracca L, Battistini R, Rossini I, Croci L, Losio MN. **Noroviruses in seafood: a 9-year monitoring in Italy**. *Foodborne Pathogens and Disease*, 2013, 10, 6, 533-539
19. Pérez-Sautu U, Sano D, Guix S, Kasimir G, Pintó. RM, Bosch. A: **Human norovirus occurrence and diversity in the Llobregatrivercatchment, Spain.** *Enviromental Microbiology*, 2012, 4, 2, 494-502
20. Pontrelli G, Boccia D, DI Renzi M, Massari M, Giugliano F, Celentano LP, Taffon S, Genovese D, DI Pasquale S, Scalise F, Rapicetta M, Croci L, Salmaso S: **Epidemiological and virological characterization of a large community-wide outbreak of hepatitis A in southern Italy.** *Epidemiology and Infection***,** 2006, 136, 8, 1027–1034
21. Rajko-Nenow P, Keaveney S, Flannery J, O'Flaherty V, Doré W. **Characterisation of norovirus contamination in an Irish shellfish ery using real-time RT-q PCR and sequencing analysis**. *International Journal of Food Microbiol*, 2012, 160, 2, 105-112
22. Richards GP, McLeod C, Le Guyader FS. **Processing strategies to inactivate enteric viruses in shellfish.** *Food and Environmental Virology*, 2010, 2, 3, 183-193
23. Robertson BH, Jansen. RW, Khanna. B, Totsuka. A, Nainan. OV, Siegl. G, Widell. A, Margolis. HS, Isomura. S, lto. K, lshizu. TS, Moritsugu. Y, Lemon. SM: **Genetic related ness of hepatitis A virus strains recovered from different geographical regions.** *Journal of General Virology*, 1992, 73, 1365-1377.
24. Sánchez G, Pintó RM, Vanaclocha H, Bosch Albert. **Molecular Characterization of Hepatitis A Virus Isolates from a Transcontinental Shellfish-Borne Outbreak.** *Journal of Clinical Microbiology*, 2002, 40, 11, 4148–4155
25. Scientific Opinion on Norovirus (NoV) in oysters: methods, limits and control options. EFSA Panel on Biological Hazards (BIOHAZ). *EFSA Journal*, 2012, 10, 1
26. Shieh YC, Khudyakov YE, Xia G, Ganova-Raeva LM, Khambaty FM, Woods JW, Veazey JE, Motes ML, Glatzer MB, Bialek SR, Fiore AE. **Molecular confirmation of oystersas the vector for hepatitis A in a 2005 multistate outbreak.** *Journal of Food Protection*, 2007, 70, 1, 145-150
27. Suffredini E, Corrain C, Arcangeli G, Fasolato L, Manfrin A, Rossetti E, Biazzi E, Mioni R, Pavoni E, Losio MN, Sanavio G, Croci L. **Occurrence of entericviruses in shellfish and relation to climatic-environmental factors.** *Letters in Applied Microbiol*, 2008, 47, 5, 467-474
28. Suffredini E, Magnabosco C, Civettini M, Rossetti E, Arcangeli G, Croci L. **Norovirus contamination in different shellfish species harvested in the same production areas.** *Journal of applied Microbiol*, 2012, 113, 3, 686-692
29. Terio V, Martella V, Moschidou P, Di Pinto P, Tantillo G, Buonavoglia C. **Norovirus in retail shellfish.** *Food Microbiology*, 2010, 27, 1, 29–32
30. Ueki Y, Shoji M, Suto A, Tanabe T, Okimura Y, Kikuchi Y, Saito N, Sano D, Omura T. **Persistence of caliciviruses in artificially contaminated oysters during depuration.** *Applied and Environmental Microbiology*, 2007, 73, 17, 5698-5701
31. Westrell T, Dusch V, Ethelberg S, Harris J, Hjertqvist M, Jourdan-da Silva N, Koller A, Lenglet A, Lisby M, Vold L. **Norovirus outbreaks linked to oyster consumption in the United Kingdom, Norway, France, Sweden and Denmark, 2010.** *Euro surveillance*, 2010, 15, 12.