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Original article

STUDY ON NOROVIRUS CONTAMINATION OF LIVE BIVALVE MOLLUSCS USING REAL-TIME PCR

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Summary

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Foodborne and waterborne viruses are a major cause of human morbidity. Of them, noroviruses are recognised as the leading causative agents of sporadic infections and epidemic outbreaks of acute gastroenteritis in humans. Contaminated food products and water are the main source of infection with noroviruses. The infection of bivalve molluscs with human pathogenic viruses occurs by faecal contamination in the production coastal waters. In this study, 47 samples of live bivalve molluscs, including 15 samples of cultivated mussels (*Mytilus galloprovincialis*) and 32 samples of wild mussels (*Tapes decussatus*), collected from the Bulgarian and Mediterranean coasts, respectively, were submitted to RT-real-time TaqMan PCR to detect the presence of noroviruses genotype GI and GII. Norovirus genotype GII was found in 11 (23.4%) of all the samples tested. A single mollusc sample (2.1%) was positive for both norovirus genotypes. Our results demonstrated that shellfish intended for sale on the Bulgarian market might pose a potential risk for acquiring norovirus infection. Thus, food safety quality control of shellfish by optimised and standardised methods for detection of foodborne viruses, including noroviruses, should be urgently implemented in Bulgaria.

Key words: genotypes GI and GII, live bivalve molluscs, noroviruses, real-time PCR

INTRODUCTION

Foodborne and waterborne viruses are a serious food safety concern posing a real risk to human health. According to the European Food Safety Authority (EFSA) report for 2014, viruses including hepatitis A virus (HAV) and noroviruses (NoV) are the most commonly detected pathogens implicated in foodborne outbreaks (20.4%).

Norovirus is recognised as a leading cause of acute non-bacterial gastroenteritis outbreaks among all age groups worldwide, affecting millions of people annually (Kroneman *et al.*, 2006). Norovirus virion is 27–35 nm in diameter, and is composed of a single-stranded positive-sense RNA genome encapsidated in a protein capsid with icosaedral symmetry.

Noroviruses belong to the *Norovirus* genus within family *Caliciviridae* and are classified into 7 genogroups (from GI to GVII). Members of genogroups GI, GII and GIV are known to infect humans, with GII strains being the most prevalent worldwide (Kageyama *et al.*, 2004).

Norovirus infection occurs after ingestion of 10 to 100 viral particles (Graham *et al.*, 1994). The main route for virus transmission is the faecal-oral one, most commonly via consumption of naturally contaminated raw or undercooked food (mussels, vegetables, fruits) or food contaminated by infected people during processing, food preparation etc. (Korsun *et al.*, 2008; Omoe, 2009).

The main source of NoV infections for human are bivalve molluscs – various species of cultivated and wild mussels, clams, oysters etc., which are traditionally consumed raw or undercooked. Contamination of bivalves occurs in coastal water areas through the discharge of treated or untreated wastewaters. Molluscs are filter feeding organisms capable to ingest contaminated phyto- and zooplankton and concentrate pathogens dispersed in the water. It has been reported that after 4–5 h in contaminated water, the bioaccumulation of the virus in molluscs could exceed 1,000 particles (Greening *et al.*, 2001). The contamination of molluscs is further facilitated by the fact that noroviruses remain viable for a long time in fresh and salt waters. Different experiments have confirmed that their infectivity is preserved at 4 ^{o}C over 2 months, and at room temperature – over 2 weeks.

Noroviruses are commonly detected in bivalve molluscs. Monitoring performed by EFSA reports their presence in up to 60% of samples investigated (EFSA, 2011). Annual reports of the Rapid Alert System for Food and Feed provide proofs for NoV-contaminated seafood, posing a potential risk to human health.

During the last 20 years, number of methods for detection of NoV in clinical samples have been developed (including electron microscopy, immunochromatography, immunoenzyme assays), which however turned out to be inappropriate for testing of contaminated foodstuffs due to their low sensitivity. Therefore, the different variants of RT-PCR are nowadays approved as "gold standard" for detection of noroviruses in contaminated food/water. In the practice, RT-PCR is mainly used both for qualitative NoV detection and for quantitation of virus copies in the tested specimens (Suffredini *et al.*, 2011; Persson *et al.*, 2018). Since 2013 this method has been standardised and introduced in the international standards ISO/TS 15216-1:2013 and ISO/TS 15216-2:2013, last revised in 2017.

The high epidemiological risk and the absence of consistent research studies on the contamination of cultured and wild mussels in our country motivated the present survey which was aimed at evaluation of norovirus contamination of live cultivated mussels from the Bulgarian Black Sea coast and wild mussels from the Turkish Mediterranean coast and determination of distribution of detected noroviruses by genogroups.

MATERIALS AND METHODS

Specimens

The study was conducted on 47 samples live mussels intended for distribution in the retail stores. Fifteen samples, consisted of cultivated mussels (*Mytilus galloprovincialis),* were collected from a farm located at the southern Black Sea Coast, and another 32 samples were wild bivalves (*Tapes decussatus)* collected

from the northeastern Mediterranean coast of Turkey. Each sample comprised a pool of at least 10 live bivalves with intact shells. Samples were transported at 4 °C and stored at –24 ºC until molecular analysis performance.

Primary processing of bivalve specimens

After cleaning and washing, molluscs were opened with sterile instruments. The digestive glands (hepatopancreases) were separated in sterile Petri dishes, and carefully homogenized. Two grams from each pool were weighed in a centrifuge tube, and lysed with 2 mL proteinase K (0.1 mg/mL, Thermo Fisher Scientific). Incubation was done at 37 °C for 60 min in a thermoshaker (Eppendorf Mastercycler 5331), followed by vortexing and centrifugation at 3000×g for 1 min. Supernatants were collected in new Eppendorf tubes and incubated again at 65°C for 15 min in a water bath for enzyme inactivation. After another centrifugation at 3000×g, 3 min, supernatants were tested for presence of noroviruses.

Extraction of norovirus RNA

Real-time TaqMan PCR preceded by reverse transcription step was used for detection of norovirus contamination of bivalves according to the protocol of the European network for surveillance of norovirus diseases (NoroNet) kindly provided by Dr. Andrej Steyer, Slovenia.

Three hundred µL of supernatants were used for norovirus RNA extraction by PureLink Viral RNA/DNA kit (Invitrogen, LifeTechnology), as per manufacturer's instructions. Extracted norovirus RNA was stored at 4 °C for 24 h or frozen at –70°C until used.

Real-Time RT-PCR

Primary denaturation of extracted RNA was done at 95 °C for 5 min, followed by cooling for 3 min. The reverse transcription was performed at 37 °C for 60 min in a thermocycler (Eppendorf Mastercycler 5331) using random hexamers (Invitrogen). Detection and genogroup identification of noroviruses was achieved by real-time TaqMan PCR using two primer pairs and probes targeted to a highly conservative region of the gene coding the RNA-dependent RNA polymerase of genogroups GI and GII. The reaction was run in 25 µL final volume using 5 µL cDNA of each sample and 20 µL reaction mixture composed of TaqMan Universal PCR Master mix (Applied Biosystems), genotype-specific primers COG1F/ COG1R for GI and COG2F/COG2R for GII, and FAM-TAMRA labelled probes for genogroups GI and GII – respectively RING1(a)-TP and RING1(b)-TP and RING2-TP (Invitrogen) (Kageyama *et al.*, 2003). The fifty amplification cycles were performed on MJ Research Opticon 2 (BioRad) in the Virology department of the National Centre for Infectious and Parasitic Diseases, Sofia. Negative (distilled water) and positive controls were also run.

Reference materials

The following human norovirus strains with genogroup and genotype identity confirmed by sequencing were used as positive controls:

- for genogroup GI – Norovirus/Hu/GI.4/BG629/2009/BUL (GenBank accession number KP123636.1);
- for genogroup GII – Norovirus/Hu/GII- 2006b/BG752/2009/BUL (GenBank accession number KP123642.1)

RESULTS

The real-time RT-PCR analysis of 47 live mussel samples detected noroviruses in 11 samples (23.4%). Ten samples contained only NoV from genogroup GII (90.9%), and one of samples was positive for both genogroups (9.1%) (Table 1).

Out of the 15 samples of cultured bivalves, 8 were NoV GII-positive (53.3%) and one (6.7%) was positive for both NoV GI and NoV GII. Only two samples (6.3%) from the 32 tested wild bivalves were positive for noroviruses from genogroup GII.

The positive signal (Ct value) in TaqMan PCR analysis of live mussels from the Black Sea and Mediterranean Sea varied from 29.1 and 45.5 cycles (Fig. 1). Samples were divided into two main groups: high positive with Ct value <40 (within the range 25–38 cycles) and low positive with Ct value >40 (40–48 cycles).

DISCUSSION

The current study describes the first investigation on norovirus contamination of cultured and wild bivalve molluscs using RT/real-time PCR in Bulgaria. From a total of 47 pools of hepatopancreases analysed, 11 samples (23.4%) yielded positive results. This rate is comparable to the data reported by Henigman *et al.* (2015) for norovirus contamination of 24.6% of

Table 1. Presence of noroviruses in hepatopancreases of live bivalve molluscs of different origin

		Norovirus genogroup			Total
		GI	GII	GI+GII	
Cultured bivalves (n=15)	Number (%) of positive samples	0 (0%)	8 (53.3%)	1 (6.7%)	9 (60.0%)
Wild bivalves (n=32)	Number (%) of positive samples	0 (0%)	2 (6.3%)	0 (0%)	2 (6.3%)
Total	Number (%) of positive samples	0 (0%)	10 (21.3%)	1 (2.1%)	11 (23.5%)

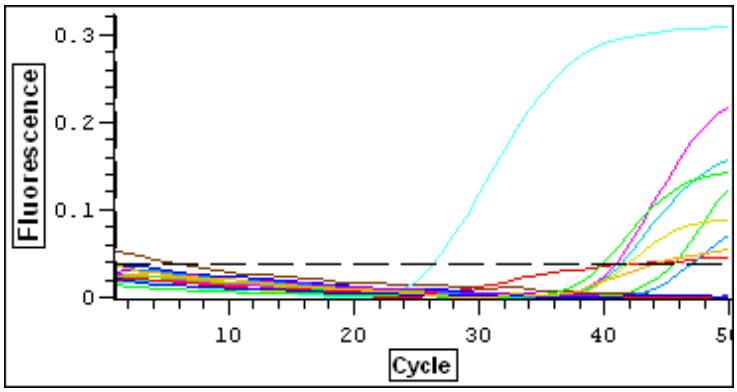

Fig. 1. Results from TaqMan RT-PCR with primers for detection of noroviruses of genogroup GII in live bivalve molluscs.

bivalve shellfish samples collected from the Adriatic Sea coast of Slovenia and 25% of mussels cultured in coastal areas of Sardinia (Marceddu *et al.*, 2017). A number of analyses conducted in mussel farms from different regions of Italy confirmed norovirus contamination rates between 4.1% and 57.7% in the studied mollusc samples (Pepe *et al.*, 2012; Pavoni *et al.*, 2013; Suffredini *et al.*, 2014). Similar data were reported in a UK study in which contamination rate of bivalve molluscs reached even 76% (CEFAS, 2011; 2014). Our study demonstrated that mussels farmed in Bulgarian Black Sea aquatory were contaminated by noroviruses and could pose a potential risk for consumers' health. Numerous researchers showed the relationship between consumption of molluscs originating from areas with faecal contamination and foodborne outbreaks in humans. For instance, Cheng *et al.* (2005) confirmed virus RNA in 10% of samples with more than 98% homology between norovirus RNA sequence isolated from oysters and a sequence detected in patients with gastrointestinal illness. In 2002, more than 14 clusters of norovirus gastroenteritis with more than 400 affected people resulting from consumption of contaminated oysters cultured in a French mussel farm were registered in France and Italy (Doyle *et al.*, 2002). In 2010, contaminated molluscs cultivated in France, UK and Ireland, and distributed in retail stores of several European countries (Sweden, Norway, Denmark, the UK and France) were incriminated as a cause of an international outbreak involving over 65 epidemic clusters of patients (Westrell *et al.*, 2010).

It is well known that shellfish farms are under constant risk for viral and bacterial contamination when sewage system damage or efflux of sewage/waste waters occurs, in case of natural disasters (e.g. flood), septic tanks leaks/overflows, storm water runoff, recreational activities etc.

Our findings demonstrated different level of contamination in cultured vs wild mussels. Sixty percent of the cultured bivalve samples collected from Bulgarian farms were positive for norovirus RNA (n=9), while 2 (6.3%) out of the 32 wild mussels samples collected from the Mediterranean coast were norovirus-contaminated. Data suggested a high extent of water contamination in areas for live molluscs culturing on Bulgarian Black Sea coast.

The current EC legislation requires a mandatory microbiological monitoring of *E. coli* as indicator of bacteriological quality of molluscs and aquatory for their production (Anonymous, 2004; 2005). Related studies have shown that post-harvest treatment techniques such as depuration or relaying effectively reduce *E. coli* counts within the set limits but do not effectively remove or inactivate noroviruses (EFSA, 2011). Savini *et al.* (2009) calculated the efficacy of depuration systems when mussels were norovirus positive and detected virus RNA in 1 out of 67 non-depurated and 1 out of 29 depurated live mussel samples. The authors did not observe any statistically significant differences between the extent of contamination of depurated and non-depurated mussels confirming that depuration methods/systems were not efficient enough for NoV decontamination. Gyawali *et al.* (2019) summarised data for the efficiency of post-harvesting depuration and relaying techniques of mussels, and concluded that their application according to EC legislation did not reduce NoV contamination.

There is a need of implementation of regulations pertaining to the continuous control on water quality in industrial aqua-

tory and to laboratory testing of shellfish, both locally produced and imported, intended for Bulgarian consumers.

A future investigation on identification of risk factors for pollution of coastal waters, monitoring of norovirus concentrations in these areas, and identification of risk periods (seasonal prevalence, association with outbreaks etc.) would be of great value. A study of Mladenova *et al.* (2008) carried out among hospitalised patients with acute diarrhoea reported the presence of noroviruses genogroup GII in 15.2% (72/474) of human faecal samples. Most patients were registered as tourists during the summer months, which allowed assuming that consumption of bivalve molluscs was the most probable cause of the illness. Regardless of the lack of direct relationships with food consumption, this study confirmed the leading role of noroviruses in the etiology of acute gastroenterites in Bulgaria and the significance of seafood in their transmission.

Noroviruses from genogroup GII were detected in all tested mussel pools, except for a single sample of Bulgarian bivalves that was also positive for NoV GI. Similar data were reported also by Vilarino *et al.* (2009) in a study on the prevalence of noroviruses in wild and cultured bivalve molluscs. They demonstrated presence of NoV genogroup GII in 53.7% of samples, whereas GI was found in only 7.3%. Yilmaz *et al.* (2010) also detected more frequently genogroup GII (4.5% of samples) but none of samples was positive for GI. The contamination of molluscs with specific genotypes reflects their public circulation, as bivalves are contaminated from human faeces in their environment. In fact, some analyses have indicated that NoV GII causes more than 89% of sporadic and outbreak-related cases in humans, while NoV GI has detected only in 11% of the people infected (Vega *et al.*, 2014). Although the reasons for the wide spread of NoV GII were not entirely clear, different virulence of viruses and their route of transmission, different affinity of mussel tissues for virus accumulation etc. could be possibly implicated (Croci *et al.*, 2007; Suffredini *et al.*, 2011). However, other studies did not provide evidence for genotype-related differences in norovirus contamination of shellfish. For instance, a survey on 4 bivalve mollusc species cultured in class B areas in northwestern Spain, Polo *et al.* (2015) established high contamination rates without statistically significant differences between cultured and wild mussels (61.4% vs 54.3%). The authors reported a slight prevalence of NoV GI (32% vs 26% NoV GII). With respect to the more accurate evaluation of seafood contamination in Bulgaria, a more detailed study on bivalve molluscs along with analysis among people affected by norovirus infection is necessary.

It should be noted that the present study has some limitations. Although RT/real-time PCR, the gold standard for NoV detection in contaminated foodstuff was used in our study, only undiluted samples from hepatopancreases of bivalves were analysed. This approach carries a risk of assay inhibition due to the presence of inhibitors in molluscs tissues for RT and PCR steps and thus, from obtaining a false negative result. Some studies have shown that the parallel testing of an undiluted sample with the same sample at tenfold dilution increased substantially the percentage of NoV detection in many foodstuffs. Furthermore, the molecular RT/real time PCR technique is partly limited by the genetic diversity of noroviruses (LeGuyader *et al.*, 2009). The NoV group comprises exceptionally diverse viruses that are constantly evolving via two pri-

mary mechanisms – point mutations and recombination. These genetic changes could induce mutations in primer-binding sites and lack of detection with used primer pairs, e.g. could result in false negative results. In addition, although highly sensitive, the molecular techniques developed so far for detection of NoV in food products have a specific sensitivity threshold. Foods with very low NoV contamination like molluscs could be reported as NoV-negative and at the same time induce disease in men because as few as 16–20 viral particles are sufficient for infection to occur. According to the international standard ISO/TS 15216-1:2017 developed by the Expert group of European Union Reference Laboratory for monitoring of bacterial and viral contamination in bivalve molluscs, the proposed limit of detection in seafood is 200 genome copies per one gram end product (mussels, oysters etc.) which implies the need from quantification of noroviruses in bivalve molluscs (CEFAS, 2013).

CONCLUSION

The protocol used for extraction and detection of NoV GI/GII by RT/real-time PCR in live bivalve molluscs is rapid, highly sensitive and appropriate for their systemic virological monitoring.

Norovirus RNA was detected in 23.4% of tested live mussel samples intended for placing on the market which makes them potentially risky as sources of norovirus gastroenteritis outbreaks.

Cultured and wild mussel samples were positive only for noroviruses from genogroup GII, except for a single Bulgarian sample that contained noroviruses from both tested genogroups.

Our results demonstrated that a permanent and consistent control on norovirus contamination of coastal areas used for farming bivalve molluscs should be implemented, as well as mandatory testing for norovirus contamination of molluscs intended for consumer markets in Bulgaria.

REFERENCES

Anonymous, 2004. Regulation (EC) № 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption, *Official Journal of the EU,* L 139/206.

Anonymous, 2005. Commission Regulation (EC) № 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Official Journal of the EU,* L 338/1.

Campos, C. J. & D. N. Lees, 2014. Environmental transmission of human noroviruses in shellfish waters. *Applied and Environmental Microbiology,* **80**, 3552–3561.

CEFAS, 2011. Investigation into the prevalence, distribution and levels of norovirus titre in oyster harvesting areas in the UK. Report by CEFAS to the Food Standards Agency, report number FS235003 (P01009), August 2011. https://www.food.gov.uk/print/pdf/node/754 (15 April 2019 date last accessed).

CEFAS, 2014. A critical review of the current evidence for the potential use of indicator species to classify UK shellfish production areas. Final report, project FS512006. https://www.food.gov.uk/sites/default/files/media/document/865-1-1607_FS512006_VMc Farlane.pdf (15 April 2019 date last accessed).

CDC, 2007. Norovirus activity – United States, 2006–2007. *Morbidity and Mortality Weekly Report (MMWR)*, **56**, 842–846.

Cheng, P., D. Wong, T. Chung & W. Lim, 2005. Norovirus contamination found in oysters worldwide. *Journal of Medical Virology,* **76**, 593–597.

Croci, L., M. N. Losio, E. Suffredini, E. Pavoni, S. Di Pasquale, F. Fallacara & G. Arcangeli, 2007. Assessment of human enteric viruses in shellfish from the northern Adriatic sea. *International Journal of Food Microbiology*, **114**, 252–257.

Doyle, A., D. Barataud, A. Gallay, J. M. Thiolet, S. Le Guyaguer, E. Kohli & V. Vaillant, 2002. Norovirus foodborne outbreaks associated with the consumption of oysters from the Etang de Thau, France, December 2002. *European Communicable Disease Bulletin*, **9**, 24–26.

EFSA, Panel on Biological Hazards (BIOHAZ), 2011. Scientific opinion on an update on the present knowledge on the occurrence and control of foodborne viruses. *EFSA Journal,* **9**, 2190.

EFSA, Panel on Biological Hazards (BIOHAZ), 2014. Scientific opinion on the risk posed by pathogens in food of non-animal origin. Part 2 (*Salmonella* and *Norovirus* in berries). *EFSA Journal,* **12**, 3706.

Graham, D., X. Jiang, T. Tanake, A. Opekum, H. Madore & M. Estes, 1994. Norwalkvirus infection of volunteers: New insights based on improved assays. *Journal of Infectious Diseases*, **170**, 34–43.

Greening, G., M. Mirams & T. Berke, 2001. Molecular epidemiology of Norwalk-like viruses associated with gastroenteritis outbreaks in New Zeland. *Journal of Medical Virology,* **64**, 58–66.

Gyawali, P., F. Graham, D. McCoubreyc & J. Hewitta, 2019. Norovirus in shellfish: An overview of post-harvest treatments and their challenges. *Food Control,* **99**, 171–179.

Henigman, U., M. Biasizzo, S. Vadnjal, I. Toplak, M. Gombač, A. Steyer, M. Poljšak Prijatelj, M. Ambrožič, I. Fonda, A. Kirbiš, & D. Barlič-Maganja, 2015. Molecular characterisation of noroviruses detected in mussels (*Mytilus galloprovincialis*) from harvesting areas in Slovenia. *The New Microbiologica,* **38**, 225–233.

Kageyama, T., M. Shinohara, K. Uchida, Sh. Fukushi, F. Hoshino, Sh. Kojima, R. Takai, T. Oka, N. Takeda & K. Katayama, 2004. Coexistence of multiple genotypes, including newly identified genotypes in outbreaks of gstroenteritis due to Norovirus in Japan. *Journal of Clinical Microbiology,* **42**, 2988–2995.

Korsun, N., Z. Mladenova & P. Teoharov, 2008. Viral infections transmitted through food or water. *Medical Review (Sofia)*, **44**, 13–18 (BG).

Kroneman, A., H. Vennema, J. Harris, G. Reuter, C.-H. von Bonsdorff, K.-O. Hedlund, K. Vainio, V. Jackson, P. Pothier, J. Koch, E. Schreier, B. Böttiger & M. Koopmans, 2006. Increase in norovirus activity reported in Europe. *European Communicable Disease Bulletin,* **11**, E061214.1.

LeGuyader, F., J.-C. Le Saux, K. Ambert-Balay, J. Krol, O. Serais, S. Parnaudeau, H. Giraudon, G. Delmas, M. Pommepuy, P. Pothier & R. Atmar, 2008. Achivirus, norovirus, astrovirus, enterovirus and rotavirus involved in clinical cases from French oyster-related gastroenteritis outbreak. *Journal of Clinical Microbiology,* **46**, 4011–4017.

Marceddu, M., S. Lamon, S. G. Consolati, S. Ciulli, R. Mazza, A. Mureddu & D. Meloni, 2017. Determination of *Salmonella* spp., *E. coli* VTEC, *Vibrio* spp., and Norovirus GI-GII in bivalve molluscs collected from growing natural beds in Sardinia (Italy). *Foods*, **6**, doi: 10.3390/ foods6100088.

Mladenova, Z., N. Korsun, T. Genova, I. DiBartolo, L. Fiore & F. Ruggeri, 2008. Prevalence and molecular epidemiology of noroviruses detected in outbreak and sporadic cases of acute gastroenteritis in Bulgaria. *Journal of Medical Virology,* **80**, 2161–2168.

Omoe, H., 2009. Food poisoning and infectious disease caused by norovirus – situation of the outbreaks and countermeasures in Japan. *Science and Technology Trends*, **30**, 8–22

Palfrey, R., M. Harman & R. Moore, 2011. Impact of waste water treatments on removal of noroviruses from sewage. DEFRA R and D Technical Report WT0924/TR, http://randd.defra.gov.uk/Document.aspx?Document=ImpactofWastewaterTreatmentsonRemovalofNorovirusesfromSewagefinal.pdf (13 May 2019 date last accessed).

Pavoni, E., M. Consoli, E. Suffredini, G. Arcangeli, L. Serracca, R. Battistini, I. Rossini, L. Croci & M. N. Losio, 2013. Noroviruses in seafood: a 9-year monitoring in Italy. *Foodborne Pathogens and Disease*, **10**, 533–539.

Pepe, T., I. Ventrone, E. Suffredini, M. Ceruso, L. Croci, A. Anastasia & M. Cortesi, 2012. Norovirus monitoring in bivalve molluscs harvested and commercialized in Southern Italy. *Journal of Food Protection,* **75**, 976–981.

Persson, S., R. Eriksson, J. Lowther, P. Ellströmb, M. Simonsson, 2018. Comparison between RT droplet digital PCR and RT real-time PCR for quantification of noroviruses in oysters. *International Journal of Food Microbiology,* **284**, 73–83.

Polo, D., M. F. Varela & J. L. Romalde, 2015. Detection and quantification of hepatitis A virus and norovirus in Spanish authorized shellfish harvesting areas. *International Journal of Food Microbiology*, **193**, 43–50.

Savini, G., C. Casaccia, N. Barile, M. Paoletti & Ch. Pinoni, 2009. Norovirus in bivalve molluscs: A study of efficacy of depuration system. *Veterinaria Italiana,* **45**, 535–539.

Suffredini, E., T. Pepe, I. Ventrone & L. Croci, 2011. Norovirus detection in shellfish using two Real-Time RT-PCR methods. *The New Microbiologica*, **34**, 9–16.

Suffredini, E., L. Lanni, G. Arcangeli, T. Pepe, R. Mazzette, G. Ciccaglioni & L. Croci, 2014. Qualitative and quantitative assessment of viral contamination in bivalve molluscs harvested in Italy. *International Journal of Food Microbiology*, **184**, 21–26.

Vega, E., L. Barclay, N. Gregoricus, S. H. Shirley, D. Lee & J. Vinjé, 2014. Genotypic and epidemiologic trends of norovirus outbreaks in the United States, 2009 to 2013. *Journal of Clinical Microbiology*, **52**, 147–155.

Vilariano, M., F. Le Guyader, D. Polo, J. Schaeffer, J. Kröl & J. Romalde, 2009. Assessment of human enteric viruses in cultured and wild bivalve molluscs. *International Microbiology,* **12**, 145–151.

Westrell, T., V. Dusch, S. Ethelberg, J. Harris, M. Hjertqvist, N. Jourdan-da Silva, A. Koller, A. Lenglet, M. Lisby & L. Vold, 2010. Norovirus outbreaks linked to oyster consumption in the United Kingdom, Norway, France, Sweden and Denmark, 2010. *European Communicable Disease Bulletin,* **15**, 1–5.

Yilmaz, H., K. Bostan, N. Turan, K. Muratoglu, A. Yilmaz, A. Ozkul, B. Kocazeybek & Ch. Helps, 2010. Real-time PCR detection of norovirus in mussels collected from the Bosphorus in Instanbul, Turkey. *Food and Environmental Virology,* **2**, 64–68.

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