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Molecular characterisation of noroviruses detected in mussels (*Mytilus galloprovincialis*) from harvesting areas in Slovenia

Urška Henigman[1], Majda Biasizzo[1], Stanka Vadnjal[1], Ivan Toplak[2], Mitja Gombač[3], Andrej Steyer[4], Mateja Poljšak Prijatelj[4], Mateja Ambrožič[5], Irena Fonda[6], Andrej Kirbiš[1†], Darja Barlič Maganja[7†]

[1]Institute for Food Hygiene, Veterinary Faculty, University of Ljubljana, Ljubljana, Slovenia;
[2]Institute for Microbiology and Parasitology, Virology Unit, University of Ljubljana, Ljubljana, Slovenia;
[3]Institute of Pathology, Forensic and Administrative Veterinary Medicine, Veterinary Faculty, University of Ljubljana, Slovenia;
[4]Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia;
[5]Food Science and Technology Department, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia;
[6]Fonda.si d.o.o. Fonda s.r.l., Liminijanska cesta 117, Portorož, Slovenia;
[7]Faculty of Health Sciences, University of Primorska, Izola, Slovenia
[†]These authors contributed equally to this work

SUMMARY

Noroviruses are a leading cause of viral gastroenteritis in humans and are responsible for many outbreaks worldwide. Mussels are one of the most important foodstuffs connected with norovirus outbreaks, also resulting in multinational dimensions. Two hundred and thirty-eight (238) samples of mussels (*Mytilus galloprovincialis*) were collected in periods between the years 2006-2008 and 2010-2012 to study the prevalence of noroviruses (NoVs) from harvesting areas along the Adriatic coast of Slovenia. Between 2006 and 2008, 9.1% to 24.6% of mussel samples tested by specific GI and/or GII real-time RT-PCR methods were found to be positive for NoVs while between 2010 and 2012 the percentage of NoV positive samples varied from 12.5% to 22.2%. At the nucleotide level within the *RdRp* gene fragment the genetic diversity of NoVs detected in mussels ranged between 78.8-81.0% nucleotide identity among GII strains (92.1-99.6% within the GII.P4 genotype), 100% nucleotide identity among GI and 58.4-60.2% among GI and GII strains. Nine of the NoV strains detected from mussels were genotyped as GII.4, while two samples were within GI.P2 and one was a positive sample within genotype GII.P21. This study confirmed that mussels are a potential source of the NoV infection. The detected NoVs share the same topology on the phylogenetic tree within the NoV strains detected in water samples and human patients, not only from Slovenia but also from many different countries worldwide. We can assume that mussels in harvesting areas are not only contaminated from the surrounding area but also by contaminated water and sewage from large transport ships, which are regularly present in the area.

KEY WORDS: Mussels, Norovirus, RT-PCR.

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INTRODUCTION

Shellfish are a major risk factor for food-borne outbreaks and are often associated with outbreaks of viral disease (Koopmans and Duizer, 2004; Le Guyader *et al.,* 2008; Guillois-Becel *et al.,* 2009, Suffredini *et al.,* 2011). Although harvested shellfish should be in a clean environment, faecal pollution from different sources of discharges, disposal from boats, ballast water and contaminated river discharges can contaminate the areas (Santo Domingo and Edge, 2010). Contamination of shellfish with NoVs can occur when living in polluted water. As mussels feed and so ingest viruses due to their filter feeding activity, shellfish serve as a vehicle of NoV infections (Lees, 2000, Le Guyader *et*

Corresponding author
Urška Henigman
Institute for Food Hygiene, Veterinary Faculty
University of Ljubljana, Gerbičeva 60
1000 Ljubljana, Slovenia
E-mail: Urska.Henigman@vf.uni-lj.si.

al., 2003). The main factors associated with an elevated incidence or persistence of NoV contamination beside winter months are also increased rainfall and low temperatures (Campos and Lees, 2014).
NoVs are classified into five genogroups (GI to GV) of which GI and GII are most commonly associated with human infections. Genogroups are subdivided into different genotypes (Kageyama *et al.,* 2004). Genotype determination and detection of variants within genotype are useful to monitor the global spread of noroviruses (Ramirez *et al.,* 2008). In Slovenia a high prevalence of enteric viruses was detected in water sources, groundwater and surface water. Among those, noroviruses (GI and GII) were also present in environmental samples (Steyer *et al.,* 2011). The epidemic data caused by noroviruses in Slovenia have been available since the year 2000 (Poljšak Prijatelj *et al.,* 2001), but the source of the infection was usually not identifiable (Zimšek *et al.,* 2003; Štrumbelj *et al.,* 2003; Grmek Košnik *et al.,* 2007). In a study conducted in Slovenia, NoVs were detected from samples of surface and groundwater with genetic characteristics reflecting those from human clinical cases (Steyer *et al.,* 2011).
The aim of our study was to examine the mussels for the presence of NoVs, to establish their prevalence from harvesting and non-harvesting area in Slovenia and to compare the NoV sequences obtained from mussels with those obtained from human and water sources in Slovenia and worldwide whether there might be a linkage.

MATERIAL AND METHODS

Two hundred and thirty-eight (238) samples of Mediterranean mussels (*Mytilus galloprovincialis*) were tested for the presence of NoV. Samples were collected in two periods from 2006 to 2008 and from 2010 to 2012 at three harvesting areas in Slovenia: Seča, Strunjan and Debeli Rtič (Figure 1). The sampling scheme from the Seča harvesting area, where 98 samples were taken, was as follows: 23, 25, 23, 14, 10 and 3 samples per year. From the Strunjan harvesting area 80 samples were taken (10, 20, 21, 12, 12, 5) and from Debeli Rtič 54 (11, 16, 13, 9, 5, 0), respectively (Table 1). Five samples from wild

FIGURE 1 - *Location of Slovenia on a map of Europe (upper part of the picture), Slovenian coastal waters with the three harvesting areas of Mediterranean mussels (Mytilus galloprovincialis) - Seča, Strunjan and Debeli Rtič and one non-harvesting area near Piran. The coastline of Slovenia is 46.6 km long.*

living mussels were collected in the area near Piran (in 2007 two, and in 2008 three samples) and one sample collected near Strunjan in 2008 were also included in our study. Mussels were collected throughout the whole year to cover all the months. For each sample, the digestive glands of eight mussels were removed from the body and homogenized with *minimum essential media* - MEM (Gibco®, Invitrogen, USA) in the ratio 1:2 to obtain an emulsified suspension. RNA was isolated from digestive gland suspensions using RNeasy® Plus Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.
Real-time RT-PCR was performed with the primer pair COG1R/COG1F and probe RING1 for the specific detection of NoV from genogroup GI and primer pair COG2R/COG2F, probe RING2 for GII, targeted sequences at the ORF1-ORF2 junction region (Kageyama *et al.,* 2003), which is the most conserved region in the NoV genome (Katayama *et al.,* 2002).
One step real-time RT-PCRs were performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, USA) and were as follows: reverse transcription at 50°C for 15 min and denaturation at 95°C for 2 min, following 45 cycles of denaturation at 95°C for 15 s and annealing extension at 56°C for 45 s.

TABLE 1 - *Mediterranean mussels tested for NoVs in the years from 2006 to 2008 and from 2010 to 2012.*

Samples		*Detected genogroup by real-time RT-PCR*			*N. of samples positive by real-time RT-PCR (%)*	*N. of samples negative by real-time RT-PCR*	*Samples positive by real-time RT-PCR regarding sampling area*				**Number of samples positive by RT-PCR*
Year	*All*	*GI*	*GII*	*GI and GII*			*Seča*	*Strunjan*	*Debeli Rtič*	*Piran*	
2006	44	0	3	1	4 (9.1)	40	2/23	1/10	1/11	0	1
2007	63	0	4	5	9 (14.3)	54	1/25	4/20	3/16	1	6
2008	61	1	6	8	15 (24.6)	46	2/23	5/21	7/13	1	9
2010	35	0	4	2	6 (17.1)	29	0/14	4/12	2/9	0	3
2011	27	0	6	0	6 (22.2)	21	3/10	2/12	1/5	0	1
2012	8	0	1	0	1 (12.5)	7	0/3	1/5	0/0	0	0
Total	238	1	24	16	41 (17.2)	197	8/98 (8.2%)	17/80 (21.2%)	14/54 (25.9%)	2/6 (33.3%)	20

*Only samples positive by real-time RT-PCR were tested by conventional one-step RT-PCR.

The RNA extracted from samples was amplified again in a conventional one-step RT-PCR using SuperScript® Onestep RT-PCR with Platinum® *Taq* (Life Technologies, Germany) and primer pair JV12Y/JV13I (Vennema *et al.,* 2002) for targeting part of the polymerase gene (ORF1). The cycling conditions were as follows: reverse transcription at 45°C for 45 min and denaturation at 94°C for 2 min following 50 cycles of denaturation at 94°C for 30 s; annealing at 40°C for 1 min and extension at 68°C for 2 min with final extension 68°C for 7 min.

Results were interpreted on the basis of the specific size of RT-PCR products (326 bp) by 1.8% agarose gel electrophoresis and a 100 bp DNA ladder. The specificity of the RT-PCR products was confirmed by direct Sanger sequencing. The obtained sequences have been deposited in GenBank with accession numbers JN040477-JN040486 and KF953824-KF953825.

Twelve (12) NoV sequences obtained from mussels (Figure 2) in this study were compared with 18 sequences previously determined and detected from environmental and clinical human samples published by Steyer *et al.* (2011). In addition, reference sequences for specific norovirus genogroup/genotype were downloaded from GenBank and, for comparison some norovirus sequences from GenBank showing the highest identity after BLAST search for sequences obtained in this study were selected for the analysis. Multiple sequence alignment and phylogenetic analysis was performed in MEGA software version 5 with ClustalW alignment algorithm, using the default parameters. The evolutionary relationship of taxa was inferred using Neighbor-Joining method and evolutionary distances were computed using p-distance method. Taxa clusters were tested with bootstraps of 1000 replicates. Norovirus genotype for the sequences from this study was determined online with the Norovirus Automated Genotyping tool (Kroneman *et al.,* 2011).

RESULTS

Real-time RT-PCR and conventional one-step RT-PCR

In total 41 samples (17.2%) tested positive for NoVs (only GI or GII or both in the sample) by the real-time RT-PCR method. The obtained cycle threshold (Ct) values for positive samples were from Ct 33 to 41. From 41 samples positive by real-time RT-PCR, a specific RT-PCR product of the polymerase gene (ORF1) was successfully amplified from only 20 samples (Table 1). Noroviruses of GI and GII were frequently detected as a simultaneous contamination between 2006 and 2010, while in 2011 and 2012 only seven positive samples containing NoVs from genogroup GII were detected. Between 2006 and 2008, 9.1% to 24.6% of mus-

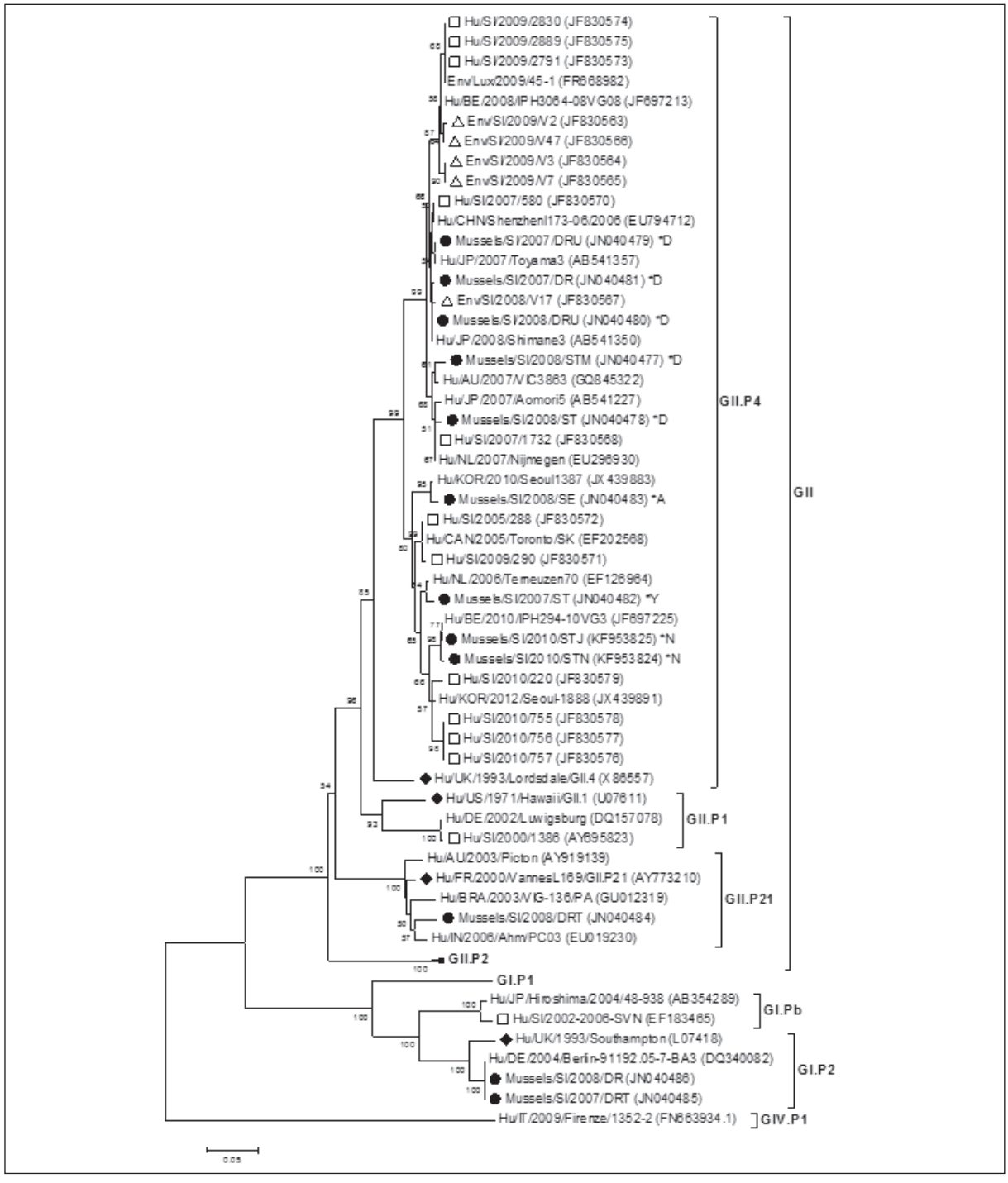

FIGURE 2 - *Phylogenetic comparison of NoV sequences detected from Mediterranean mussels (●) in Seča, Strunjan and Debeli Rtič (this study), surface water samples (Δ) and NoV sequences obtained from people suffering from gastroenteritis (□) in Slovenia (Steyer et al., 2011) and 21 closely related sequences obtained from the GenBank. Reference strains (♦) are marked with scale below (e.g. Norwalk is reference strain for I.1 genotype). The genetic relationship is based on a neighbouring joining method of the 279- nucleotide sequence of the polymerase gene. Variants of GII.4 genotypes are marked with asterisks and a letter: variant Den_Haag_2006b (*D), Apeldoorn_2007 (*A), Yerseke_2006a (*Y), New_Orleans_2009 (*N).*

sel samples tested positive, while between 2010 and 2012 the percentage of NoV positive samples was from 12.5% to 22.2% (Table 1).
In total, 98 samples from the Seča harvesting area were examined with eight of them (8.2%) found to be positive. The percentage of positive samples varied from 4.0% to 30.0% in the period from 2006 to 2011, while in 2010 and 2012 no NoV positive samples were detected. From 80 samples originating from the Strunjan harvesting area, NoVs were detected in 17 samples (21.2%) with the prevalences from 10.0% in 2006 to 33.3% in 2010. Among 54 samples from the Debeli Rtič harvesting area 14 (25.9%) samples were contaminated with NoVs. The percentage of positive samples ranged from 9.1% to 53.8% in 2008 while in 2012 no sample from that area was analysed. Wild mussels were examined from non-harvesting areas, five samples from the area near Piran and one sample from the area near Strunjan. NoVs were detected in only two samples (33.3%) from the area near Piran.

Comparison of NoV strains detected in mussels, water and clinical samples in Slovenia and globally

From 20 RT-PCR positive samples only 12 sequences of 279 nucleotides were obtained. Nine sequences (75.0%) were classified as genotype GII.4, one (8.3%) as GII.P21 and two (16.7%) as GI.P2 (Figure 1). High genetic diversity with 78.8-81.0% nucleotide identity among GII strains (92.1-99.6% within the GII.P4 genotype), 100% nucleotide identity among GI and 58.4-60.2% among GI and GII strains was detected.
The polymerase gene nucleotide sequences obtained from this study were compared with sequences already detected in Slovenia and sequences available in GenBank. The phylogenetic analysis showed close relationships among NoV sequences detected from Slovenian mussels to sequences from clinical and environmental samples in Slovenia (Steyer *et al.,* 2011) and sequences found worldwide. NoV strains detected in mussels harvested at Debeli Rtič in 2007 and 2008 (Mussels/SI/2007/DRU, Mussels/SI/2008/DRU and Mussels/SI/2007/DR) show 99.3-99.6% sequence similarity to clinical strain (Hu/SI/2007/580) and strain (Env/SI/2008/V17) detected in 2008 from surface water in strains Slovenia, whilst strains Mussels/SI/2008/DRU was 100% identical with many strains in GenBank (representative Hu/JP/2008/Shimane3) from Japan, China and Taiwan. Isolate Strain Mussels/SI/2007/DRU shares 99.3 and 99.6% sequence similarity with strains from Japan (Hu/JP/2007/Toyama3) and China (Hu/CHN/2006/Shenzhen173-06), respectively. The NoV strain s isolated from surface water in Slovenia (Env/SI/2009/V3) shares 98.9-99.3% sequence similarity with two strains from Luxembourg and Belgium (Env/Lux/2009/45-1 and Env/BE/2008/IPH3064-08VG08) and are at the amino acid level 100% identical (data not shown). Two strains from the Strunjan harvesting area (Mussels/SI/2008/STM and Mussels/SI/2008/ST) share 98.2-98.9% sequence similarity with the strain (Hu/SI/2007/1732) isolated from a patient in Slovenia and 98.2-99.3% sequence similarity with strains from Japan, Australia and Netherlands (Hu/JP/2007/Aomori5, Hu/AU/2007/VIC3863, Hu/NL/2007/Nijmegen).
The only strain detected from the Seča harvesting area (Mussels/SI/2008/SE) shares 96.4-96.8% sequence similarity with two human strains detected in 2005 in Slovenia (Hu/SI/2005/288 and Hu/SI/2005/290) and even higher (98.9%) similarity with a number of Korean strains (representative Hu/KOR/2010/Seoul1387).
Three strains from the Strunjan harvesting area (Mussels/SI/2010/STN, Mussels/SI/2010/STJ and Mussels/SI/2007/ST) share 96.1-97.1% sequence similarity with four strains detected from patients with gastroenteritis in Slovenia in 2010. The latter strain shows higher sequence similarity (98.9%) to the human (clinical) strain (Hu/NL/2006/Terneuzen70) from the Netherlands. The human strain detected in Belgium (Hu/BE/2010/IPH294-10VG3P4) and strain from the Strunjan harvesting area (Mussels/SI/2010/STN) share 99.3% nucleotide identity.
The strain (Mussels/SI/2008/DRT) from the Debeli Rtič harvesting area was not related to any NoV strain previously detected in Slovenia; it is more similar to Indian and Brazilian strains (Hu/IN/2006/Ahm/PC03/GII.P21, Hu/BRA/2003/VIG-136/PA). Two strains detected in 2007 and 2008 from the Debeli Rtič harvesting area (Mussels/SI/2007/DRT and Mussels/SI/2008/DR) are 100% identical to each other and show the closest (86%) nucleotide identi-

ty to the human strain (Hu/SI/SI-2002) detected in Slovenia and are 100% homologous with the German human strain (Hu/DE/2004/Berlin/91192.05-7-BA3) (Figure 2). The sequence analysis of the polymerase gene region of NoV strains detected in mussels harvested in Slovenian coastal waters revealed five different variants of GII.P4 strains. In 2007 the GII.P4 Yerseke_2006a variant was detected in Strunjan (Mussels/SI/2007/ST). In 2007 and 2008 five strains were classified as Den Haag 2006b variant; two of them originated from the Strunjan harvesting area (Mussels/SI/2008/STM, Mussels/SI/2008/ST) and three from the Debeli Rtič harvesting area (Mussels/SI/2007/DRU, Mussels/SI/2007/DR and Mussels/SI/2008/DRU). In 2008 the strain from the Seča harvesting area (Mussels/SI/2008/SE) was classified as the GII.P4 Apeldoorn_2007 variant. In 2010 two identical strains detected in mussels from the Strunjan harvesting area (Mussels/SI/2010/STN and Mussels/SI/2010/STJ) were classified as the GII.P4 New_Orleans_2009 variant (Figure 2).

DISCUSSION

Mussels were sampled throughout the year during a five-year period to obtain information on the NoV contamination of shellfish harvested in Slovenia, and also to determine sequence similarities among the strains isolated from shellfish with other NoV strains isolated in Slovenia. All the samples originating from Slovenian producing areas are classified as potentially contaminated (class B), meaning that mussels have to undergo a process of depuration before they can be sold on the market.

However, shellfish originating from harvesting areas in the Italian part of the Adriatic sea are mainly classified as class A (Delibera Friuli 124/2010 Allegato A). The percentage of contaminated mussels from the Slovenian harvesting areas was lower (17.2%) compared to Italian data from 2011, where contamination was 34.4% (Suffredini *et al.,* 2011). Another study in Italy carried out from 2011 to 2012 showed 6.8% samples to be positive for NoV GI and 11.9% to be positive for GII (Fusco *et al.,* 2013). Results of the study carried out in France also differ from year to year - results from a three-year study showed 35.0% of contaminated mussels (Le Guyader *et al.,* 2000). However, reports from 2010-2011 showed a surprisingly low contamination of shellfish in the market - only 9.0% (Schaeffer *et al.,* 2013).

Among mussels collected in Slovenian coastal waters, contamination with NoVs was highest at the Debeli Rtič harvesting area (25.9%; 14/54). In Strunjan, 21.2% (17/80) of mussels tested positive for NoVs whereas in Seča contamination was detected in only 8.1% (8/98) of the sampled mussels (Table 1). It is difficult to evaluate the exact degree of wild mussel contamination because only a small number of wild samples (6) were collected.

By real-time RT-PCR, on average 17.2% (41/238) of mussels were found contaminated with NoVs, 2.4% (1/41) of them were classified as GI, 58.5% (24/41) were classified as GII, while in 39.0% (16/41) both genogroups (GI and GII) were detected (Table 1). A study investigating the characteristics of infections caused by NoV in Slovenia over a period from 2000 to 2007 also showed that the majority of the outbreaks were caused by NoV GII (88.2%), while 10.9% of the outbreaks were caused by NoVs GI (Zimšek Mijovski, 2010). Among all gastroenteritis outbreaks, 89.8% were caused by NoVs and 70.3% of outbreaks occurred in kindergartens, schools, nursing homes and hospitals. The main source of infection was direct contact from person to person. Only in 8.0% of cases was food the suspected vehicle, whilst water was the suspected source in 2.2% (Zimšek Mijovski, 2010). To the authors' knowledge it has not yet been reported that shellfish are a source of outbreaks in Slovenia.

With a rate of 68.2%, genotype II.4 is the leading strain causing outbreaks, not only in Slovenia (Zimšek Mijovski, 2010) but also worldwide with a rate of more than 80.0% (Lindesmith *et al.,* 2012). Also in our study the predominant genotype detected in mussels was GII.P4 75.0% (9/12), followed by GI.P2 16.7% (1/12) and GII.P21 8.3% (1/12). Results of a large comprehensive study showed that genotype II.4 was responsible for most of the human outbreaks (52.0%), followed by genotype II.b and II.7. The proportion of genogroup I was higher in bivalve molluscs than in infected humans. The two most frequently detected genotypes in foodborne ou-

tbreaks were I.2 and I.4 (Verhoef *et al.,* 2010). The rapid evolution of GII.4 noroviruses results in the successive emergence of new variants that have been observed since 2002 (Siebenga *et al.,* 2008). In 2006 the GII.4 variants in Europe and the USA were Laurens (2006a) and Minerva (2006b) (Siebenga *et al.,* 2008). In October 2009 a new variant, GII.4.2009 New Orleans, emerged and became the predominant outbreak strain, while Minerva circulated at lower levels (Vega *et al.,* 2011). The results of this study are in agreement with the appearance of the circulating strains - variant Den Haag 2006b was circulating in the period 2007-2009 (Eden *et al.,* 2010), while New Orleans 2009 was predominant in 2009-2012 (Vega *et al.,* 2011). During 2012 it was replaced by Sydney 2012 (Eden *et al.,* 2014), which is an emergent GII.4 variant detected in Slovenia in August 2012 from a child suffering from diarrhoea (unpublished data). The authors assume that in the (near) future this strain might also be detected in mussels from Slovenian coastal waters. For a better overview of the sequences, we would have to include a higher number of the sequences.

Sequence similarity among strains detected in mussels, isolated from sources of drinking and surface water and human clinical samples from Slovenia, was 95% at the nucleotide level and 100% at the amino acid level (data not shown). On the other hand, an even greater percentage of similarity was shown within the human strains worldwide. A sequence analysis showed an up to 100% match at the nucleotide level with strains isolated from around the globe (Japan, China, Korea, India). A high variability of the sequences was probably due to the small number of sequences that were obtained over a five-year period. Surface water samples in Slovenia were contaminated in 41.3% (26/63) of cases with NoV GII, in 33.3% (21/63) with GI, while drinking water had NoV in 2.2% of samples (Steyer *et al.,* 2011). In regard to the high contamination of surface water samples, it could be expected that even in shellfish, due to their filter feeding activity, the percentage of contamination would be similar, if not even higher. It has already been reported that after the treatment process wastewaters are also the main source of surface water contamination (Simmons and Xagoraraki, 2011). In the past municipal wastewater from the towns of Koper, Piran and Izola were discharged into the sea via the Rižana river and submarine pipes located 3.5 km and 300 m from the coast, respectively (Malačič *et al.,* 2000). In 2009, two renovated wastewater treatment plants in Koper and Piran started to operate but systematic monitoring of NoVs had not been carried out. Wastewaters from Izola are now collected in the Koper treatment plant and discharged into the Rižana river. The percentage of contaminated mussels in our study varied from 9.1% to 24.6% and did not differ greatly throughout these periods (Table 1). Many other unidentified sources may contribute to the input of waste into the sea and could be one of the main sources of seawater contamination with enteric viruses. Although NoV outbreaks are reported year-round they peak during months of cold weather (Mounts *et al.,* 2000). The occurrence of NoV human infections in Slovenia is not as seasonal as in other European countries. However, half of human infections appear in the colder months of the year (Zimšek Mijovski, 2010). In this study contaminated mussel samples originated in the winter months. Only one contaminated sample dated from July (data not shown). It would be interesting to monitor enteric viruses, at least in the rivers Rižana, Drnica and Badaševica, which flow into the Adriatic Sea, to evaluate a possible correlation between enteric virus detection and concentration in rivers and consequently the detection rate of those viruses in mussels.

If such a correlation is present, it would be necessary to implement preventive measures to decrease the possibility of enteric viruses being released into rivers and consequently to the sea. Phylogenetic analysis of the present study revealed that strains isolated from mussels (harvested in Debeli Rtič from 2007 and 2008) showed the highest (99.6%) nucleotide identity with only one strain (Env/SI/2008/V17) detected from surface water in Slovenia. Similarity among GII strains isolated from mussels and human strains was lower, 96.1-98.9%, while among GI sequence similarity was 86.0%. The analysis was limited to only 12 mussel strains. In order to obtain a better overview more strains should be included in the analysis.

Genetically identical strains (Mussels/SI/2008/DR and Mussels/SI/2007/DRT) detected in dif-

ferent sampling periods were also found, reflecting the longer retention of a certain strain on the site, or contamination with identical viral strains in both sampling periods. The first hypothesis is more likely, since some studies on contaminated groundwater suggest that NoV (GI) can remain detectable for more than three years (Seitz *et al.,* 2011).

In the harvesting area near Debeli Rtič, a higher percentage of NoVs (25.9%) was detected. The reason for that could be found in the location, as this area is at the most northern point where the main Adriatic Sea current flows. The fact that the NoV strains isolated from Mediterranean mussels showed a high relatedness to the GII.4 clinical strains, strains isolated from waters in Slovenia and strains from the world, indicates that shellfish harvesting areas are contaminated with water and sewage from surrounding areas.

The authors assume that the higher levels of contamination in Debeli Rtič can also be attributed to intensive shipping in this area, discharges of wastewaters, the river estuary and the main sea current. The consumption of shellfish from Slovenian harvesting areas presents a health risk to consumers. Although the majority of the shellfish is cooked, there is a need to develop a risk assessment, taking into account all the possible factors that could have an impact on preventing possible outbreaks caused by NoV contamination.

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