Norovirus detection in shellfish using two Real-Time RT-PCR methods

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SUMMARY

Shellfish are recognized as a potential vehicle of viral diseases. The aim of the present study was to determine the ability of two real-time RT-PCR methods (an in-house method and a commercial kit) for detecting Norovirus (NoV) belonging to genogroups GI and GII in shellfish. The analyses were performed both on a Norovirus Reference Panel (NRP), consisting of synthetic RNA, and on naturally contaminated mussels. For the experiments carried out on the NRP a statistically significant difference (χ^2=8.03) was shown between the results obtained by the two methods. The in-house real-time RT-PCR allowed the detection of all genotypes belonging to GI and GII, while the commercial kit was not suitable for the detection of the majority of the GI sequences constituting the panel. No significant difference was instead detected in the experiments carried out on shellfish, where the presence of GI was always concomitant with GII. Both methods were suitable for detection of NoV in shellfish, however the in-house real-time RT-PCR method had the advantage of differentiating GI and GII contamination. As regards the shellfish analysed, a considerable frequency of NoV contamination (34.4% of the samples) was detected, with a predominance of NoV GII.

KEY WORDS: Norovirus, Shellfish, Real time RT-PCR

Received August 10, 2010 *Accepted* October 29, 2010

INTRODUCTION

Noroviruses (NoV), belonging to the family of Caliciviridae, are considered the leading cause of non bacterial human gastroenteritis in developed countries (Koopmans and Duizer, 2004; Atmar and Estes, 2006; Svraka *et al.,* 2007). The NoV genome contains a 7.5 kb single-stranded, positive sense RNA, organised in three open reading frames (ORFs). ORF1 encodes a non-structural protein, ORF2 encodes the major capsid protein (VP1), and ORF3 encodes a small capsid protein (VP2) (Bertolotti-Ciarlet *et al.,* 2003). Because of their high genetic diversity NoVs are classified into five genogroups (GI to GV), of which GI and GII are most commonly associated with human infections. NoV GI and GII are further subdivided into different genotypes (at least 14 for GI and 17 for GII) based on capsid and RdRp sequences (Kageyama *et al.,* 2004). The NoV classification is in constant evolution with the discovery of new variant strains. Although many genotypes may cocirculate in a specific area, the GII genotypes seem to have a prominent epidemiological role in human gastroenteritis and, in particular, the recently identified GII.4 variants (Kroneman *et al.,* 2006; Phan *et al.,* 2006; Mesquita and Nascimento, 2009). Shellfish play an important role in the transmission of noroviruses, as filtering of large volumes of seawater contaminated from fecal waste may result in accumulation of these pathogens to considerable levels in shellfish tissue (Metcalf *et al.,* 1979; Rippey, 1994; Burkhardt and Calci, 2000). Thus consuming raw or improperly cooked shellfish is a major risk factor for food-borne outbreaks (Koopmans and Duizer, 2004; Prato *et al.,* 2004; Huppatz *et al.,* 2008; Le Guyader *et al.,* 2008; Guillois-Becel *et al.,* 2009).

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NoVs can only be detected by molecular approaches since they do not grow in cell culture (Duizer *et al.,* 2004) and in the past decade a large number of methods have been developed (Atmar *et al.,* 1995; De Medici *et al.,* 2004). The use of these methods, despite the limitations due to the lack of international standardization, has led to increased knowledge on the incidence of enteric viruses associated with shellfish-related outbreaks (Sanchez *et al.,* 2002; Gallimore *et al.,* 2004; Prato *et al.,* 2004) and their circulation in different areas (Le Guyader *et al.,* 2000; Formiga-Cruz *et al.*, 2002; Myrmel *et al.,* 2004).

Many studies report the predominance of NoV GII in shellfish contamination (Myrmel *et al.,* 2004; Loisy *et al.,* 2005; Suffredini *et al.,* 2008), in line with the apparent worldwide dominance of this genogroup in fecal samples compared to GI strains, based on molecular epidemiology studies of outbreaks (Fankhauser *et al.,* 1998; Kawamoto *et al.,* 2001; Lopman *et al.,* 2002). The reason for this is unknown, although possible explanations include differences in biological properties of NoVs, such as virulence, routes of transmission, or stability of the virus in the environment, probably combined with differences in the efficacy of methods for detection of the genogroups. Many authors have in fact hypothesized the inability of various methods and primers to identify the majority of shellfish contaminated with NoV GI (Lees, 2000; De Medici *et al.,* 2004; Lopman *et al.,* 2004).

Despite the genetic diversity of the NoVs, broadly reactive real-time reverse transcription PCR methods (rRT-PCR) have been developed (Kageyama *et al.,* 2003; Loisy *et al.,* 2005; Trujillo *et al.,* 2006; da Silva *et al.,* 2007; Gentry *et al.,* 2009). However an important limitation of these in-house methods is that they are labour-intensive and time-consuming. In the recent years, some commercial techniques based on end point or real-time RT-PCR (rRT-PCR) have been proposed rather than using in-house assays for detection of enteric viruses in clinical and environmental samples (Pillet *et al.,* 0000). The authors report that such methods are able to detect a broad range of strains, combining high sensitivity and specificity.

The aim of the present study was the evaluation of the performance of two rRT-PCR methods, an in-house method (method A) and a commercial kit (method B), for the detection of GI and GII noroviruses in shellfish. The experiments were performed both on a Norovirus Reference Panel (NRP), consisting of synthetic RNA, and on naturally contaminated mussels.

MATERIALS AND METHODS

Viruses and NoV RNA

NoV positive fecal specimens

The NoV genotypes GI.4 and GII.1 originated from stool samples collected during two outbreaks. Fecal suspensions were prepared in phosphate-buffered saline (PBS) (10% w/v). After centrifugation (3000 *g* for 20 min) the supernatants were aliquoted and frozen at -80°C. These were used as positive controls for method A.

Norovirus reference panel

The Panel was kindly provided by Rijksinstituut voor Volkgezondheit en Milieu (RIVM, The Netherlands) as desiccated synthetic RNA stabilized with yeast tRNA.

The panel included the three NoV genogroups that are found in humans (GI, GII and GIV) with various genotypes (Table 1).

Shellfish

A total of 90 mussel samples (*Mytilus galloprovincialis*), collected from different harvesting areas of Campania region in the Tyrrhenian Sea, were shipped to the laboratory in insulated boxes and were examined for NoV contamination.

Sample preparation

Norovirus Reference Panel

Dried RNA samples were resuspended in 100 µl of eluition buffer (molecular grade water, 200 U ml^{-1} RNAse inhibitor, 3 mM dithiothreitol) and stored at -80°C until testing.

Shellfish. For viral extraction ten mussels were selected from each sample, and the digestive gland was aseptically dissected and finely chopped with a sterile razor. A 2 gram aliquot was mixed with 2 ml of proteinase K solution (0.1 mg ml^{-1}), incubated at 37°C in a shaking incubator for 60 min, and then in a water-bath at 60°C for 15 min to inactivate the enzyme. The samples were then centrifuged at 3000 *g* for 5 min and the supernatant (approximately 3.0 ml)

TABLE 1 - *Results from the Norovirus Reference Panel (NRP) analysed by the in-house and the commercially available rRT-PCR assays.*

	Genotype	*In-house rRT-PCR*		*Commercial rRT-PCR*
		NoV GI	*NoV GII*	
GI.1	Norwalk	27.39	>45.0	31.04
GI.2	Whiterose	17.09	>45.0	>45.0
GI.2	Southampton	16.75	>45.0	>45.0
GI.3	Birmingham	15.77	>45.0	>45.0
GI.4	Malta	15.08	>45.0	>45.0
GI.5	Musgrove	33.60	>45.0	>45.0
GI.6	Mikkeli	17.11	>45.0	>45.0
GI.7	Winchester	12.60	>45.0	>45.0
GI.10	Boxer	13.84	>45.0	>45.0
GII.1	Hawaii	>45.0	12.77	14.97
GII.2	Melksham	>45.0	12.04	34.11
GII.3	Toronto	>45.0	14.14	>45.0
GII.4	Grimsby	>45.0	12.46	14.19
GII.6	Seacroft	>45.0	13.31	33.23
GII.7	Leeds	>45.0	17.76	15.77
GII.10	Erfurt	>45.0	11.16	13.56
GIIb	GGIIb	>45.0	11.17	13.01
GIIc	GGIIc	>45.0	12.67	14.81
GIV*	Alphatron	33.19	26.44	29.23

*Results obtained using a template concentration 3 log higher than for the other NRP samples. Analysis on dilution equivalent to those of the other samples provided *Ct*>45.0.

was recovered. Viral RNA extraction was performed on 500 µl of supernatant using the Mini Mag Nuclisens Magnetic Extraction kit (bioMerieux, France) according to the manufacturer's instructions; nucleic acids were recovered in 100 µl of elution buffer. Samples' RNA, with the addition of RNase inhibitor (1U μl^{-1}) were stored at -80°C until testing.

Real Time PCR methods

Method A - In-house one-step rRT-PCR

A protocol (da Silva *et al.,* 2007) based on two separate reactions for the detection respectively of GI and GII strains was used. Before use in the study, the method was subjected to in-house validation; specificity of the amplification (100% for both reactions) was evaluated on several viral and bacterial nucleic acids and tLOD (10 target copies/reaction) was verified on synthetic standardized template.

The primers and probes used to detect GI were: forward primer QNIF4 (5'-CGC TGG ATG CGN TTC CAT-3'), reverse primer NVILCR (5'-CCT TAG ACG CCA TCA TCA TTT AC-3') and probe NVILCRpr (5'-TGG ACA GGA GAY CGC RAT CT-3'). Primers and probe targeting GII were: forward primer QNIF2 (5'-ATG TTC AGR TGG ATG AGR TTC TCW GA-3'), reverse primer COG2R (5'-TCG ACG CCA TCT TCA TTC ACA-3') and probe QNIFS (5'-AGC ACG TGG GAG GGC GAT CG-3') (Y=C or T; R=A or G; N=A,C,G, or T; W=A or T). The two probes were labelled with 6-carboxyfluorescein (FAM) at the 5' end and with 6-carboxytetramethylrhodamine (TAMRA) at 3' end. Reverse transcription and PCR were performed on an ABI Prism 7700 SDS detector (Applied Biosystems) using the Platinum Quantitative RT-PCR ThermoScript™ one-step system (Invitrogen, France) and the following reaction mix: 2X thermoScript reaction mix (12.5 µl), 900 nM reverse primer, 500 nM forward primer, 250 nM Taqman probe, 1X ROX reference dye and 0.5 µl of ThermoScript Plus/ Platinum enzyme mixture. Five µl of sample nucleic acid extract were added per well (final reaction volume 25 µl). The amplification conditions were: reverse transcription for 60 min at 55°C followed by 5 min at 95°C and 45 cycles of 15 s at 95°C, 1 min at 60°C, and 1 min at 65°C.

Two negative controls (molecular grade water) were added in each run. For positive controls, the NoV-containing fecal samples described above (GI.4 and GII.1) were extracted using the Mini Mag Nuclisens Magnetic Extraction kit and tested undiluted and in tenfold dilutions. Tests were performed in quadruplicate and two dilutions (*Ct* value 30.08±0.51 for GI and *Ct* value 29.62±0.76 for GII) were selected as positive controls. Runs containing the samples were considered acceptable when *Ct* value for positive controls fell in the

above defined range and no amplification was detected in negative controls.

Method B - Commercial kit rRT-PCR

The AnDiaTec Norovirus Real Time PCR (Argene, France), based on a single reaction for the non-differential detection of both GI and GII NoV, was used according to the manufacturer's instructions. Specific primers and Taqman probes labelled with FAM, an internal control (probe labelled with VIC/HEX), positive and negative controls were included in the kit. The amplification conditions were: reverse transcription for 30 min at 45°C, followed by 2 min at 95°C and 45 cycles of 20 s at 95°C, 1 min at 53°C, and 2 min at 72°C. The reaction was performed on a Stratagene Mx3005P.

All shellfish samples nucleic acids were tested undiluted and diluted 1:10 to evaluate the effect of RT-PCR inhibitors; amplification efficiency (E) in undiluted sample was calculated according to the formula $E=2^{-\Delta Ct}$, with $\Delta Ct = Ct_{\text{undiluted}} - (Ct_{\text{1:10 dilution}} - 3.3)$. All amplification reactions were run in duplicate in two independent assays and a third repetition was performed if there were discordant results. Samples with a *Ct* value below 45 in at least two replicates and no evidence of amplification of the negative controls were considered positive.

Statistical analysis

The results obtained by the two rRT-PCR protocols were analysed by the McNemar test with Yates' correction for continuity. The threshold value of the χ^2 (chi-square) statistics for data distribution with one degree of freedom was conventionally fixed as 3.84 ($p<0.05$).

RESULTS

Norovirus Reference Panel

Analysis of the results of the two rRT-PCR methods using the Norovirus Reference Panel (Table 1) showed that using the in-house assay (method A), that applies two separated reactions for NoV GI and GII, all genotypes constituting the panel were correctly detected, whereas with the commercial kit (method B), based on a single reaction for GI and GII, 8 of 9 GII genotypes were correctly detected, but only one genotype (GI.1)

TABLE 2 - *Results from the mussels samples analysed by the in-house and the commercially available rRT-PCR assays.*

Sample	*In-house rRT-PCR (Ct ± SD)*		*Commercial rRT-PCR (Ct ± SD)*
	NoV GI	*NoV GII*	
1	33.65±1.45	29.64±0.26	37.61±0.41
2	32.89±1.01	28.92±0.29	35.58±0.52
3	32.72±1.13	28.45±0.47	35.69±0.23
4	32.63±0.52	28.58±0.13	39.18±0.38
5	32.59±0.46	28.29±0.27	35.03±1.06
6	33.69±1.37	29.15±0.31	35.64±0.29
7	34.32±1.57	29.03±0.41	36.42±1.13
8	32.64±0.13	28.22±0.60	35.41±1.52
9	32.10±0.38	28.83±0.39	34.72±0.47
10	>45.00	35.40±1.65	36.63±1.13
11	>45.00	>45.00	36.03±0.27
12	>45.00	32.46±0.12	35.86±0.52
13	>45.00	31.80±0.57	39.96±0.60
14	>45.00	33.42±0.75	>45.00
15	>45.00	32.45±2.49	>45.00
16	>45.00	37.02±1.07	>45.00
17	38.66±0.46	32.06±0.96	35.12±0.67
18	>45.00	32.52±0.52	36.63±0.27
19	>45.00	>45.00	35.17±0.55
20	>45.00	35.15±1.35	37.67±1.01
21	>45.00	33.28±1.04	38.82±0.29
22	>45.00	32.30±0.23	38.84±0.25
23	>45.00	32.64±0.60	35.15±1.03
24	>45.00	31.29±1.08	40.30±0.41
25	>45.00	29.61±0.55	35.36±0.23
26	>45.00	30.50±0.67	36.66±0.60
27	>45.00	30.99±0.55	34.37±0.13
28	>45.00	29.71±0.23	37.15±0.31
29	>45.00	29.17±0.40	35.73±0.29
30	>45.00	30.25±0.88	35.17±0.55
31	>45.00	29.89±0.40	35.99±0.29
32 to 90	>45.00	>45.00	>45.00
positive samples	29 (32.2%)		28 (31.1%)

of genogroup I provided a positive result (*Ct*=31.04). The statistical analysis performed by McNemar test showed a significant difference (χ^2=8.03) between the two methods.
The RNA of the GIV was detected by both methods when tested at high concentrations and, in the case of method A, positive results were obtained by both protocols for GI and GII. No amplification of GIV sequence was instead detectable using template concentrations comparable to those applied for GI and GII.

Naturally contaminated mussels
Among the 90 shellfish samples collected from the Campania region, a total of 31 (34.4%) were positive for the presence of NoV (29 positive according to method A and 28 using method B) (Table 2). In particular, two samples (n° 11 and 19) that were negative for both GI and GII by method A were positive by method B (providing *Ct* values respectively of 36.06 and 35.17) while an additional three samples (n° 14 to 16) were positive for NoV GII using method A, with *Ct* values respectively of 33.42, 32.45, and 37.02, but gave negative results with method B. The method A, providing distinct results related to the presence of NoV GI and/or GII in the samples, showed that all positive samples contained NoV GII and, among these, 10 were also positive for NoV GI. There were no statistically significant differences between the two methods by the McNemar test.
The comparison of the results obtained on undiluted samples and their tenfold dilution showed no relevant amplification inhibition. With the exception of samples that, presumably due to low contamination level, were detected positive only in undiluted samples, ΔCt between the two dilutions was always ≥3.0 (equivalent to a theoretical amplification efficiency of at least 80% in the undiluted samples).

DISCUSSION

The availability of sensitive and efficient methods for directly detecting enteric viruses in shellfish is an important tool for the prevention of food-borne viral diseases. The various methods described over the past 15 years demonstrate that it was possible to detect viruses, nevertheless there are problems due to the low levels of virus contamination and the presence of interfering substances that inhibit molecular detection. Furthermore, because of the high genetic variability of NoV, it has become even more important to have a widely applicable technique for detection of the genomic variants in shellfish.
Regarding the results obtained with the Norovirus Reference Panel, the performance of the two methods used (A and B) showed a statistically significant difference (χ^2=8.03). Method A provided better results allowing detection of genotypes belonging to GI and GII constituting the panel, while method B was not suitable for detection of the majority of the NoV GI. It may be hypothesized that the results were influenced by the use of synthetic RNA constructed using only selected genomic regions of NoV, although - according to available information - such selection included all the representative sequences for diagnostic applications (RdRp, capsid gene, ORF1-ORF2 overlapping region, etc.).
The experiments carried out on shellfish did not show a significant difference between the two methods, providing opposing results only in 5 of 90 cases (5.6%; two false negative results with method A and three false negative results with method B). Furthermore, it was not possible to evaluate the performance of the two methods for the detection of NoV GI, because all samples contaminated by this genogroup were also positive for NoV GII.
Concerning the applicability of laboratory methods, the characteristic rapidity and simplicity of preparation of the PCR reaction mix is an advantage of using commercial kits. Moreover, the reaction mixture contains an internal control, which allows determination of inhibition of target amplification due to matrix interference. Generally, method B provided higher *Ct* values than method A for the same samples, but it is possible that the differences are due to the use of two different real-time PCR instruments.
Based on the results of the current study, both methods appear to be suitable for detection of NoV in shellfish, however the in-house rRT-PCR (method A) has the advantage of being able to differentiate genotypes NoV GI and GII. The ability to differentiate the two genogroups allows the study of the different circulation patterns of the different genotypes. In fact, molecular epidemiology studies of outbreaks (Fankhauser *et al.*,

1998; Kawamoto *et al.,* 2001; Lopman *et al.,* 2002) show an apparent worldwide dominance of NoV GII the over GI strains in human fecal samples, also in terms of concentration (Victoria *et al.,* 2009), also suggesting that the higher GII viral load could facilitate the transmission of the virus. On the other hand, da Silva *et al.* (2007) reported that GI may be more resistant to breakdown during wastewater treatment, and this higher resistance could explain the fact that GI is more often implicated in food or water-related outbreaks than GII (Kageyama *et al.,* 2004; Maunula and Von Bonsdorff, 2005; Blanton *et al.,* 2006; Le Guyader *et al.,* 2006).

In the current study, 34.4% of the analysed shellfish samples from the Campania region were positive for NoV GI and/or GII, indicating a widespread diffusion of NoVs in the samples' harvesting areas.

The higher prevalence of NoV GII may be due to a different affinity of the two genotypes to mussel tissue that may influence the ability to bioaccumulate in shellfish, as others have reported (Comelli *et al.,* 2008).

Further analyses are needed to evaluate NoV contamination in the different harvesting areas of the Campania region taking into account circulating viral genotypes, possible pollution sources, and atmospheric conditions. This information will allow the development of control strategies for NoV to assure the availability of a safe product for the consumer.

ACKNOWLEDGEMENTS

This work has been partially supported by: RFPS-2007-7-645333 "P5 Risk based microbiological criteria: a tool for consumers protection and intervention strategies optimisation" 7th European Framework projects: Grant agreement 222738; "Selection and improving of fit-for-purpose sampling procedures for specific foods and risks" (www.baselineeurope.eu).

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