Genotyping of GII.4 and GIIb norovirus RT-PCR amplicons by RFLP analysis

Stefania Ramirez a, Giovanni M. Giammanco a, Simona De Grazia a, Claudia Colomba b, Vito Martella c, Serenella Arista a,∗

a Department of Hygiene and Microbiology, University of Palermo, via del Vespro 133, 90127 Palermo, Italy
b Institute of Infectious Diseases and Virology, University of Palermo, Piazza Montalto 8, 90134 Palermo, Italy
c Department of Animal Health and Well-being, University of Bari, S.p. per Casamassima km 3, 70010 Valenzano, Bari, Italy

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Abstract

GII.4 and GIIb/Hilversum norovirus (NoV) strains appear to have a prominent epidemiological role in outbreaks or sporadic cases of human gastroenteritis. Sequence analysis, although laborious, is the reference method used for characterization of noroviruses. In this study a screening test is proposed to characterize GIIb and GII.4 NoVs based on restriction fragment length polymorphism (RFLP) analysis of amplicons obtained from the RNA-dependent RNA polymerase (RdRp) region. Virtual analysis of 793 RdRp sequences of GGI and GGII NoVs, retrieved from GenBank, and representative of global geographical origins on a long-time period, permitted the selection of four restriction enzymes, XmnI, AhdI, BstXI, and AcuI, suitable for correct identification of GIIb and GII.4 NoV genotypes. Experimental analysis by the RT-PCR RFLP analysis of 41 NoV strains detected in Palermo during the years 2002–2005 allowed to recognize all the Italian strains as belonging to GIIb/Hilversum or GII.4, and sequence analysis confirmed these results. The PCR-RFLP protocol developed in this study proved to be a simple and reliable proxy for sequence-based classification of the GIIb/Hilversum and GII.4 NoV variants displaying high specificity (100%) and sensitivity (94%).

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Keywords: Norovirus; Characterization; GIIb/Hilversum strain; GII.4 genotype; Restriction fragment length polymorphism (RFLP)

1. Introduction

Noroviruses (NoVs) are non-enveloped viruses within the Caliciviridae family possessing a 7.7–8 kb single stranded RNA of positive polarity (Fauquet et al., 2005). They have been recognized as causative agents of epidemic gastroenteritis in humans of all age groups and of acute sporadic gastroenteritis in infants and young children (Hutson et al., 2004; Monica et al., 2007; Zintz et al., 2005). NoVs are heterogeneous genetically as a result of accumulation of point mutation and recombinational events (Ambert-Balay et al., 2005; Bull et al., 2005; Reuter et al., 2005, 2006; Rohayem et al., 2005). They have been tentatively classified into five genogroups (GGI–GGV) on the basis of partial or complete sequences of the capsid gene. Eight different genotypes of GGI NoVs, 17 of GGII and a single genotype of GGIV affect humans (Zheng et al., 2006).

Genotype determination and detection of variants within a genotype are useful in order to monitor the global spread of noroviruses and to trace epidemics. The availability of genotyping data collected worldwide is necessary for the design of effective vaccination strategies against norovirus disease.

Although many genotypes of human NoVs may co-circulate in a particular geographic area, only a few appear to have a prominent epidemiological role in human gastroenteritis (Ambert-Balay et al., 2005; Blanton et al., 2006; Ike et al., 2006; Lindell et al., 2005). In particular, GGI NoVs account for at least the 90% of the NoV isolates, and the majority of episodes of diarrhoea by GGI NoV strains are caused by GII.4 and by GIIb/Hilversum strains (Ambert-Balay et al., 2005; Bull et al., 2006; Ike et al., 2006; Lindell et al., 2005; Ramirez et al., 2006; Sanchez-Fauquier et al., 2005). Since the detection of the GII.4 prototype strain Lordsdale/1993/UK, several GII.4 NoV vari-
The role as cause of gastroenteritis of children has been highlighted more frequently in children than in adults, and its prominent al., 2005, 2006). The GGIIb/Hilversum strain has been detected 2005; Reuter et al., 2005), seems to have emerged as a con-
out the European continent (Buesa et al., 2002; Lindell et al., France (Ambert-Balay et al., 2005) and then detected through-
the NoV GGIIb/Hilversum strain, first identified in 2000 in Europe, from July to December 2006 (Gallimore et al., 2006). The NoV GGIIb/Hilversum strain, first identified in 2000 in 
Europe (Ambert-Balay et al., 2005) and then detected through-
out the European continent (Buesa et al., 2002; Lindell et al., 2005; Reuter et al., 2005), seems to have emerged as a con-
sequence of recombination with other GGI NoVs (Reuter et al., 2005, 2006). The GGIb/Hilversum strain has been detected more frequently in children than in adults, and its prominent role as cause of gastroenteritis of children has been highlighted (Lindell et al., 2005; Ramirez et al., 2006).

In the NoV genome, the RdRp region contains highly con-
served motifs and represents the best target for the construction of broadly reactive primers that may be applied as a molec-
ular tool for the detection of a variety of NoV genogroups, genotypes, and variants. Although the phylogenetic classifica-
ton of noroviruses is determined through characterization of the gene (ORF2) encoding the capsid (Vinje et al., 2004), molecular assays based on the RdRp fragment (ORF1) represent a tool for comparison of large data sets and may be regarded as a good proxy for strains characterization by sequence analysis of short amplicons (Ike et al., 2006; Lindell et al., 2005; Reuter et al., 2006; Vinje et al., 2003).

Restriction fragment length polymorphism analysis RFLP has been applied to PCR amplicons for routine characterization of a variety of micro-organisms, including bacteria (Leao et al., 2005; Prammananan et al., 2006), fungi (Rodriguez-Nava et al., 2006), and enteric viruses such as adeno viruses and rotaviruses (De Jong et al., 1999; Iturriza-Gomara et al., 2002; O’Halloran et al., 2002; Rodriguez-Castillo et al., 2006). In comparison with sequence analysis, PCR-RFLP is a less laborious, quick method of wide applicability.

In the present study, a PCR-RFLP assay was designed and evaluated as a screening test to characterize GIIb/Hilversum and GII.4 NoVs. Virtual analysis of 793 RdRp sequences of GGI and GGII NoVs retrieved from GenBank has been used for the selection of the suitable restriction enzymes. The reliability of the protocol is evaluated on NoV strains isolated from 2002 to 2005 in Palermo, Italy, from children admitted to hospital with gastroenteritis.

2. Materials and methods

2.1. Virtual restriction analysis

2.1.1. Selection of the sequences

A series of separate BLAST searches for nucleotide–nucleotide comparison was undertaken on GenBank (ncbi.nlm.nih.gov/BLAST/) on 15 February 2007, using as a query the reference sequences of the polymerase gene of the prototype Lordsdale and of the two most circulating GII.4 clusters, FarmingtonHill and Hunter (X86557/Lordsdale/1993/UK, AY502023/FarmingtonHill/2002/US, DQ078801/Hunter532D/ 2004/AU), and of the GIIb/Hilversum strain (AY773210/ Vannes/2000/FR). The first 250 unique sequences, showing the best matching quality score, were retrieved for each query, together with the prototype sequences of known genogroups and of the circulating genotypes available on public databases.

All the sequences were aligned to the respective NoV geno-
type reference sequence using Clustal W (Thompson et al., 1994) and the nucleotide sequence similarities were calculated on the longest common fragment. For the attribution to a genotype, Vinje’s criteria (a similarity in the polymerase region of equal or more than 85% or 90% for GGI and GGII, respectively) (Vinje and Koopmans, 2000) were used and all the sequences that did not match such criteria were deleted from the analysis. A 327 bp long fragment (from 4279 to 4606 nucleotide position, when referred to Lordsdale/1993/UK X86557) was used, and all the sequences that did not cover at least the 70% of the central portion of the segments were deleted.

2.1.2. Selection of restriction enzymes

A list of restriction enzymes was retrieved from the http://rebase.neb.com/cgi-bin/nebenzlist site. A total of 217 enzymes were tested by virtual digestion of all the selected NoV sequences using a home-made software (available from the authors upon request). By using this approach, the presence and position of restriction sites was screened in all the selected NoV RdRp sequences. Enzymes were selected based on the ability to distinguish selectively GIIb and GII.4 NoVs from GI and other GII NoV strains and to discriminate between GIIb and GII.4 NoVs.

2.2. Experimental restriction analysis

2.2.1. RNA extraction and RT-PCR

NoV RNA was extracted from 200 µl of 10% faecal sus- pension in minimum essential medium balanced salt solution (MEM) using guanidinium isothiocyanate/silicae according to the procedure of Boom et al. (1990). RNA was eluted in 50 µl RNase-free H2O in the presence of RNasin (0.2 µg/µl, Promega Corporation, Madison, WI, US) and used in reverse transcription (RT)-PCR.

RT-PCR was performed with specific primers JV12/JV13 that amplify a 327 bp fragment of the RdRp region (region A) (Vinje and Koopmans, 1996) using Superscript III One step (Invitrogen, Paisley, UK).

2.2.2. Samples

Forty-one NoV-positive faecal samples, collected in the years 2002–2005 from children <5 years age, admitted with acute gastroenteritis to the “G. Di Cristina” Children’s Hospital of Palermo, were included in this study. Sixteen, of these 41 samples, contained GIIb/Hilversum NoV strains, 12 GII.4 strains, and 2 GGI strains, as previously iden-
tified by sequence analysis (Ramirez et al., 2006). An additional 11 samples, positive for NoVs by RT-PCR but not submitted previously to strain characterisation, were also tested.

2.2.3. Restriction analysis

Briefly, 10 µl of each PCR product was digested in a final volume of 20 µl with restriction enzymes XmnI, BstXI (Promega Corporation), AcdI, and AhdI (New England Biolabs, Beverly, MA, US), following manufacturers’ instructions. The digestion products were electrophoresed in 3% agarose gel stained with ethidium bromide (0.5 µg/ml) and photographed after transillumination by UV light.

2.3. Sequence analysis

Partial sequences of the RdRp of the 11 NoV strains not characterized previously were determined. The amplicons obtained with primers JV12/JV13 were purified through spin-columns (Microcon50, Millipore, Bedford, MA, US) and submitted to sequence analysis (MWG-biotech, Ebersberg, Germany). The nucleotide sequences were aligned and analysed using Clustal W (Thompson et al., 1994).

3. Results

3.1. Selection of NoV sequences for virtual analysis

A total of 793 RdRp sequences, representative of the various NoV strains identified thus far, were selected from GenBank on the basis of their similarity with respect to at least one reference GGI or GGII sequence representative of the different genotypes (Vinje and Koopmans, 2000) and of their length (at least 70% of the entire 327 bp fragment chosen for the analysis). Table 1 shows the reference sequences used for the selection and the number of sequences retrieved for each genotype. One hundred and two sequences were GGI NoV strains identified from 1969 to 2005, while 691 sequences were GGII NoV strains isolated from 1972 to 2006.

3.2. Virtual restriction analysis

Out of 217 enzymes tested, 196 were found to have restriction sites in the RdRp gene fragment of NoV strains. Of these, 40 enzymes displayed only 1 or 2 restriction sites or gave a restriction fragment shorter than 295 bp.

Six of the 40 enzymes were able to cut at least 90% of the GIIb sequences. Among these, AcdI and BstXI digested and differentiated unequivocally 95/100 GIIb sequences from all other NoV genotypes. AcdI produced two fragments of 130 and 197 bp, while BstXI gave 145 and 182 bp restriction products.

The combined use of three enzymes, XmnI, BstXI, and AcdI, was necessary to identify 280 out of 299 (93.6%) GII.4 sequences without any false positive result. XmnI digested 291/299 (97.3%) GII.4 sequences, yielding a pattern of 126 and 201 bp that was also generated with GII.1, GII.6, and GII.12 sequences. The second enzyme, BstXI, did not digest GII.4 sequences but was able to cut 62 of 65 (95.4%) sequences belonging to genotypes other than GII.4 previously cut by XmnI. After this analysis only one GII.6 and two GII.12 sequences were recognized erroneously as GII.4 and not typed correctly. For this reason the third enzyme, AcdI, was used. It did not discriminate between GII.4 and GIIb sequences, but was able to cut 62 of 65 (95.4%) sequences belonging to genotypes other than GII.4 previously cut by XmnI.

In Table 2 the sequence fragment recognized by each of the restriction enzymes used and its position in reference to the
Lordsdale/1993/UK X86557 are shown. The substitutions observed in the 5 GIIB and in the 19 GI.4 sequences that were not typed by the restriction analysis are shown in Tables 3 and 4, respectively.

### 3.3. Experimental restriction analysis

On the basis of the results obtained by the virtual analysis, the enzymes AhdI, XmnI, BstXI, and AcuI were used for PCR-RFLP analysis of 41 NoV strains identified in Palermo in 2002–2005. Twenty-one strains produced the specific GIIB restriction pattern from the combined use of AhdI and BstXI enzymes, and 18 strains were recognized as GI.4 by XmnI and AcuI digestion (Fig. 1). Two NoV GGI strains were tested as controls and, among the four enzymes, only AcuI was able to digest one of the two strains producing a peculiar and easily distinguishable two bands pattern. In all of the cases sequence analysis confirmed the typing obtained by PCR-RFLP analysis.

### 4. Discussion

The role of NoVs as a major cause of gastroenteritis in humans is well established. Large-scale application of RT-PCR and sequence analysis to the study of NoV epidemiology have allowed the definition of their role as enteric pathogens, to trace the origin of gastroenteritis outbreaks and to monitor the dynamics of evolution of NoV strains on a global scale. Due to their highly contagious nature, and to their economic impact on public health, NoVs have been classified by the Centers for Disease Control and Prevention and National Institute of Allergy and Infectious Diseases as Category B Priority Pathogens (CDC, 2010).

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### Table 2

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Recognized sequence</th>
<th>Position</th>
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<tr>
<td>AhdI</td>
<td>5′-GACNNN^_NGTC-3′</td>
<td>4403–4413</td>
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<tr>
<td>BstXI</td>
<td>5′-CCANNNNN^_NNTGG-3′</td>
<td>4416–4427</td>
</tr>
<tr>
<td>XmnI</td>
<td>5′-GAANN^_NTTC-3′</td>
<td>4400–4409</td>
</tr>
<tr>
<td>AcuI</td>
<td>5′-CTGAAG(N)16^_3′</td>
<td>4536–4557</td>
</tr>
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</table>

*a* Cutting position of the restriction enzyme.

### Table 3

<table>
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<tr>
<th>GenBank code</th>
<th>% homology</th>
<th>Origin</th>
<th>Year of isolation</th>
<th>Enzyme failing to type correctly</th>
<th>Position of substitutions in the recognition sequence for the enzyme</th>
</tr>
</thead>
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<tr>
<td>AB242258</td>
<td>99</td>
<td>Japan</td>
<td>2004</td>
<td>AhdI</td>
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<td>2001</td>
<td>AhdI</td>
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<td>Norway</td>
<td>2004</td>
<td>AhdI</td>
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<tr>
<td>AV919139</td>
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<td>Australia</td>
<td>2003</td>
<td>BstXI</td>
<td>4417 C → T</td>
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<tr>
<td>AB231357</td>
<td>97</td>
<td>Japan</td>
<td>2003</td>
<td>BstXI</td>
<td>. C → T</td>
</tr>
</tbody>
</table>

*a* Homology was calculated on the reference GIIB Vannes strain.

### Table 4

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<tr>
<th>GenBank code</th>
<th>% homology</th>
<th>Origin</th>
<th>Year of isolation</th>
<th>Enzyme failing to type correctly</th>
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<td>2002</td>
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<td>4408 T → C</td>
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<tr>
<td>DQ157118</td>
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<td>Netherlands</td>
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<td>2002</td>
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<td>1998</td>
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<td>&lt;1999</td>
<td>BstXI</td>
<td>4417 T → C</td>
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</table>

*a* Homology was calculated on the reference GI.4 Farmington strain.
Fig. 1. Patterns of cleavage obtained with AhdI (panel a, lanes 2–7), BstXI (panel a, lanes 8–13), XmnI (panel b, lanes 2–7), and AcuI (panel b, lanes 8–13) endonucleases. Lanes 2–3 and 8–9 GIIb/Hilversum NoV amplicons; lanes 4–5 and 10–11 GII.4 Italian strains and lanes 6–7 and 12–13 GI Italian strains. The low range Mass ruler DNA Ladder (Fermentas, Burlington, ON) was used as a size marker in lane 1 of both migrations.

Only specialized laboratories undertake molecular characterization of NoV strains by sequence analysis, since dedicated instruments and skilled technical capacity are needed. Here a simple and rapid screening test, based on PCR-RFLP and within the reach of many diagnostic laboratories, was developed to recognize the two NoV strains predominant in Europe, GIIb/Hilversum and GII.4 (Buesa et al., 2002; Ike et al., 2006; Lindell et al., 2005; Lopman et al., 2004; Ramirez et al., 2006; Reuter et al., 2005; Vainio and Myrmel, 2006), and to reduce the number of samples to submit to sequence analysis. Because an affordable screening test must guarantee a 100% specificity, a typing protocol was developed on the basis of 793 NoV RdRp sequences available in GenBank and representative of the global NoV circulation over a 37 years period (1969–2006), by evaluating the virtual restriction patterns produced by 217 endonucleases. Computer-assisted restriction analysis of the selected sequences showed that the combined use of two enzymes, AhdI and BstXI, was able to correctly characterize 95% of the GIIb/Hilversum NoVs. Three enzymes, BstXI, XmnI, and AcuI, were necessary to identify correctly 93.6% of 299 GII.4 sequences. The GIIb and GII.4 NoV strains that failed to be identified showed nucleotide substitutions in the regions recognized by the restriction enzymes. The GIIb unrecognized strains were mostly circulating in 2003 and 2004 in remote countries such as Japan (AB242258, AB231356), Norway (Vainio and Myrmel, 2006), Australia (Bull et al., 2006), and Germany (AF409066), while the atypically restricted GII.4 sequences were heterogeneous temporally and geographically (Ike et al., 2006; Iritani et al., 2003; Lindell et al., 2005; Zintz et al., 2005, and GenBank accession numbers cited in Table 3). Point mutations in template RNA such as those affecting restriction endonuclease recognition sites in cDNA can also lead to lack of recognition of template RNA or ss cDNA by PCR primers, a problem that has been well documented for a number of RNA viruses and particularly for rotaviruses (Adah et al., 1997; Iturriza-Gomara et al., 2000).

Both in GIIb and GII.4 virtual typing, the use of the four selected enzymes guaranteed 100% specificity, but the use of more endonucleases, excessively increasing the cost and the complexity of the method, would be necessary to enhance the sensitivity of the analysis.

The applicability for NoV typing by PCR-RFLP analysis proposed was tested experimentally on 41 NoV strains detected in Palermo in the years 2002–2005 and compared to sequence analysis. The selected enzymes allowed recognizing all the Italian strains as belonging to GIIb/Hilversum or GII.4, and sequence analysis confirmed these results. The predominance of GIIb and GII.4 NoVs (95.1%) in the area of Palermo demonstrated further the wide circulation of such NoV strains in Europe (Buesa et al.,
If PCR-RFLP is applied to trace the circulation of the main genotypes of noroviruses in a geographical area, our results demonstrate that this type of information can be obtained. Undoubtedly, the assay is not able to detect all norovirus variants within a genotype and to differentiate between them and sequencing should be used in order to monitor the spread of antibody-escape mutants, which may arise over time and could eventually result in epidemic disease.

In conclusion, the PCR-RFLP protocol developed in this study for NoV characterization proved to be a simple and reliable proxy for the detection of the GIIb/Hilversum and GII.4 variants, as confirmed by full nucleotide sequencing. Although the method has only 94% sensitivity (mainly due to well-documented point mutations in restriction enzyme recognition sites of ampiclons), it has its value as a screening test for many laboratories without sequencing equipment and its application could contribute to acquire a more precise picture of the circulation of norovirus strains worldwide and to ascertain the dominating role of the GIIb/Hilversum and GII.4 variants. The availability of genotyping data from developing countries would be of help for the design of effective vaccination strategies for noroviruses.

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