

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF NOROVIRUS IN SÃO PAULO STATE, BRAZIL

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ABSTRACT

Norovirus (NoV), previously called Norwalk-like virus, represents an important group of human pathogens associated with outbreaks of nonbacterial gastroenteritis. Epidemiological surveys of outbreaks have shown that the most important routes of transmission are person-to-person contacts and contaminated food and water, with the virus affecting adults and children. NoV is classified into genogroups, being genogroups GI, GII and GIV found in humans. In view of the high genetic diversity of NoV and the lack of information about this virus in Brazil, the aim of the present study was the molecular characterization of NoV isolated from diarrheic stool samples of patients from São Paulo State. In this study, 204 stool specimens collected during diarrhea outbreaks were analyzed by RT-PCR, and 12 were sequenced for genogroup confirmation. One-step PCR was performed in order to amplify the B region of ORF 1 using the MON 431, 432, 433 and 434 primer pool. From total, 32 (15.7%) stool specimens were positive for NoV genogroup GII. Comparison of the sequences of the PCR products to GenBank sequences showed 88.8% to 98.8% identity, suggesting the presence of genogroup GII in gastroenteritis outbreaks in São Paulo State.

Key words: Norovirus, Genogroup GII, Molecular characterization.

More than one billion cases of acute diarrhea are estimated to occur annually in children and adults worldwide. The estimated annual mortality associated with gastroenteritis is six million people (20). Various infectious agents can cause manifestations of gastroenteritis, including bacteria, viruses and parasites.

Among virus, norovirus (NoV) are the leading cause of acute nonbacterial human gastroenteritis worldwide responsible for 80-90% of the reported outbreaks (17). The virus is transmitted through contaminated food and water or person-to-person contact by the fecal-oral route. These viruses are highly contagious and infections may occur in sporadic cases or in large outbreaks of acute diarrhea on hospital wards, hospitals, schools, universities, camps, cruises, hotels, and restaurants (15). NoV infections cause symptoms of severe vomiting, watery diarrhea, nausea, abdominal cramps, fever, and general malaise (14,33).

NoV, previously called Norwalk-like virus, is the current denomination of a genus of viruses, which were known until recently as caliciviruses (referring to the family *Caliciviridae*) and as Small Round Structured Viruses due to their morphology (19). NoV virions present a simple structure consisting of a capsid and nucleic acid, and measure about 27 to 30 nm in diameter. The NoV genome consists of a single-strand of positive-sense RNA organized into three open-reading frames (ORFs). ORF 1 encodes nonstructural proteins such as RNA-dependent RNA polymerase, ORF 2 encodes viral capsid protein 1, and ORF 3 encodes a small capsid protein (viral protein 2) associated with stability of viral capsid protein 1 (2,9,10,13,31). The complete genome comprises approximately 7.5 kb and consists of 45 to 56% cytosine + guanine (C + G) (5).

Molecular analyses of the polymerase and capsid regions indicated the presence of five genogroups (GI, GII, GIII, GIV and GV) in NoV strains isolated from different hosts (16). Genogroups

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GI, GII and GIV were detected in humans and genogroups GIII and GV in bovine and mouse, respectively (12). GI and GII seem to be the two major genogroups of worldwide spread and are further subdivided into more than 25 different genotypes (32). The majority of newly emerging NoV strains belongs to GII.4 (Genogroup II genotype 4) and has a global presence associated with gastroenteritis outbreaks in United States, Brazil, Canada, China, Germany, Australia and others (2).

Little is known about the circulation of NoV in Brazil, especially in São Paulo State (27). The need for studies identifying and characterizing the genogroups of this etiological agent associated with outbreaks of nonbacterial acute diarrhea motivated the present study.

A total of 204 stool samples were sent for Enteric Virus Laboratory of Adolfo Lutz Institute to rotavirus diagnosis from diarrhea outbreaks that occurred in the State of São Paulo at the years 1995, 2004, 2005 and 2006. All samples showed rotavirus negative results and were selected by clinical data to NoV diagnosis, to represent restrict (hospital and healthcare) and opened community. The standard virus was identified in human faecal specimens characterized previous like NoV genogroup GII by PCR, hybridization and nucleotide sequencing (29).

Study methods were approved by the Ethical Committee in Human Research of Adolfo Lutz Institute.

Primers set used in the present study were deduced from the 3' end conserved genome region of Pol ORF 1 (7): Mon 431 (sense, 5' - TGG ACI AGR GGI CCY AAY CA - 3'), Mon 432 (sense, 5' - TGG ACI CGY GGI CCY AAY CA - 3'), Mon 433 (anti-sense, 5' - GAA YCT CAT CCA YCT GAA CAT - 3') and Mon 434 (anti-sense, 5' - GAA SCG CAT CCA RCG GAA CAT - 3'), it consists of an amplification of both GI and GII genogroups. NoV ssRNA was extracted directly from stool by the Trizol® reagent (Invitrogen, Carlshad, CA, USA) (5), according to the manufacturer's instructions and it was stored at -70°C. RT-PCR was performed by one step method (1,11). PCR mix contained in 50 µL: 1.25 mM of each deoxynucleotide (dATP, dGTP, dCTP, dTTP), 0.1 M DTT, 1X 5X buffer [TRIS-HCL 300 mM pH 9.0 and 62.5 mM (NH₄)₂ SO₄], 0.5X 10X buffer [TRIS-HCL 100 mM pH 9.0, KCl 500 mM and Triton X-100 1%], 1.25 mM MgCl₂, 20 µM of pool of primers MON 431, MON 432, MON 433 and MON 434 (7), 1 U SuperScript™ II RNase H-Reverse Transcriptase (Invitrogen™), 2 U Platinum® Taq DNA Polymerase (Invitrogen™), RNAsin (Invitrogen™). As template was used 5 µL of ssRNA. The amplified products were analyzed by electrophoresis in 1.5% agarose gel, containing 0.5 µl ethidium bromides and visualized under UV illumination (24). The expected length for PCR products, synthesized with pool MON was 213bp.

Genogroup IV was not evaluated in this study. This Genogroup is rare and no standard primes were available. As a perspective for further study, we suggest the investigation of this particular Human Genogroup to enhanced our findings.

Thirty-two (15.7%) samples were identified as NoV genogroup GII by RT-PCR using the MON 431-433 primer pool.

Twelve samples were selected for sequencing in order to confirm NoV genogroup. These were chosen once appeared clean PCR products (without additional bands) and representatives of different origins and years.

The nucleotide sequence of NoV genogroup II was determined directly from a PCR product, after purification with silica based membranes (Concert™ rapid PCR Purification System Germany, US, UK. (GIBCO BRL®). Sequencing reactions were performed by using the Kit ABI Prism® Big Dye™ Terminator Cycle Sequencing, Ready Reaction (Applied Biosystems, Foster City, EUA) according to the manufacture instructions. The primers (Mon 431 and Mon 433), and of 1 to 10 pmol DNA concentration were utilized in the reactions. The extension products (172 nucleotides) were purified by Centri-cep columns (Princeton Adelpia, Ni) and the nucleotide sequences determined in an automated 377 ABI sequencer (PE Applied Biosystems, Inc., USA).

The nucleotide sequences were edited using the Chromas program. The programs Editseq and Megalign (DNASTAR, Inc., Madison, Wis. USA) were used to deduce the amino acid sequences and to calculate the identity between the analyzed sequences. Comparative sequence analysis of nucleotide and amino acids sequences of NoV genogroups GII and GI were done by using Clustal X program, developed by Thompson *et al.* (28). The software MEGA (Molecular Evolutionary Genetics Analysis) version 2.1: (18) was used for edition and documentation.

The sequences determined in this study were deposit in GenBank with the accession numbers EF442174 (IAL-C16-BRA) to EF442185 (IAL-C178-BRA). Sequences of genogroup GII: DQ397325-BRA (Leite *et al.*, unpublished) and AF414414-USA (1) and genogroup GI: DQ386978-BRA (4) retrieved from GenBank were used in comparative sequence analysis.

Comparison of the sequences determined in this study with sequences available in GenBank was the method of choice for genome analysis and characterization of NoV genogroups according to the proposed classification (>85% similarity for GI samples and >90% similarity for GII samples) (13,31,34).

All twelve samples analyzed by gene sequencing confirmed that they belong to genogroup GII that is considered the most prevalent strain worldwid. The sequences of the samples identified in each outbreak showed a high degree of similarity (Fig. 1). Comparison of the sequences showed 88.9 to 100% similarity among them and 93 to 100% similarity when compared to NoV genogroup GII sequence (DQ397325-BRA) available in GenBank. The samples IAL-C16, IAL-C65 and IAL-C70 are from the same community (Praia Grande city) showed 97.7%, IAL-C174, IAL-C176 and IAL-C178 from the same healthcare demonstrated 100% and IAL-C19, IAL-C22, IAL-C104, IAL-C112 and IAL-C152 from hospital present 88.9 to 100% of similarity among them.

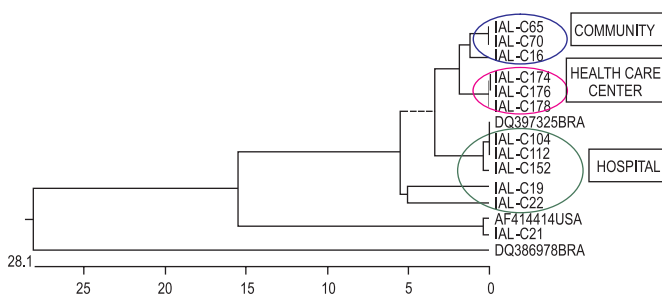


Figure 1. Dendrogram indicating similarity of nucleotide sequences of norovirus samples obtained of acute diarrhea outbreaks in São Paulo State where compared with sequences from GenBank. The scala indicates the number of divergent nucleotide residues.

However, the results of the analysis of sample IAL-C21, showed a high degree of similarity compared to sample AF414414-USA/GII (99.4%) concomitantly with a low degree of similarity compared to the other samples analyzed in the present study (Fig. 1), suggesting the presence of genogroup GII variant. The occurrence of a NoV GII.4 variant associated with outbreaks of gastroenteritis has been reported recently (2,4,26). As perspective for further study, we suggest analyzes of the VP1 region of ORF2 in order to identify the variants and to confirm our finding.

As expected, the strain DQ386978-BRA of genogroup GI demonstrated low similarity (50.9 to 58.5%) compared with studied samples.

Recent Brazilian studies regarding NoV circulation identified GII as the most common genogroup (3,8,21,23,30). In a study conducted from 1995 to 1999, Castilho *et al.* (2006) detected GII.4 in São Paulo State associated with acute gastroenteritis.

These results are demonstrating that NoV was the ethiological agent responsible for those acute gastroenteritis cases and the sequence analysis revealed that the circulating strain was NoV GII confirming the worldwide distribution of this genotype.

The fact that NoV cannot be cultured and the diversity of its genome make the development of a sensitive detection method difficult. However, detection of a low viral load is important for the epidemiological study of outbreaks, especially when the origin of an outbreak is analyzed retrospectively, when some symptoms of the patients may have disappeared and when excretion levels of NoV are low.

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RESUMO

Identificação e caracterização molecular de norovírus em surtos de gastroenterites no Estado de São Paulo

Norovírus (NoV), anteriormente chamado *Norwalk-like* vírus, constituem um importante grupo de patógeno humano associado a surtos de gastroenterites não bacterianas. Levantamentos epidemiológicos de surtos demonstram que as mais importantes vias de transmissão são contatos pessoa-pessoa, água e alimentos contaminados, afetando adultos e crianças. Os NoV são classificados em genogrupos, sendo os genogrupos GI, GII e GIV encontrados em humanos. A grande diversidade genética dos NoV e a falta de informação sobre esses vírus no Brasil determinaram o tema deste estudo que teve como objetivo a identificação e caracterização molecular de NoV em amostras fecais de pacientes com gastroenterites, provenientes de surtos de diarréia no estado de São Paulo. Neste estudo, 204 amostras de fezes coletadas durante surto de diarréia foram analisadas por RT-PCR, e 12 foram seqüenciadas para confirmar o genogrupos. O método *one step* PCR foi empregado para a amplificação da região B da ORF1 com o *pool* de *primers* MON 431, 432, 433 e 434. Do total, 32 (15,7%) amostras foram positivas para norovírus genogrupos GII. A comparação das seqüências dos produtos de PCR às seqüências do *GenBank* demonstrou 88,8% a 98,8% de identidade, sugerindo a presença de norovírus genogrupos GII em surtos de gastroenterites no Estado de São Paulo.

Palavras-chave: Norovírus, Genogrupos GII, Caracterização Molecular.

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