ORIGINAL ARTICLE

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SCREENING OF FECAL SAMPLES FROM ASYMPTOMATIC CHILDREN, FOR NOROVIRUS DETECTION, USING A THIRD GENERATION ENZYME IMMUNOASSAY COMMERCIAL KIT

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ABSTRACT

Norovirus is the leading cause of non-bacterial acute gastroenteritis outbreaks worldwide. Recently, third generation Enzyme Immunoassay (EIA) commercial kits have been developed, and controversial results have been obtained by different studies regarding the sensitivity and specificity of these assays. Therefore, the aim of this study was to test 60 fecal samples, previously tested as positive by RT-PCR for caliciviruses (40 norovirus-positive and 20 sapovirus-positive samples), for qualitative determination of genogroup I and II noroviruses by a commercial EIA kit (RIDASCREEN® Norovirus (C1401) 3rd Generation, R-Biopharm, Darmstadt, Germany). The samples were obtained from 30 children aged less than five years, mostly asymptomatic, who attend a day-care center in Goiânia, Goiás, Brazil. The results conferred a positivity rate for NoV of 35% and a specificity rate of 100% for the EIA, when compared to the RT-PCR. The test also failed to detect samples that were positive for GI.1 and GI.4 norovirus. The presumably lower viral load of asymptomatic children might be related to the poor sensitivity. Our results reinforce the notion that screening of samples by molecular assays, especially of samples that might have a low number of viral particles such as those obtained from asymptomatic patients, should not be replaced by the use of EIA kits.

KEY WORDS: Asymptomatic children; enzyme immunoassay; Norovirus; sensitivity; specificity.

RESUMO

Triagem de amostras fecais de crianças assintomáticas utilizando-se um *kit* comercial de Elisa 3ª geração

Os norovírus são a principal causa de surtos de gastroenterite aguda não bacteriana em todo o mundo. Recentemente, *kits* comerciais de Enzima Imunoensaio (EIE) têm sido desenvolvidos, e resultados controversos sobre a sensibilidade e especificidade desses ensaios foram obtidos por diferentes estudos. Portanto, o objetivo deste trabalho foi testar 60 amostras fecais para a

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determinação qualitativa de norovírus dos genogrupos I e II por meio de *kit* comercial de EIE (RIDASCREEN® Norovirus (C1401) 3rd Generation, R-Biopharm, Darmstadt, Germany). Previamente testadas, elas se mostraram positivas para calicivírus por RT-PCR (40 positivas para norovirus e 20 positivas para sapovirus). As amostras foram obtidas de 30 crianças menores de 5 anos de idade, predominantemente assintomáticas, que frequentavam uma creche em Goiânia, Goiás, Brasil. Os resultados revelaram índices de 35% de positividade para os norovírus e de 100% de especificidade para o EIE quando comparado a RT-PCR. O teste também falhou em detectar amostras que eram positivas para norovírus GI.1 e GI.4. A carga viral, presumidamente mais baixa, das crianças assintomáticas pode estar relacionada com a baixa sensibilidade. Os resultados reforçam o entendimento de que a triagem de amostras por ensaios moleculares não deve ser substituída pelo uso de *kits* de EIE, especialmente quando se tratar de amostras que, presumidamente, apresentem um baixo número de partículas virais como as obtidas de pacientes assintomáticos.

DESCRITORES: Crianças assintomáticas; ensaio imunoenzimático; Norovírus; sensibilidade; especificidade.

INTRODUCTION AND OBJECTIVES

Noroviruses (NoV) are important etiological agents of acute gastroenteritis (AGE), causing over 50% of all outbreaks in the world (11). It is estimated that they are also responsible for more than 260 million infections a year in different parts of the world (7).

The *Norovirus* constitutes a distinct genus of the *Caliciviridae* family which is composed of five genera. However, only calicivirus (CV), members of the *Norovirus* and *Sapovirus* genera are of human importance (12). Both genera are divided into five genogroups (GI-V), and only NoV (genotypes from genogroups I, II and IV) infect humans (27). Up until now, 35 NoV genotypes are recognized (11), based on the complete sequence of VP1, the major capsid protein. However, a new classification based on both VP1 sequence and the nearly complete polymerase gene from the first open reading frame has been proposed (15).

The NoVs are transmitted by the fecal-oral route, through person-to-person contact, or by the ingestion of contaminated food or water (8). Fomites, and even aerosols formed during vomiting episodes are also potential sources of infection (17).

These agents infect people of all ages, throughout the year, with outbreaks being common in semi-closed environments with agglomeration of people such as hospitals, schools, nursing homes, and day-care centers (14). Infection is generally self-limiting, and the main associated symptoms are: vomiting, diarrhea, nausea, and abdominal pain. The NoVs have also been detected in samples from asymptomatic children (18, 20); however, these samples presumably have lower viral load, compared to samples from symptomatic patients (2, 16).

The great genomic and antigenic variability of the NoVs has hampered the development and standardization of diagnostic and typing methods (26). They also have never been cultured in vitro, and there is still no consensus about an ideal experimental animal model (5, 23, 25). All of these factors have made it

very difficult to study these agents (1, 22). Currently, although there is still a lack of a standardized system for CV strain characterization, the Polymerase Chain Reaction Post Reverse Transcription (RT-PCR) followed by Genomic Sequencing or Real-time PCR technologies are currently the methods of choice for CV detection and molecular characterization (1, 10).

Recently, third generation Enzyme Immunoassay (EIA) commercial kits have been developed, and could represent a rapid and efficient alternative for the detection of NoV. However, controversial results have been obtained by different studies regarding the sensitivity and specificity of these assays (3, 6, 24).

In this study the kit evaluated was the (RIDASCREEN® Norovirus (C1401) 3rd Generation, R-Biopharm, Darmstadt, Germany) that determines NoV antigens of genogroups I and II. The manufacturers claim a sensitivity rate of 78.6-93.3% and a specificity of 100%, and state that the panel used to evaluate the kit did not comprise all NoV genotypes. They also declare that a negative result could be due to intermittent viral excretion, inappropriate sampling time and also low virus load in the sample.

Therefore, the aim of the study is to test the sensitivity and specificity of a third generation EIA commercial kit for the qualitative detection of NoV genogroup I and II strains in fecal samples previously typed by RT-PCR and Genomic Sequencing.

MATERIALS AND METHODS

Study material

The study material consisted of 60 fecal samples, previously tested and negative for adenovirus, astrovirus, rotavirus (data not published), that were positive for norovirus (N=40) or sapovirus (N=20) (20) obtained from 30 children aged less than five years, mostly asymptomatic (95%), that attended a day-care center in Goiânia, Goiás. The fecal samples were collected, from October 2009 to October 2011, only when the legal guardian agreed and signed the informed consent form. Fecal samples were kept at -20ºC in the Virology Laboratory until further processing. The study was approved by the Ethics Committee of the Federal University of Goiás (protocol: 087/2009).

Enzyme Immunoassay

The panel samples were submitted to screening, for qualitative determination of genogroups I and II noroviruses, by a commercial Enzyme Immunoassay kit specific for norovirus antigen detection (RIDASCREEN® Norovirus (C1401) 3rd Generation, R-Biopharm, Darmstadt, Germany), following the manufacturer's instructions.

Briefly, fecal samples were diluted in dilution buffer provided in the kit and 200 µL of each dilution was pipetted into their respective microwell of NoV "Virus-like particles" pre-sensitized ELISA plates that were incubated at 37°C for one hour. Plates were washed, five times, with a washing buffer, and 100 µL of the conjugated 1 (MAb against genogroups I and II NoV) were added, followed by 1 hour incubation at 37 °C. After a new washing step, 100 µL of the conjugated 2 (Anti Mouse-IgG-HRP labeled) were added, followed by 30 minutes incubation at 37 °C. Plates were washed again and 100 µL of the substrate (TMB + H_2O_2) was added. After 15 minutes at room temperature, reaction was stopped by addition of stopping solution (1N H_2SO_4). The optical density of each sample was obtained using a wavelength of 450 nm in an ELISA reader (Behring- Marburg, Germany). Samples were considered positive and negative when their optical density was 10% higher or lower than the cut-off value, respectively.

Sensitivity and Specificity

Sensitivity of the assay was defined as the percentage of norovirus-positive samples by the "Gold-standard" method (RT-PCR) that were also detected by the EIA kit. Specificity was defined as the percentage of sapovirus-positive samples by RT-PCR that were negative by the EIA kit.

RESULTS

The results of the EIA, in comparison with the results previously obtained with the molecular analysis (21) are depicted in Table 1.

Table 1. Positivity rates for NoV using a commercial EIA kit, in comparison to RT-PCR results.

Virus	RT-PCR positive samples (N)	EIA		Positivity rates (%)
		Positive	Negative	
NoV G.I	4	1	3	25
NoV G.II	34	12	22	35
NoV G.I+II	2	1	1	50
Total	40	14	26	35

From the 60 fecal samples previously positive for CV by RT-PCR, four (6.7%) were positive for genogroup I NoV (GI NoV), 34 (56.7%) were positive for GII NoV, 2 (3.3%) were positive for GI and GII NoV, and 20 (33.3%) were positive for sapovirus (SaV).

The concordance of the results between the EIA and RT-PCR techniques was: 25% for GI NoV (1/4), 35% (12/34) for GII, and 50% (1/2) for mixed infections (GI and GII NoV). Therefore, 14 of the 40 previously NoV-positive samples by RT-PCR, were also detected by the EIA, conferring a positivity rate of

NoV of 35%. Considering the genomic sequencing results, it was observed that the NoV genotypes: GI.7, GII.1, GII.2, GII.6 were detected by the EIA kit; however, the test failed to detect samples positive for GI.1 and GI.4 NoV.

Of the 20 SaV-positive samples, none was positive by EIA.

DISCUSSION AND CONCLUSION

The positivity rate for NoV found in this study was 35%. In previous reports, samples from patients with AGE have been tested for NoV detection using third generation antigen-based kits, resulting in variable sensitivity rates, from 61 to 92% (9, 13, 19, 24). In this study, although the viral load of the samples was not determined, the low positivity for NoV could be partially explained by the fact that 95% of the samples tested were obtained from asymptomatic children, probably presenting a low number of viral particles in their feces (2, 16).

The 100% specificity rate found in this study is similar to rates (83 to 98%) reported by previous studies that have also used a third generation EIA kit (9, 13, 19, 24).

We believe that the low number of fecal samples used to evaluate the commercial kit sensitivity in this study was compensated by the fact that in this study half of the samples had been submitted to genomic sequencing, and therefore their genotype was known, unlike previously published studies (4, 9, 19) that have characterized the NoV-positive samples in genogroups but not in specific genotypes. The fact that the NoVs have high genomic and antigenic variability also makes the detection of all genotypes by commercial EIA kits difficult (9).

Only SaV-positive samples, that were also negative for adenovirus, astrovirus and rotavirus, were used, when compared to other studies (13, 19) that have used samples that were only known to be negative for NoV by RT-PCR. Therefore, the panel of samples chosen in this study to evaluate the sensitivity of the assay could be considered advantageous because the SaV belongs to the same family of the NoV, being genetically and antigenically similar to them.

The results from this study confirm the notion that tests based on antigen detection are useful for NoV screening during outbreaks, especially samples that have a high number of viral particles such as those obtained from patients presenting with acute gastroenteritis; however they suggest that a negative result by EIA does not eliminate the possibility of NoV infection.

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