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Short communication

Evaluation of a commercial enzyme immunoassay for the detection of norovirus antigen in fecal samples from children with sporadic acute gastroenteritis

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Abstract

The Ridascreen® Norwalk-like virus enzyme immunoassay was compared with (RT)-PCR on 92 stool samples collected from children with sporadic acute gastroenteritis. Homogenization and pre-dilution of the whole stool sample resulted in high specificity (97.5%) and moderate sensitivity (60%). This assay may be useful to screen outbreaks for norovirus, but limited to detect the virus in sporadic cases of diarrhea.

Keywords: Norovirus; EIA; (RT)-PCR; Diarrhea

Noroviruses (NoVs) are the leading cause of nonbacterial gastroenteritis outbreaks in children and adults worldwide (Clark and McKendrick, 2004), and probably represent the second most common etiological agent of sporadic diarrhea in young children after rotavirus (Bereciartu et al., 2002; Boga et al., 2004; Negishi et al., 2004). A major obstacle in establishing the diagnosis of NoV infection has been the absence of a rapid and sensitive diagnostic method suitable for use in public health laboratories and hospitals. This has been due, in part, to the extensive genetic and antigenic diversity of this group of virus. On the basis of genetic analysis, NoVs have been classified into two major genogroups (GI and GII). Within the genogroups, strains can be further divided into at least 22 genetic clusters or genotypes (Vinjé et al., 2004). NoVs were first detected by electron microscopy, but more recently reverse transcription (RT)-PCR has become the standard test for the diagnosis of these pathogens. Although the (RT)-PCR assay has improved the sensitivity of detection, this usually needs to be confirmed by nucleotide sequencing or blot hybridization. These two methods are expensive, time-consuming and not practical in a diagnostic laboratory. Moreover, the design of broadly reactive oligonucleotide primers directed to specifically detect NoVs genotypes is difficult and may differ between laboratories (Vinjé et al., 2003). These difficulties point towards the development of a faster and simpler method, such as an enzyme immunoassay (EIA). Recently, a number of EIA kits for the detection of NoV antigen in stool samples have been commercialized. However, low sensitivities were reported for these commercial assays (Burton-MacLeod et al., 2004; Richards et al., 2003), including the more recently introduced Ridascreen® Norwalk-like virus test (R-Biopharm, Darmstadt, Germany) (Dimitriadis and Marshall, 2005; Schmid et al., 2004). In the present study, a re-evaluation of the performance of the Ridascreen® commercial EIA for antigen norovirus detection in stool samples from children with acute gastroenteritis was carried out, after introducing modifications in sample preparation.

Ninety-two fecal samples from randomly selected children <5 years of age who sought care for diarrhea at the Children's Hospital "Dr. Jorge Lizarraga", Valencia, Venezuela between January 2001 and November 2003 were analyzed. Fecal samples were collected within 72 h after the onset of symptoms and stored at −20 °C until testing. The Ridascreen® Norwalk-like virus enzyme immunoassay is based on monoclonal antibodies against specific norovirus antigen bound on to the surface

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of microwell strips to capture the antigen in stool samples. For the sample preparation, a pre-dilution (1:3 w/v) of the original stool sample in distilled water was made and these homogeneous aliquots were tested by EIA and (RT)-PCR. Since it has been reported that virus particles may be unevenly distributed in the specimen (Wünsch et al., 2005), homogenization of the whole sample, prior to testing, could result in an improvement in the performance of the immunoassay. The samples were further processed according to the manufacturer's instructions. Briefly, 100 μl of a fecal suspension (1:6 v/v) in the sample diluent buffer provided was added to each well. After 1 h at room temperature, the microwells were washed five times with 300 μl of wash buffer. Two drops of enzyme conjugate were added to each well and further incubated at room temperature for 30 min. After five washes, specifically bound conjugate was determined by the addition of a chromogenic substrate. The absorbance was measured at 450 nm. Specimens with an absorbance value equal or greater than the cut-off value (absorbance value of the negative control + 0.15) were considered positive. Each sample was tested twice to determine reproducibility of the kit. The results of the EIA were compared with a conventional (RT)-PCR assay using primers pair GLPSG1, GLPSG2/YGDD1 which amplifies a region of the norovirus gene encoding the RNA-dependent RNA polymerase (Green et al., 1995). For (RT)-PCR, nucleic acids from clarified fecal samples were extracted with Trizol/LS (Gibco BRL, MD, USA) and positive samples were confirmed by direct sequencing. Products were sequenced in both directions using the same primers used in the (RT)-PCR. The amplified sequences were edited using the BioEdit Sequence Alignment Editor program, Version 7.0.4.1 (Isis Pharmaceuticals, Carlsbad, CA) and compared to those of HuCVs strains deposited in the GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). In order to compare the detection limits of the two methods, 10-fold serial dilutions (1:6, 1:60, 1:600, 1:6000) of three positive stool samples were prepared. Each dilution was simultaneously tested by EIA and (RT)-PCR assay. For statistical analysis, the kappa value was calculated to compare the reproducibility of the EIA. This value expresses the agreement between two observations on the same subject compared with the agreement that would be expected by chance. A kappa of 0 denotes no agreement beyond what is expected by chance and a kappa of 1 denotes perfect agreement (Landis and Koch, 1977). The EIA was performed twice. Both assays met the published quality control criteria with the negative control giving OD values of <0.2 OD units and the positive control values of >0.5 OD units. Samples with equivocal results (range within 10% above or below the cut-off value) in any of the EIAs were considered negative as the results in the other EIA and the (RT)-PCR assays were also negative. "True" positive samples were considered as those specimens that were positive by the (RT)-PCR assay. The sensitivity of the EIA test was defined as the percentage of true-positive specimens found to be positive by the EIA kit. Specificity was defined as the percentage of specimens that were not true-positives and were identified as negative by the EIA test. The agreement was the percentage of the results that were fully concordant, with the true-positive samples being positive by EIA and the true negative samples being negative by EIA.

Table 1
Norovirus test results in 79 specimens

EIA[a]	(RT)-PCR	No. of specimens ($n = 79$)
+	+	6
−	+	4
+	−	3
−	−	66

[a] Only samples with concordant results ($n = 79$).

Norovirus antigen was detected by both EIAs in 9 samples, 70 were negative in both tests and 13 samples gave discrepant results. This gave an agreement of 85.9% (kappa = 0.4966) between the EIA assays. Only samples with concordant results in the EIA were compared to the (RT)-PCR used as a reference method. A summary is shown in Table 1. A total of 10 samples were positive by (RT)-PCR; 6 were recognized by the EIA test (sensitivity, 60%). Three samples, which tested negative twice by (RT)-PCR, were considered positive by the EIA (specificity, 95.7%) (Table 2). Nevertheless, the possibility of degradation of the viral RNA in these samples cannot be excluded. The agreement between the EIA and the (RT)-PCR was 91.1% (72/79). Sequences were obtained for 9 of the 10 (RT)-PCR positive samples (one sample did not yield enough material for sequencing). Comparison of the sequences with strains deposited in GenBank clearly indicated that the amplicons correspond to human noroviruses. For the 10-fold dilution series, the EIA was able to detect norovirus antigen only in the 1:6 dilution of the samples, whereas the (RT)-PCR detected norovirus RNA in all the dilutions prepared (data not shown). All the samples had been tested previously by EIA for the identification of rotavirus (Salinas et al., 2004). To further test the specificity of the Ridascreen® assay, the results of the rotavirus and norovirus EIAs were compared. There was no concordance in the results between the norovirus and rotavirus tests (kappa = 0), clearly indicating that the Ridascreen® EIA does not cross-react with rotavirus.

The data support previous observation that norovirus circulate frequently and may be an important cause of sporadic diarrhea in Venezuela (Pujol et al., 1998; Rodríguez-Guillén et

Table 2
Specificity and sensitivity of the Ridascreen® Norwalk-like virus EIA for the detection of norovirus antigens in stool samples

Reference	Sample source (n)	Sensitivity	Specificity
Dimitriadis and Marshall (2005)	Outbreak (130)	71/100 (71%)[a]	14/30 (47%)[a]
Schmid et al. (2004)	Outbreak (38); sporadic (14)	9/26 (34.6%)	17/26 (65.3%)
This work	Sporadic (92)	6/10 (60%)	66/69 (95.7%)

[a] For single specimen diagnosis.

al., 2005). In the present study, the Ridascreen® test showed less sensitivity but comparable specificity than the (RT)-PCR. Differences in sensitivity may be due to limitations in the EIA to detect norovirus genetic variants, as has been reported for other commercial EIAs (Burton-MacLeod et al., 2004), to the inability of the EIA to detect positive samples with low viral load, or to a combination of both factors. Narrow specificity of noncommercial EIA assays for norovirus antigen detection has been reported (Jiang, 2003). On the other hand, the introduction of a modification of the original manufacturer's protocol, pre-dilution in distilled water of the fecal samples to obtain homogenous distribution of the antigen, resulted in an improvement of the specificity and comparable or superior sensitivity of the test compared to previous reports (Dimitriadis and Marshall, 2005; Schmid et al., 2004). It is likely that sample dilution reduced the binding of fecal debris which resulted in significantly improved specificity without loss of sensitivity. In general, the Ridascreen® assay shows a performance comparable or superior to that reported for other commercial assays (Burton-MacLeod et al., 2004; Richards et al., 2003) (Table 2). The present study reinforces the concept that commercial kits may be useful for investigating outbreaks of gastroenteritis, but are of limited use when testing individual stool samples. Furthermore, these results and others (Burton-MacLeod et al., 2004; Dimitriadis and Marshall, 2005; Richards et al., 2003) indicate that commercial EIAs still require improvement to match the performance of (RT)-PCR in routine norovirus detection.

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References

Bereciartu, A., Bok, K., Gómez, J., 2002. Identification of viral agents causing gastroenteritis among children in Buenos Aires, Argentina. J. Clin. Virol. 25, 197–203.

Boga, J.A., Melón, S., Nicieza, I., de Diego, I., Villar, M., Parra, F., de Oña, M., 2004. Etiology of sporadic cases of pediatric acute gastroenteritis in Asturias, Spain, and genotyping and characterization of norovirus strains involved. J. Clin. Microbiol. 42, 2668–2674.

Burton-MacLeod, J.A., Kane, E.M., Beard, R.S., Hadley, L.A., Glass, R.I., Ando, T., 2004. Evaluation and comparison of two commercial enzyme-linked immunosorbent assay kits for detection of antigenically diverse human noroviruses in stool samples. J. Clin. Microbiol. 42, 2587–2595.

Clark, B., McKendrick, M., 2004. A review of viral gastroenteritis. Curr. Opin. Infect. Dis. 17, 461–469.

Dimitriadis, A., Marshall, J.A., 2005. Evaluation of a commercial enzyme immunoassay for detection of norovirus in outbreak specimens. Eur. J. Clin. Microbiol. Infect. Dis. 24, 615–618.

Green, S.M., Lambden, P.R., Deng, Y., Lowes, J.A., Lineham, S., Bushell, J., Rogers, J., Caul, E.O., Ashley, C.R., Clarke, I.N., 1995. Polymerase chain reaction detection of small round-structured viruses from two related hospital outbreaks of gastroenteritis using inosine-containing primers. J. Med. Virol. 45, 197–202.

Jiang, X., 2003. Development of serological and molecular tests for the diagnosis of calicivirus infections. In: Desselberger, U., Gray, J. (Eds.), Viral Gastroenteritis. Elsevier Science B.V., pp. 505–522.

Landis, J.R., Koch, G.G., 1977. The measurement of observer agreement for categorical data. Biometrics 33, 159–174.

Negishi, S.O., Nguyen, T.A., Phan, T.G., Ushijima, H., 2004. Molecular epidemiology of viral gastroenteritis in Asia. Pediatr. Int. 46, 245–252.

Pujol, F.H., Vasquez, G., Rojas, A.M., Fuenmayor, M.E., Loureiro, C.L., Pérez-Schael, I., Estes, M.K., Liprandi, F., 1998. Norwalk virus infection in Venezuela. Ann. Trop. Med. Parasitol. 92, 205–211.

Richards, A.F., Lopman, B., Gunn, A., Curry, A., Ellis, D., Cotterill, H., Ratcliffe, S., Jenkins, M., Appleton, H., Gallimore, C.I., Gray, J.J., Brown, D.W.G., 2003. Evaluation of a commercial EIA for detection Norwalk-like virus antigen in faeces. J. Clin. Virol. 26, 109–115.

Rodríguez-Guillén, L., Vizzi, E., Alcalá, A.C., Pujol, F.H., Liprandi, F., Ludert, J.E., 2005. Calicivirus infection in human immunodeficiency virus seropositive children and adults. J. Clin. Virol. 33, 104–109.

Salinas, B., González, G., González, R., Escalona, M., Materán, M., Pérez-Schael, I., 2004. Epidemiologic and clinical characteristics of rotavirus disease during five years of surveillance in Venezuela. Pediatr. Infect. Dis. J. 23, S161–S167.

Schmid, M., Oehme, R., Schalasta, G., Brockmann, S., Kimming, P., Enders, G., 2004. Fast detection of noroviruses using real-time PCR assay and automated sample preparation. BMC Infect. Dis. 4, 15–22.

Vinjé, J., Vennema, H., Maunula, L., von Bonsdorff, C.H., Hoehne, M., Schreier, E., Richards, A., Green, J., Brown, D., Beard, S.S., Monroe, S.S., de Bruin, E., Svensson, L., Koopmans, M.P.G., 2003. International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses. J. Clin. Microbiol. 41, 1423–1433.

Vinjé, J., Hamidjaja, R.A., Sobsey, M.D., 2004. Development and application of a capsid VP1 (region D) based reverse transcription PCR assay for genotyping of genogroup I and II noroviruses. J. Virol. Meth. 116, 109–117.

Wünsch, J., Kiourkenidis, G., Fischer, R., Leidinger, H., Sander, P., 2005. Ridascreen® norovirus. Suitable conditions to compare results obtained by EIA and PCR to reliably diagnose norovirus infections in stool specimen. In: Proceedings of the XIX International Congress of Clinical Chemistry IFCC/AACC, Orlando, USA.