MEDICAL MICROBIOLOGY

Detection of Rotavirus in Stool Samples of Gastroenteritis Patients

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In this pilot study, we examined 100 stool samples from patients with gastroenteritis to determine the presence of Rotavirus using immunoassays and molecular diagnostic methods. When the samples were analyzed by enzyme-linked immunoabsorbent assay (ELISA), we found 11 Rotavirus-positive samples. However, using molecular techniques (reverse transcriptase-polymerase chain reaction (RT/PCR]), we identified 51 positive samples for Rotavirus. These results corroborate the generally accepted concept that molecular techniques are more sensitive than serological diagnostic tests. In addition, our data suggests that Rotavirus is an important etiologic agent of gastroenteritis in the local pediatric population. More extensive studies are necessary to determine the prevalence of Rotavirus in Puerto Rico in order to design effective control measures to protect our population against this pathogen.

Key words: Enteric pathogen, ELISA, Gastroenteritis, Giardia lamblia rotavirus, RT/ PCR

Viruses can often cause an infection of the intestinal tract, called gastroenteritis. This term is commonly used to describe the irritation and inflammation of the inside lining of the stomach and intestines. This condition commonly causes nausea, vomiting, diarrhea, abdominal distress or bloating, fever and flu-like symptoms, such as headache, joint pain or muscle soreness. Virus infection is commonly acquired after eating and drinking contaminated food or water (1). Gastroenteritis is a more serious illness in infants, young children and persons who are unable to care for themselves, such as disabled or elderly, due to the risk of dehydration from loss of fluids. They may need to be hospitalized for treatment to correct or prevent dehydration. If the lost fluids are not replaced, dehydration can be severe and fatal. The public health burden of infectious diarrhea is substantial, particularly among children from both, developed and developing countries (2). In United States, more than 1.5 million episodes of infectious diarrhea occur each year, causing approximately 200,000 hospitalizations and 300 deaths (3). Worldwide, gastroenteritis accounts for 1.5 billion cases and 2 millions of deaths among children aged < 5 years (3).

Viruses were first recognized to be etiologic agents of gastroenteritis in the 1930s when a filterable agent that caused diarrhea in rabbits was discovered (4). Different viruses have been identified in stool samples from humans with diarrhea, including Rotaviruses, Enteric Adenoviruses, Caliciviruses and Astroviruses. In young children (< 5 years old), gastroenteritis is caused mainly by Rotavirus infection. Infections in adults in close contact with infected children are also common. In industrialized countries, the incidence of Rotavirus illness is approximately 9.1 million cases annually, of which 223,000 need hospitalization (5). Rotavirus diarrhea is even more prevalent in developing countries, causing 130 million cases each year and 1.9 million hospitalizations. More importantly, of the 2 million fatal cases reported annually worldwide due to gastroenteritis, 440,000 are caused by Rotavirus (5). Human rotaviruses were first detected by electron microscopy (EM) observation in biopsy specimens from duodena of children with acute diarrhea (6). Six distinct rotavirus groups (A through F) have been identified serologically on the basis of common antigens. Group A rotaviruses is the most common cause of infantile gastroenteritis worldwide. The mode of transmission of these viruses is probably person-to-person. Although, foodborne and waterborne transmission of these viruses have also been reported (7,8).

Diagnosis of gastroenteritis is usually made by the patient 's medical history and physical exam. In some cases, the individual's stool or serum may be tested. The

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traditional methods that have been used to identify viral agents that cause diarrhea are electron microscopy (EM), coupled with immunologic techniques (e.g. immune EM) and serologic assays such as Enzyme-Linked Immunosorbent Assay (ELISA). However, new molecular technologies such the Polymerase Chain Reaction (PCR) are valuable tools to detect the presence of the pathogen's nucleic acid in clinical specimens. PCR is routinely used to identify the etiological agent during investigations of outbreaks because it is more sensitive than the classical detection methods (9,10). The improved sensitivity of this method is very helpful when the sample is taken late in the course of the illness and/or the concentration of the causative agent is low.

There is no epidemiological data about the incidence and prevalence of viral pathogens that cause diarrhea in Puerto Rico. Clinical microbiology laboratories screen routinely only for few bacterial pathogens such as *Shigella* and *Salmonella* spp., and most enteric viral pathogens are probably not detected. The main objective of this work was to do a preliminary study on the incidence of one of the viral pathogens, Rotavirus, in stool samples from patients with gastroenteritis. ELISA and reverse transcriptase PCR (RT/PCR) were the two methods used to detect Rotavirus antigen and nucleic acid, respectively. In addition, we studied the co-infection status of the patients by screening for *Giardia lamblia* and *Cryptosporidium parvum*. One hundred samples from gastroenteritis patients were examined using both methods and we detected 11 and 50 samples positive for Rotavirus by ELISA and RT/PCR, respectively. Coinfection with *Giardia* was detected in 23 cases.

Materials and Methods

Specimen. One hundred-stool specimens were obtained from patients with symptoms of gastroenteritis. The Guaynabo Clinical Laboratory provided these samples after the routine laboratory analysis. Upon receipt, a small aliquot (1-3g stool) was transferred to sterile containers and stored at -20°C as soon as possible. Each sample was coded and the only information provided by the laboratory was age and sex of the patients. This study was conducted under the institutional IRB approval.

Controls. Ten stool samples from healthy donors were also collected as controls for this study. Absence of gastrointestinal symptoms was the selection criteria when these samples were collected. In addition, five different Rotavirus strains (WA, DS1, ST3, US1205, and P strain), kindly provided by Dr. Roger Glass, Division of Viral and Rickettsial Diseases, CDC, Atlanta, were used as positive controls for the RT/PCR reaction. RT/PCR reactions were also monitored by the inclusion of no DNA samples to control for possible contamination.

ELISA. Detection of Rotaviruses was performed using the commercially available kit IDEA Rotavirus from DAKO Diagnostic (Carpinteria, CA). The procedure was performed following the manufacturer's instructions. Briefly, microwells were coated with virus-specific antibodies to capture viral antigen in the solid phase. A 10% stool suspension was added to the wells and incubated for 1 hour at 37°C. Wells were washed four times prior to the addition of the Rotavirus-specific rabbit antibody. After a second wash, peroxidase-conjugated anti-rabbit IgG was used for detection. After the final wash, the 2-part 3,3'-5, 5' tetramethylbensidine (TMB) chromogen was added to the wells and a blue color was produced in the presence of virus antigen. Optical density (OD) was measured at 450nm using a BioRad (Hercules, CA) microplate reader model 680.

Viral Culture. Rotavirus strains were cultivated on MA104 cells, which were grown in Minimal Essential Medium (MEM) supplemented with 10 % fetal bovine serum (FBS) at 37°C, in a 5 % CO_2 atmosphere. Different Rotavirus strains (1.7×10^5 pfu/ml) were trypsinized and subsequently used to infect confluent MA104 cell cultures. Infected cultures were incubated at 37°C for 4 days or until cytophatic effects were observed.

Viral RNA isolation and RT/PCR. Viral RNA was isolated using the QIAamp viral RNA kit (Qiagen, Chatsworth, CA). Briefly, 0.5 ml of stool sample was suspended in 0.89 % NaCl up to 5 ml. The solution was clarified by centrifugation for 20 min at 4000 rpm and the supernatant was filtered using a 0.22 µm filter. The filtrate was used as starting material for the purification procedure, which was performed as recommended by the manufacturer. Briefly, 140ml of filtrate was mixed with 560ml of buffer AVL and incubated at room temperature for 10 min. Then, 560ml of ethanol was added to the sample solution and transferred to the QIAamp spin column. After collecting and discarding the filtrate, the column was washed twice with 500ml of buffer AW1, followed by buffer AW2. Viral RNA was eluted from the column using 60ml of buffer AVE. A 5µl aliquot of viral RNA was used for the RT/PCR reaction. RT-PCR was performed using the Titan One Tube RT/PCR system (Roche Diagnostic, Boehringer Mannheim, Germany) and a modified version of the amplification protocol described by Rasool et al., 2002 (11). The reaction mix contained 10µl of the 5x RT/PCR reaction buffer, 1.5 mM $MgCl_2$, 5mM DTT, 200µM of dNTP, 400 µM each primer (forward primer [5'TTGCCA CCAATTCAAAATAC3'] and reverse primer [5'ATTTCGGACCATTTATAACC3']), 10µl RNAse inhibitor (Sigma, St. Louis, MO), and 1µl of AMV RT and

expand High Fidelity enzyme mix, in a final volume of 50 µl. The amplification protocol included a reverse transcription step at 50°C for 30 min., 40 cycles of denaturation at 94°C for 1 min., annealing at 42°C for 1 min., and extension at 68°C for 1 min, and a final extension cycle at 68°C for 7 min. The Bio-Rad Icycler, (Hercules, CA) was used for the RT/PCR reaction.

Gel Electrophoresis. Electrophoresis was performed to determine the size of the amplicon. Five µl of each PCR reaction were resolved in a 2% agarose gel and visualized in a Bio-Rad Gel Documentation EQ System (Hercules, CA). The expected size for the Rotavirus amplicon was 201 bp.

Statistical Analysis. Statistical analysis was performed with EPI-INFO version 6.0 software with a level of significance of 0.05. Sensitivity, specificity, positive and negative predictive values of the ELISA and RT/PCR methods were determined using the screening tool. In addition, Fisher exact test, and Kaplan coefficient calculation were performed to detect significant differences and the agreement in the results between the two methods. A logistic regression was also performed using XLSTAT PRO software (Version 6.0) to measure the magnitude of the association between ELISA and RT/PCR.

Results

A group of 100 stool specimens from patients with gastroenteritis was tested for the presence of Rotavirus by ELISA and RT/PCR. We found that 8 samples were positive for Rotavirus by both methods, but using RT/PCR we detected 43 additional positive samples, for a total of 51 (Table 1). In addition, three of the Rotavirus samples positive by ELISA were negative by RT-PCR (Table 1). As shown in Figure 1, PCR amplification produced a single band of the expected size (201 bp). Samples amplified from healthy donors did not produce the Rotavirus specific band (data not shown).

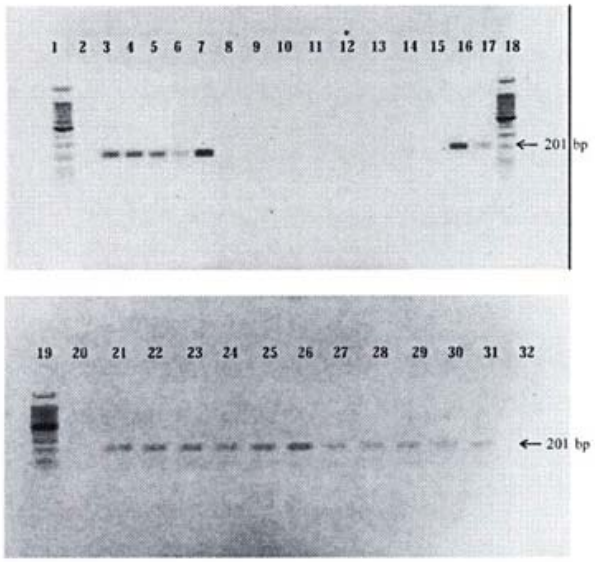
Table 1. ELISA and RT- PCR Results for Rotavirus Detection

ELISA*	RT- PCR†	No. of samples
Positive	Positive	8
Positive	Negative	3
Negative	Positive	43
Negative	Negative	46

*ELISA was performed using the commercially available kit IDEA Rotavirus from DAKO Diagnostic (Carpinteria, CA) and †RT/PCR was performed as described by Rasool et al., 2002 with minor modifications.

The age distribution of the 51 Rotavirus-positive samples is shown in Table 2. Rotavirus infection was more frequent in the 0 to 10 years age group than in older patients. In addition, the consistency of most of stool samples that were positive for Rotavirus was either soft or watery, although there were more samples with soft consistency than watery. The female to male ratio among participants with positive samples was 1.1:1 (data not shown). As expected, we found that the sensitivity of the

Figure 1. Agarose gel showing RT-PCR of Rotavirus



Rotavirus RT/PCR was performed as described by Rassol et al., 2002 with minor modifications. Lane 1, 18, 19 – 100 bp marker, Lane 2, 20, 32 – no DNA negative control; Lane 3 – positive control, WA strain; Lane 4 – positive control, SD strain; Lane 5 – positive control, ST strain; Lane 6- positive control, US1205 strain; Lane 7 – Positive control, P strain; Lane 8 –15, negative RT/PCR results; Lane 16, 17, 21-31 – positive RT/PCR results. *The band in sample #12 had a lower intensity than the US1205 strain (lane 6), the weakest positive control, therefore was considered as negative result.

Table 2. Age distribution of patients with a Rotavirus-positive sample

Age (years)	ELISA	RT-PCR
0 – 10	11	36
11 – 20	0	12
21 – 50	0	0
> 51	0	3

Table 3. Consistency of the positive Rotavirus samples

Consistency	ELISA	RT-PCR
Soft	8	40
Normal	0	5
Watery	3	4
Hard	0	1
Mucoid	0	1

RT/PCR was higher than the ELISA (51% vs. 11%, Table 4), although the specificity of both methods was the same (100%).

Table 4. Statistical analyses

Test	ELISA	RT-PCR
Sensitivity	11%	51%
Specificity	100%	100%
Positive predictive value	100%	100%
Negative predictive value	10.1%	16.9%

Some positive samples contained more than one microorganism that can cause gastroenteritis. ELISA done to detect the parasites *Giardia lamblia* and *Cryptosporidium parvum* revealed that 23 Rotavirus-positive samples were also positive for *Giardia lamblia* (Table 5). However, samples co-infected with Rotavirus and *Cryptosporidium parvum* were not detected (data not shown).

Table 5. Dual infections with Rotavirus and *Giardia lamblia*

ELISA * *G. Lamblia*	ELISA Rotavirus	RT- PCR Rotavirus	No. of samples
Negative	Positive	Negative	2
Negative	Positive	Positive	7
Negative	Negative	Positive	21
Positive	Positive	Negative	1
Positive	Positive	Positive	1
Positive	Negative	Positive	22
Positive	Negative	Negative	14
Negative	Negative	Negative	32

*ELISA for Giardia detection was performed using the commercially available kit GIARDEIA for Antibodies Inc. (Davis, CA)

Discussion

Gastroenteritis is the leading cause of pediatric morbidity and mortality in developed and developing countries (3). Rotaviruses cause the majority of diarrhea cases in infants and children. In a recent study, group A *Rotaviruses*, human *Caliciviruses*, *Astroviruses* and *Adenovirus* types 40 and 41 were detected by ELISA or PCR-based techniques in 61 %, 14 %, 6 %, and 3% of stool samples from children with gastroenteritis (12).

In our study, 11 samples were positive for Rotavirus by ELISA. However, when tested by RT/PCR, Rotavirus infection was detected in 51 samples. Our data showed that only 8 samples were positive by both methods, which shows low agreement in the results between the two methods (observed agreement = 54% and Kappa coefficient = 0.09, p-value = 0.054). In addition, we found three potential false positive samples by ELISA since the RT/PCR result was negative. The OD readings of these samples were within 10% of the cutoff value, suggesting that these samples gave false-positive results. Some of the factors that may have contributed to the low detection levels by ELISA included improper sample handling and storage and/or collection of samples at later stages of the disease. Since the endpoint virus concentration detected by the IDEA Rotavirus kit is 7×10^5 virions per ml, it is possible that some of our samples contained low virus concentrations and thus were not detected by ELISA. Although in this study the sensitivity of both methods was not determined by serial dilutions of fecal specimens, others studies have found that RT/PCR was 100 to 1000 times more sensitive than ELISA for Rotavirus detection (13, 14). In accordance to these results, we found that RT/PCR was more sensitive than ELISA. The probability to obtain a positive result with RT/PCR was 3 times higher than using ELISA (Odd ratio = 2.98, 95% confident intervals 0.74 – 11.98). However, the difference in sensitivity did not reach statistical significance (Fisher exact p = 0.2) probably due to the small number of samples tested in this study.

Infections with *Giardia lamblia* were found in 23 of the Rotavirus-positive samples. Mixed infections with these enteric pathogens were previously reported in Brazil (15) and Pakistan (16). This is an important result because it demonstrates that mixed infections also contribute to the development of gastroenteritis in Puerto Rico. *Giardia lamblia* has a worldwide distribution and is often the etiologic agent of outbreaks of gastroenteritis and traveler's diarrhea. Some persons have large numbers of parasites attached to the bowel wall that may cause acute or chronic diarrhea. The stool may be watery, semisolid, greasy, bulky, and foul smelling at various times during the course of the infection. The consistency of the *Giardia lamblia* and Rotavirus positive samples in this study was more soft than watery, similar to the consistency observed in most of the Rotavirus only positive samples. These results suggest that having an infection with two enteropathogens apparently did not worsen the clinical symptoms. In addition, the findings about the stool consistency were unexpected because both, Rotavirus and *Giardia lamblia,* are supposed to cause severe and watery diarrhea. It is possible that these samples were collected late in the course of the infection, when the clinical symptoms have improved.

PCR-based techniques have been developed in many research laboratories for detecting and typing viruses that cause gastroenteritis (17,18, 19). This technology has the advantage of being more sensitive than the immunoassays, as demonstrated in this study. In addition, if the amplified products are further studied by sequencing, PCR-based methods may provide more information about

the strains or variants causing the illness. Moreover, multiplex PCR reactions can be designed to detect more than one enteric pathogen simultaneously (20, 21, 22). The major drawbacks to incorporate this technology to the clinical laboratory setting are the requirement of specialized equipment (thermocycler) and the need of expertise in molecular biology. However, these obstacles should be overcome in order to take advantage of the improved sensitivity and versatility of the PCR-based techniques for clinical diagnostics of enteric pathogens.

In conclusion, our data suggests that Rotavirus is an important etiologic agent of gastroenteritis in the local pediatric population. However,more extensive studies are necessary to determine the prevalence of Rotavirus in Puerto Rico in order to design effective control measures to protect our population against this pathogen.

Resumen

En este estudio piloto, examinamos 100 muestras de heces fecales de pacientes con gastroenteritis para detectar la presencia de Rotavirus utilizando métodos de diagnóstico inmunológico y molecular. Cuando las muestras fueron analizadas por ensayo de ELISA, encontramos 11 muestras positivas para Rotavirus. Sin embargo, utilizando métodos moleculares (RT/PCR) se identificaron 51 muestras positivas para Rotavirus. Estos resultados corroboran el concepto generalmente aceptado que las técnicas moleculares son más sensitivas que los inmunoensayos. Además, nuestros datos sugieren que Rotavirus es un agente etiológico importante de gastroenteritis en nuestra población pediatrica. Son necesarios estudios más extensos para determinar la prevalencia de Rotavirus en Puerto Rico para diseñar medidas de control efectivas contra este patógeno.

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