Comparison of ELISA with shell vial cell culture method for the detection of human rotavirus in fecal specimens

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SUMMARY

The aim of the study was to compare an enzyme immunoassay method with shell vial cell culture method for detection of rotavirus in fecal specimens. In addition, the correlation between laboratory results and clinical scores of patients with gastroenteritis was evaluated.

A total of 219 fecal specimens from children (ages 3 weeks to 5 years) with acute gastroenteritis submitted to pediatric emergency room were evaluated by both ELISA and shell vial cell culture. A Vesikari score was used for assessing the severity of the illness.

Among 219 stool samples tested, 107 (48.9%) were determined to be positive. Two specimens were positive by shell vial cell culture method while they were ELISA negative. According to these results the calculated sensitivity, specificity, PPV, and NPV of ELISA were 98.1%, 100%, 100%, and 98.2%, respectively. The mean severity score for the 107 episodes of rotavirus diarrhoea was 11.0±3.6 compared to 4.5±1.9 for the 112 episodes of non-rotavirus diarrhea in the same population.

Our study indicates that ELISA, which is easier to perform, faster and cheaper than cell culture methods may be suitable for routine diagnosis of rotavirus infections. The severity of rotavirus positive gastroenteritis was significantly higher than that of rotavirus negative patients.

KEY WORDS: Rotavirus, shell vial cell culture, elisa, clinical scores

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INTRODUCTION

Rotaviruses are responsible for 30% to 60% of all cases of diarrhea in young children throughout the world. The major effect of rotavirus infections is seen in children younger than 2 years (Singh-Naz *et al*., 1990). Although rotavirus infections usually are self-limiting, deaths resulting from dehydration and electrolyte imbalance are common in developing countries (Lipson and Zelinsky Papez, 1989). The virus is responsible for an estimated 611,000 childhood deaths per year according to World Health Organization (Brooks *et al*., 2004; Parashar, 2006).

A rapid and accurate laboratory identification of rotavirus gastroenteritis remains important, as such testing assists the pediatrician in decisions of patient care and management. Currently a variety of methods are commercially available for rapid laboratory diagnosis in stool samples for rotavirus gastroenteritis, i.e., enzyme immunoassays, latex agglutination and immunochromatographic assays. These assays are easy to perform, require no special equipment and provide results in a short turnaround time, but false positive results are not uncommon (Eing *et al.,* 2001; Giordano *et al*., 2005).

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Nevertheless, electron microscopy, polyacrylamide gel electrophoresis, reverse transcriptase polymerase chain reaction and cell culture methods are used as reference methods for rotavirus detection in stool samples, because of high specificity and sensitivity. However, these methods are restricted to use because they require experienced technicians, discard tedious, expensive and time-consuming (Lipson and Zelinsky Papez, 1989; Christy *et al*., 1990; Lipson *et al*., 2001; Raboni *et al*., 2002). This study compared an enzyme immunoassay with shell vial cell culture method for detection of rotavirus in fecal specimens. In addition, we evaluated the correlation between laboratory results and clinical scores of patients with gastroenteritis.

MATERIAL AND METHODS

Patient population and specimen collection

Fecal specimens from 219 children (ages 3 weeks to 5 years, 3 weeks-24 months n=146, 25-60 months n=73) with acute gastroenteritis submitted to pediatric emergency room were included in the study. The study was performed during the winter season between September 2003 and May 2004. Stool specimens were collected in sterile containers from the patients on the first five days after the onset of diarrhea. A total of 219 fecal specimens obtained from 138 boys, 81 girls were evaluated by both ELISA and shell vial cell culture methods after direct microscopic examination and routine bacteriological analysis. Specimens received in the laboratory were temporarily maintained at 4°C for 24h and aliquots of stool specimens were stored undiluted at -80°C.

Direct microscopic examination and bacteriologic analysis

All of the 219 fecal specimens were examined by direct microscopy and were inoculated to blood; CIN, Skirrow, and EMB agar plates, incubated aerobically for 24h at 37°C, followed by identification for *Escherichia coli, Shigella spp., Salmonella spp., Yersinia spp.* and *Campylobacter spp* by routine diagnostic methods (Winn *et al.,* 2006).

Cell culture

Proteolytic activation of rotavirus was performed as described previously (Lipson 1992). Briefly, a 15% (wt/vol) stool preparation was prepared in Eagles' Minimal Essential Medium (EMEM) supplemented with 15 µg/ml trypsin (Biochrom Ag, Berlin, Germany). The suspension was vortexed and then centrifuged at 10000 *g* for 10 min. Supernatants were incubated at 37°C for 30 min, diluted 1:20 in EMEM supplemented with 2 µg/ml trypsin without fetal bovine serum, followed by inoculation into shell vial coverslip.
Vero cell line and shell vial centrifugation method were used for rotavirus isolation as described previously (Wiedbrauk D.L. and Johnston, 1993). Briefly, 0.2 ml activated fecal specimen was inoculated into shell vial containing Vero cell monolayers on the coverslip. The vials were centrifuged at 3000 *g* for 60 min at 25°C. The specimen was aspirated from each vial and replaced with an isolation medium containing 1 µg/ml trypsin-TPCK and EMEM without fetal bovine serum. The vials were incubated at 37°C for 48 h. The coverslips were fixed in chilled acetone for 10 min and stained by an indirect immunofluorescence assay according to the manufacturer's recommendations. The primary stain consisted of goat antirotavirus polyclonal antibody IgG fraction (diluted 1:5 in PBS) directed to common VP6 antigen of the group A rotavirus (Light Diagnostics, Chemicon international, USA). The second reagent consisted of goat anti-mouse IgG antibody FITC-conjugated (Light Diagnostics, Chemicon Internationale, USA). Coverslips were examined for typical fluorescent cytoplasmic inclusions and were considered positive if one or more inclusions were present.

Enzyme immunoassay

The Generic Assay (Dahlewitz, Germany) used in the study is an enzymometric one-step immunoassay for the qualitative determination of rotavirus antigen employing a solid phase immobilized polyclonal antibody (sheep) and a murine monoclonal antibodies conjugated to horseradish peroxidase. Both antibodies are directed against the group specific VP6 antigen of group A rotaviruses. The assay was run according to the manifacturer's instructions. Results were read spectrophotometrically.

Clinical scores

A Vesikari score was used for assessing the severity of the illness. Patients were analyzed for

watery diarrhoea, vomiting, fever and dehydration by the clinicians. A 0-20 point numerical score was devised according to the distribution of clinical features in the patients (Ruuska and Vesikari, 1990). Besides, clinical scores were divided in two subgroups; 1-10 points in the moderately severely ill group (Group I), and 11-20 points in the severely ill gastroenteritis group (Group II) (Joensuu *et al.*, 1997).

Statistics

Sensitivity, specificity, positive and negative predictive values, diagnostic accuracy, student t-test and chi-square were calculated according to standart statistical procedures (SPSS v 13.0).

RESULTS

Among 219 stool samples tested, 107 (48.9%) were determined to be positive. Characteristics of children and prevalence of gastroenteritis in children are summarized in table 1. Rotavirus infection was found to be more predominant in children 3 weeks to 24 months than the 25-60 months group (p=0,013). No difference was detected according to sex (p=0,471).

Of the 219 stool specimens tested 105 were positive and 112 were negative by two assays. Seven specimens which were ELISA positive with low OD values were negative by shell vial cell culture. These specimens were retested by ELISA and found to be negative. Other bacterial pathogens were not detected in these specimens. Two specimens were positive by shell vial cell culture method while they were ELISA negative. Clinical scores of these patients were 12 and 14. According to these results the calculated sensitivity, specificity, PPV, NPV, and diagnostic accuracy of ELISA were 98.1%, 100%, 100%, 98.2%, and 99.0% respectively (Table 2). Microscopic examinations of 185 specimens were found to be normal. Thirty-four (15,5%) stool specimens had leucocytes and/or erytrocytes. Twenty-six of the 34 specimens had only leucocytes, seven had both leucocytes and erythrocytes, and the remaining one had only erytrocytes by microscopic examination. Three (1.4%) of 219 stool specimens were *Salmonella enteritis, Shigella flexneri* or *Shigella dysenteriae* culture positive by routine diagnostic methods. Rotavirus was not detected in these specimens. Of the 107 rotavirus positive specimens, seven (6.5%) had leucocytes and two (1.9%) had both leucocytes and erythrocytes.

Annual rotavirus gastroenteritis peak was detected between November and March. The month-

TABLE 1 - *Characteristics of the children and the prevalence of gastroenteritis.*

	Rotavirus positive No. (%)	*Rotavirus negative No. (%)*	*Total (%)*	*p values (chi-square)*
Age				
3 weeks-24 months	80 (54.8%)	66 (45.2%)	146 (66.7%)	p=0.013
25-60 months	27 (37.0%)	46 (63.0%)	73 (33.3%)	
Sex				
Male	70 (50.7%)	68 (49.3%)	138 (63.0%)	p=0.471
Female	37 (45.6%)	44 (54.4%)	81 (37.0%)	

TABLE 2 - *Comparison of ELISA and shell vial cell culture results.*

		Shel vial cell culture		
		Positive	*Negative*	*Total*
ELISA	Positive	105	0	105
	Negative	2	112	114
Total		107	112	219

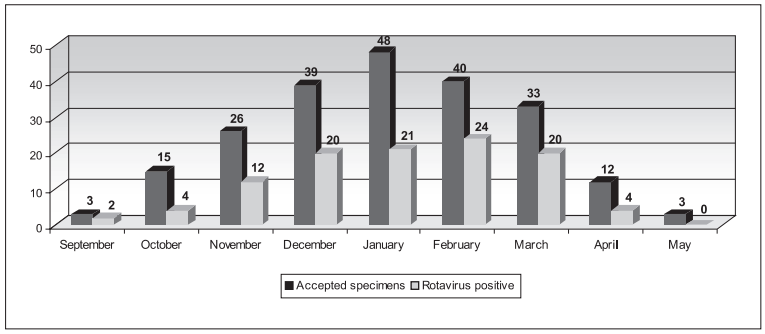

FIGURE 1 - *Monthly distribution of rotavirus positive specimens.*

ly distribution confirmed the peak activity occurring in February (Figure 1).

A 0-20 point numerical score (Vesikari) was devised according to the distribution of clinical features in the patients. Using this system, the mean severity score for the 107 episodes of rotavirus diarrhoea was 11.0±3.6 (10.3-11.7, 95% CI) compared to 4.5±1.9 (4.1-4.8 95% CI) for the 112 episodes of non-rotavirus diarrhea in the same population (p = 0.000) (Figure 2).

With regard to the clinical scores of the patients, 111 (99.1%) rotavirus negative patients were classified in group I and one patient (0.9%) in group II. Forty-six (43.0%) rotavirus positive patients were classified in group I while 61 (57.0%) of them were in group II (p=0.000) (Table 3).

DISCUSSION

This study detected rotavirus positivity in 48.9% (107/219) of the fecal specimens. The majority of the cases were in children younger than 24 months of age as indicated in previous studies (Kasule *et al.,* 2003; Frühwirth *et al.,* 2001). In contrast to Frühwirth *et al*. (2001) report, no difference was observed in rotavirus positivity according to sex.

The shell vial enhanced centrifugation method achieved successful replacement of tube culture. Duration of rotavirus isolation was decreased by activating specimens in the presence of trypsin and staining with FITC conjugated antibody. In previous studies, African green monkey kidney cells were found to be more efficient for rotavirus isolation than continuous cell lines such as CaCo-2, MA104 (Bryden *et al.* 1977; Birch *et al.,* 1983; Lipson and Zelinsky Papez, 1989; Christy *et al.,* 1990; Lipson, 1992; Lipson *et al.*, 2001). An advantage of shell vial cell culture methods is that they can be done individually. Virus isolation can be useful when low levels of infectious virus are suspected or when further work on the epidemiology or molecular biology

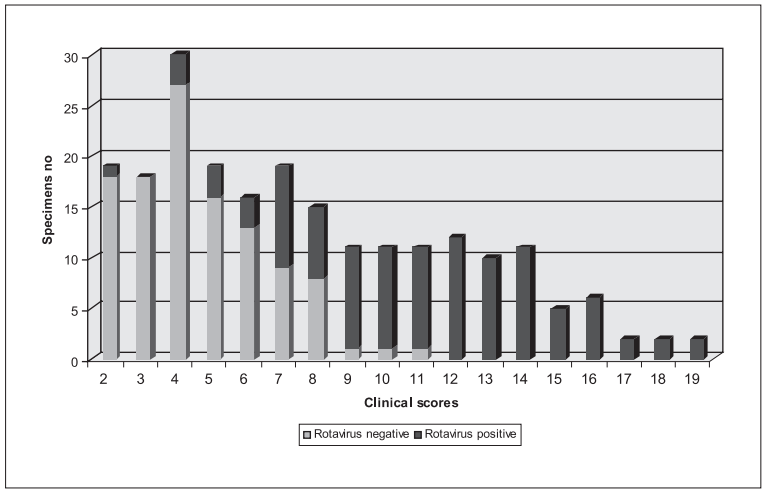

FIGURE 2 - *Distribution of the clinical scores of the children with rotavirus positive and negative results.*

TABLE 3 - *Clinical scores of the children with rotavirus positive and negative results.*

		Clinical scores			*p value*
		Group I	*Group II*	*Total*	*(chi-square)*
Rotavirus	Positive	46	61	107	0.000
	Negative	111	1	112	
Total		157	62	219	

of the isolates requires the generation of virus stocks. Shell vial enhanced centrifugation methods permit the establishment of a simple to perform, rapid, and sensitive isolation, for application in the important clinical disciplines of evaluation testing.

The results of the study indicate that ELISA is highly sensitive and specific (98.1%, 100%, respectively), but seven specimens were detected as false positives in the first run with low OD's and the clinical scores of the mentioned patients were 2 to 3. Retesting of these specimens gave negative results. A previous study reported that false positive results could be referred to cross reactivity with reovirus and/or with other components of the stool (5). This study did not test other gastroenteritis causing viruses that may cause cross reactivity. False positive results could also be obtained due to technical problems in manual ELISA. It is clear that specimens with low OD values should be retested by ELISA or tested with another method. At the same time, test results should be evaluated in relation to the clinical findings.

Another study showed that in regions with low prevalence (3.82%) of rotavirus, PPV (57.7%) of the ELISA decreased (Eing *et al.,* 2001). By making use of likelihood ratios, it is possible to revise pretest probabilities (prevalance) of an illness and convert them into significant posttest probabilities (PPV). As the disease prevalence decreases in a population, the PPV falls and the NPV reciprocally rises (Fagan normogram) (Dawson-Saunders and Trapp, 1994; Giocoli, 2000). In our region, since rotavirus prevalance is quite high (Kurugol *et al.,* 2003), antigen tests such as ELISA could be used safely.

Two cell culture positive specimens are not detected by ELISA. Most rotavirus laden specimens contain more than of 10^{10}-10^{11} particles per gram of stool at the first five days of the illness (Brooks *et al.,* 2004, Lipson *et al.,* 2001). In different studies on the rotavirus detection limits of different assays, it was demonstrated that ELISA detected 10^{6-7} and cell cultures detected 10^4 particles/gram (Ward *et al.* 1984; Christy *et al.,* 1990; Frühwirth *et al.,* 2001). In our study, specimens were obtained in the first five days after the onset of symptoms.

Nine (8.4%) out of 107 rotavirus positive specimens had leucocytes and erythrocytes on microscobic examination and no pathogen was isolated in these specimens. Enteropathogens other than *Escherichia coli, Shigella spp., Salmonella spp., Yersinia spp.* and *Campylobacter spp* which are not in the spectrum of this study could be the causative agents in the mentioned patients.

Our study observed that rotavirus infection follows discard a seasonal pattern in our region, where it is associated with cooler temperatures from November to March reaching a peak in February. The seasonal distribution of rotavirus infection during the cooler months has been reported before (Ward *et al.,* 1984).

The severity of rotavirus positive gastroenteritis was significantly higher than that of rotavirus negative patients (11.0±3.6 *v* 4.5±1.9, p=0,000). All of the patients with scores 12-19 and 96% (80/83) of the patients with clinical scores higher than 9 had rotavirus positivity. 99.1% of patients negative for rotavirus were in group 1. On the other hand, 46 patients (43.0%) in group I (7.7±2.0) were rotavirus positive, but the mean clinical scores of these patients were higher than rotavirus negative patients (4.5±1.9). A possible explanation for this may be admission of these patients to the hospital early in the course of illness. Similar results were observed in previous studies (Ruuska and Vesikari, 1990; Joensuu *et al.,* 1997; Frühwirth *et al.,* 2001).

A rapid diagnosis of rotavirus infection in patients admitted to hospital with symptoms of gas-

troenteritis would enable better treatment of the patients, such as isolation or discharge, as in many cases effective rehydration can be achieved at home, and most rotavirus infections are self-limiting. An accurate diagnosis of rotavirus is essential since it obviates the unneccessary use of antibiotic therapy. The choice of rotavirus assay will depend to a large extent on the requirements of the individual laboratory. Each laboratory must consider its needs on the basis of patient population, prevalance of disease in the region, pricing, and technical help to determine what is best for its specific environment. Our study indicates that ELISA, which is easier to perform, faster and cheaper than cell culture methods, may in this setting be sufficiently sensitive and suitable for routine diagnosis of rotavirus infections.

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