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Comparison of Six Methods for Detecting Human Rotavirus in Stools

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The following six methods for detecting rotavirus in human faecal samples were compared: electron microscopy, immune electron microscopy, immunofluorescence in cell culture, two enzyme immunoassays (Rotazyme, Enzygnost) and a latex agglutination test (Rotalex). Specimens were collected from 112 children with diarrhoea. The relative sensitivities of the different assays for human rotavirus were as follows: electron microscopy, 84%; immunofluorescence, 86%; Rotalex, 88%; Rotazyme, 89%; immune electron microscopy, 93%; Enzygnost, 98%. According to our findings Enzygnost is the most sensitive method, but Rotalex is more valuable for screening a small number of faecal samples. No false-positive results were observed in the two enzyme immunoassays or in Rotalex.

Since the discovery of human rotaviruses in the duodenal mucosa of children with gastroenteritis by Bishop et al. (1), the role of these agents in infantile gastroenteritis has been well documented (2). Human rotaviruses cannot be isolated in the usually available tissue culture, and only a few strains have been adapted for growth in vitro (3, 4). Various methods have been developed for rapid detection of rotaviruses or their antigens in faecal extracts. These include electron microscopy (5), the original diagnostic technique which is now reference method; immune electron microscopy (6); counter immunoelectrophoresis (7); complement fixation (8); immune adherence hemagglutination (9); reversed passive hemagglutination (10); fluorescent virus precipitation (11); immunofluorescence in cell culture (12); immunoassays using antibodies labeled with either radioisotopes (13), enzymes (14) or lectins (15), and antibody-coated erythrocytes (16).

The present study was undertaken to compare the ability of the following procedures to detect rotavirus in stools: electron microscopy (EM), immune electron microscopy (IEM), immunofluorescence in cell culture (IF), two commercially available enzyme-linked immunosorbent assay (ELISA) systems (Rotazyme, Abbott; Enzygnost, Behring) and latex agglutination test (Rotalex, Orion). The usefulness of the respective methods in a routine diagnostic laboratory was also assessed.

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Materials and Methods

Clinical Specimens. One hundred and twelve stool samples were collected from children between the age of six and 24 months with diarrhoea. Twenty percent (w/v) emulsions were made in minimum essential medium (MEM) and clarified by low speed centrifugation (3800 rpm) for 30 min, at 4 °C. After filtering through 0.45 μ millipore, the supernatants were stored at − 80 °C. These faecal extracts were used for EM, IEM, IF, Rotazyme, Enzygnost and Rotalex. Each sample was coded and tested without knowledge of the results obtained by the other tests.

Electron Microscopy. This was carried out as described by Ferchal et al. (17). Briefly, three hundred-mesh formwar carbon-backed copper specimen grids were floated, formwar side down, for 4 min on droplets of faecal extracts and then negatively stained for 4 min with 2% phosphotungstic acid, pH 6.2 (Merck Product). After drying, the specimens were examined with a Philips EM 300 electron microscope at magnifications ranging from 19,000 to 34,000. A negative specimen was considered as such only after examination for 20 min.

Immune Electron Microscopy. Briefly, 100 μl of faecal extract were mixed with 100 μl of a 1:50 dilution of a human serum positive for rotavirus antibody with a titer of 128, as determined by complement fixation (CF). After incubation at 4 °C for 18 h, the mixtures were centrifuged onto microscope grids for 3 min at 90,000 rpm at room temperature in a Beckman airfuge with an EM-90 rotor. After washing on three droplets of PBS, negative staining and examination of the specimens were performed as in the conventional EM procedure. Aggregation of rotaviruses was also investigated using a pool of monoclonal antibodies against the rotavirus group antigen (18).

Immunofluorescence. Monolayers of MA-104 cells, a stable cell line derived from embryonic rhesus monkey kidney, were grown on round coverslips (12 mm diameter) in flat-bottomed glass tubes. Each tube was seeded with a suspension of 200,000 MA-104 cells in 1 ml of MEM containing 10% foetal calf serum (FCS) and antibiotics (100 μg/ml of ampicillin; 200 μg/ml of kanamycin). These tubes were incubated for 24 h at 37 °C. The confluent monolayer was washed three

times with MEM containing 5 μg/ml of trypsin and then infected with 200 μl of a 1:2 to 1:10 dilution of faecal extract pretreated with 10 μg/ml of trypsin for 30 min at 37 °C. The tubes were centrifuged for 1 h at 4000 rpm at 37 °C. The cell layer was rinsed twice with MEM containing 5 μg/ml of trypsin, and 1 ml MEM (without FCS but with antibiotics) was added. After incubation for 18–24 h at 37 °C, the coverslips were fixed with acetone for 2 h at − 20 °C. A 1:25 dilution of a pool of monoclonal antibodies against the rotavirus group antigen, kindly provided by Roseto (18), was added for 1 h at 37 °C. The cell layer was then washed, stained with fluorescein-conjugated sheep anti-mouse globulin, washed again, mounted in buffered glycerol and observed in a Leitz UV microscope. SA-11 strain simian rotavirus, kindly provided by H. H. Malherbe (University of Texas Health Science Center, San Antonio, TX, USA), was used as control.

Enzyme Immunoassays. The Rotazyme test was performed as suggested by the manufacturers except that 20% faecal extracts were used. Briefly, a bead that had been precoated with an antiserum to SA-11 prepared in guinea pig was incubated for 3 h at 45 °C with 200 μl of faecal extract. The bead was then washed four times and incubated for 1 h at 45 °C with 200 μl of rabbit antirotavirus serum conjugated to horseradish peroxidase. After washing, the bead was transferred to a fresh tube and incubated for 15 min at room temperature in 200 μl of 0-ethylenediamine-2HCl substrate. The reaction was stopped by addition of 1 ml of HCl and read either visually or by a spectrophotometer at 492 nm.

In the Enzygnost test, microplate wells precoated with rabbit anti-SA-11 serum were washed three times. Twenty per cent faecal extracts (150 μl per well) were added and the plates further incubated for 2 h at 37 °C. After washing, 50 μl of calf anti-Nebraska calf diarrhoea-rotavirus (NCDV) serum conjugated to alkaline phosphatase, diluted 1:30, was added and incubated for 1 h at 37 °C. Finally the plates were rinsed six times and 100 μl of p-nitrophenyl-phosphate substrate was added for 45 min at room temperature. The reaction was stopped by adding 50 μl of 2N NaOH. Results were read both visually and at 405 nm by a spectrophotometer.

Neutralisation. Clinical specimens positive in ELISA but negative in IEM techniques were checked by a blocking test. Faecal extracts were incubated for 1 h at 37 °C with an equal volume of a 1:100 dilution of human serum positive for rotavirus antibody (CF titer: 128). The above mentioned ELISA procedures were repeated and the results compared with the faecal extract diluted 1:2 with PBS. The test was considered positive if a 50% or greater decrease in adsorbance values was noticed.

Latex Agglutination (Rotalex). Faecal extracts were diluted 1:2 in Tris buffer, pH 7.4. After standing 30 min at room temperature, these diluted specimens were centrifuged (2000 rpm) for 20 min at 4 °C. One drop of supernatant was added to one drop of latex particles, either precoated with rabbit anti-NCDV or non-immune antibodies on a glass slide. The drops were carefully mixed and tilted for 2 min. The test was positive if a distinct agglutination was observed, provided the control suspension remained milky.

Results

The six techniques used in this study were first evaluated by performing assays on a serially diluted human rotavirus (HRV)-positive faecal extract used as reference. The highest dilutions of faecal specimen detected as positive by EM, IEM, IF, Rotazyme, Enzygnost and Rotalex were 1:160, 1:2560, 1:320, 1:1280, 1:5120 and 1:640 respectively (Table 1). Thus, the sensitivity of Rotalex was four times greater than that of EM, the reference diagnostic method.

Table 1: Sensitivity of six methods for detection of human rotavirus in faecal extracts.

Methods	Highest reciprocal titer
Electron microscopy	160
Immune electron microscopy	2560
Immunofluorescence	320
Rotazyme*	1280
Enzygnost*	5120
Rotalex	640

*Cut-off values were 0.08 (Rotazyme) and 0.12 (Enzygnost).

Table 2: Accuracy of six methods in detecting human rotavirus in 112 stools of children suffering from diarrhoea.

No. of specimens	Electron microscopy	Immune electron microscopy	Immuno-fluorescence	Rotazyme	Enzygnost	Rotalex
45	+	+	+	+	+	+
1	+	+	+	−	+	+
2	−	+	−	−	+	+
2	−	−	−	+	+	−
2	−	−	−	−	+	−
1	+	+	+	−	−	−
1	−	+	+	+	+	−
1	−	+	−	+	+	+
1	−	+	−	+	+	−
56	−	−	−	−	−	−

The accuracy of these six tests in detecting rotavirus is shown in Table 2. Fifty-six faecal samples were negative in all tests. Forty-seven were positive in EM, 52 in IEM, 48 in IF, and 49 in Rotalex. All faecal samples had been tested by EM previously without filtration and none of the positive specimens became negative after filtration. Using the ELISA procedures viral antigens were detected in 50 (Rotazyme) and 55 (Enzygnost) samples. The relative sensitivity of the different diagnostic tests was 84% for EM (47/56); 86% for IF (48/56); 88% for Rotalex (49/56); 89% for Rotazyme (50/56); 93% for IEM (52/56); 98% for Enzygnost (55/56).

One specimen, positive only in EM, IEM, and IF, remained negative in ELISA and Rotalex after treatment with ethylenediamine tetraacetate (EDTA) (0.025 M), which is known to remove the HRV outercoat polypeptides and expose the group antigen (19). The possibility of a prozone effect was ruled out, since the sample remained negative when diluted.

Four specimens positive in ELISA procedures were negative in other tests, as confirmed by a blocking assay. As shown in Table 2 all specimens positive in Rotalex were recorded as IEM-positive. To investigate the reproducibility of the Rotalex test, 20 samples stored at −80 °C were retested one month later. Neither a reduction in sensitivity nor a tendency to develop non-specific agglutination was observed. Specificity of Rotalex was confirmed by clearly negative test results in 16 faecal extracts from the 112 samples containing either adenovirus (6 samples), coronavirus (5 samples), small round virus (3 samples) or astrovirus (2 samples).

Discussion

EM of negatively stained stool extracts has been the standard method for detecting rotaviruses, although it is the least sensitive. Many factors affect the reliability of this technique including virus size, concentration and purity, wetting properties of the grids used, time devoted to searching for the virus in samples, and operator skill. Although rapid, it is not suitable for examination of many specimens daily, and expensive equipment is required. However, it is a "catch-all" method; other gastroenteritis viruses may be seen, and it permits detection of dual virus infections.

IEM is more sensitive than EM. Aggregation of rotavirus by specific antibody is effective only after 60 min of incubation at 37 °C and we therefore routinely let the reaction proceed overnight. High speed centrifugation of the antibody rotavirus complex directly onto microscope grids increases the number of virus particles adsorbed on the grid. Although the IEM procedure is easy to perform, problems may be encountered such as (i) the necessity of a reliable source of antisera, well characterized as polyclonal or monoclonal against the rotavirus group antigen and (ii) a somewhat long reading period. Since virus in the IEM preparations was often present in relatively isolated clumps, a large area of the grid tended to be devoid of any virus.

IF appears to be at least as sensitive as EM, as previously described (20), but less sensitive than IEM. All the specimens diagnosed by EM, and one sample negative in EM and IF but positive by other tests, appeared to contain infectious rotavirus. The major difficulty in the IF test is the toxicity of faecal extracts which forced us to examine higher dilutions of the specimens. These three methods, EM, IEM and IF, require special and costly equipment and prove laborious when examining large numbers of samples. The need for rapid and reliable rotavirus diagnostic procedures has thus led to the development of rapid and sensitive immunoassays.

One of these, a simple latex agglutination test (Rotalex), has recently become commercially available. Latex particles are precoated with rabbit anti-NCDV antibodies and by means of cross reactivity of this antibody with HRV, agglutination becomes macroscopically evident within a minute. We did not follow the manufacturer's instruction to test stools at a 1:10 dilution in Tris. Most laboratories routinely prepare 10% or 20% (w/v) faecal extracts in PBS or MEM clarified by low speed centrifugation which can then be used in all tests. No problems were encountered using this extract which also avoids non-specific agglutination due to solid debris present in stools. Specificity of Rotalex was excellent; no false-positive reactions were observed in this study. No blocking test was required, since all the specimens positive in Rotalex were positive in IEM. Nevertheless, when the method is used alone, positive tests should be confirmed by neutralisation. Although the reproducibility was good, more studies are needed to draw a definitive conclusion. Rotalex is rapid and does not require complicated or expensive equipment. We found Rotalex to be more sensitive than EM in accordance with previous reports (21, 22). Nevertheless, its sensitivity has to be improved.

The two ELISA procedures were found to be more sensitive than EM, IF and Rotalex. Enzygnost was more sensitive than Rotazyme, but we cannot explain this discrepancy. Approximately 1% of clinical specimens negative by the IEM method were positive in ELISA. All of these specimens were confirmed by blocking assay as advocated by Sarkkinen et al. (23). Our use of a convalescent human serum for the blocking test may explain why we did not observe any false-positive results in Rotazyme or Enzygnost. The failure to detect virus by IEM in these ELISA-positive samples may be explained by the fact that human rotavirus-infected faeces contain considerable amounts

Table 3: Features of six methods for detecting human rotaviruses in stools evaluated on a scale from 1 + to 3 +, where 1 + indicates the lowest degree.

Features	Electron microscopy	Immune electron microscopy	Immuno-fluorescence	Rotazyme	Enzygnost	Rotalex
Sensitivity	1 +	3 +	1 +	2 +	3 +	2 +
Specificity	"catch-all" method	3 +	3 +	3 +	3 +	3 +
Reproducibility	3 +	3 +	3 +	3 +	3 +	3 +[a]
Tissue-consumption	1 +	1 +	1 +	2 +	2 +	3 +
Practicability	1 +	1 +	2 +	3 +	3 +	3 +
Reading	1 +	1 +	1 +	3 +[b]	3 +[b]	3 +
Efficiency for daily detection	1 +	1 +	1 +	3 +	2 +	3 +
Efficiency for large scale studies	1 +	1 +	1 +	2 +	3 +	3 +
Cost of reagents	1 +	1 +	1 +	1 +	3 +	2 +
Cost of reader	1 +	1 +	1 +	1 +[c]	1 +[c]	unnecessary

[a] More studies are needed to establish or refute the evaluation.
[b] If ELISA reader is not used.
[c] Not essential.

of the antigenic subunit associated with the inner capsid of the virus, the site of the group antigen common to all rotaviruses (24). One sample that was positive in EM, IEM and IF was negative in the two ELISA procedures on two separate occasions. An explanation might be that rotavirus is covered by antibodies (25) and inhibitors are present in the faecal sample (26). However, viruses that are morphologically indistinguishable from rotavirus but fail to react to ELISA have been detected in faecal samples from children with gastroenteritis in Washington (27), Melbourne (28) and Paris (29). Electrophoresis of the nucleic acid of one of these agents, which seem to lack the common group-specific antigen of rotaviruses, showed a unique migration pattern. Although a spectrophotometer was used for ELISA, results could be read visually with little difficulty. Nevertheless, these two sensitive methods also have disadvantages: the Rotazyme kit is expensive and Enzygnost is convenient only when large numbers of specimens must be assayed.

In conclusion, our comparison of six methods for detecting HRV has shown that, considering all features (summarized in Table 3), Enzygnost is the most sensitive technique. However, Rotalex, a simple and rapid test, is more valuable for screening a small number of rotavirus isolates per day in laboratories with limited financial means.

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