Comparison of Solid-Phase Immune Electron Microscopy by Use of Protein A with Direct Electron Microscopy and Enzyme-Linked Immunosorbent Assay for Detection of Rotavirus in Stool

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A total of 525 stool specimens collected during 1 year were examined for the presence of rotavirus by direct electron microscopy (EM), enzyme-linked immunosorbent assay (ELISA), and a solid-phase immune electron microscope method (SPIEM) utilizing protein A-coated grids for anchoring of specific viral antisera. Rotavirus was seen in 187 specimens; SPIEM detected 183 (97.8%), whereas direct EM and ELISA detected 161 (86%) and 166 (88.7%), respectively. No false-positive reactions were seen by ELISA. The sensitivity of the methods was evaluated by coded investigation of a dilution series of a positive sample, with a negative fecal specimen as diluent. SPIEM was approximately 30 times more sensitive than direct EM and 10 times more sensitive than ELISA. A study was done to compare the elapsed time for recognition of rotavirus by SPIEM and EM in 25 randomly selected positive specimens. All virus-positive specimens were detected within 2 min by SPIEM, whereas up to 9 min was required for direct EM. SPIEM with protein A is a highly sensitive method, useful for rapid detection of viruses in clinical specimens. Due to the direct visualization of virus particles by electron microscopy, there is no requirement for monospecific antisera for the method.

Human rotavirus is an important cause of gastroenteritis in infants and young children. The inability of the virus to grow efficiently in conventional cell culture systems has resulted in the utilization of diagnostic methods such as immunoelectroosmophoresis (6), radioimmunoassay (7), enzyme-linked immunosorbent assay (ELISA) (17, 19), latex agglutination (12), and immune electron microscopy (IEM) (6) for detection of virus directly in clinical specimens. Ever since rotavirus was first detected (1, 4), electron microscopy (EM) of fecal specimens has been a valuable tool for diagnosis of viral gastroenteritis. Due to the broad scope of the method, a multitude of new viruses has been detected in stools. However, the relative sensitivity of direct EM as compared with other methods, especially IEM procedures, needs to be more thoroughly investigated.

The basis of IEM methods is the observation in an electron microscope of the interaction between virus and its antibody. This permits direct visual identification of the virus by its morphology as well as immunological identification of the virus by the reacting antibody. The most commonly used IEM method is based on the formation of immune aggregates of the virus and its corresponding antibody.

Solid-phase immune electron microscopy (SPIEM) was first described in 1973 by Derrick (3), who coated EM grids with specific antibodies. The technique was recently modified by precoating of grids with staphylococcal protein A for anchoring of specific antibodies (14). The modification has been used with success for detection of rotavirus (10), as well as for characterization of adenovirus (16).

The aim of the present study was to compare the sensitivity of SPIEM (with protein A) with direct EM and ELISA for detection of rotavirus in fecal specimens.

MATERIALS AND METHODS

Clinical specimens. A total of 525 stool specimens from children suffering from acute gastroenteritis were routinely examined for the presence of rotavirus during 1 year. The specimens were prepared as 10% suspensions, shaken with glass beads, and subsequently clarified by low-speed centrifugation at 2,000 rpm for 15 min.

Purification of human rotavirus for immunization. Rotavirus was recovered from a stool specimen which had been found to contain a large number of rotavirus...
particles by EM. The stool suspension was extracted with trifluorotrichloroethane, and the virus present in the aqueous phase was subsequently centrifuged through a 40% sucrose cushion at 100,000 × g for 120 min. The virus pellet was suspended in 1 ml of 0.002 M Tris-hydrochloride buffer (pH 7.5) and centrifuged to equilibrium on a preformed CsCl gradient at 4°C for 18 h at 100,000 × g. The various bands observed were harvested and dialysed overnight against distilled water. Virus particles in the bands were determined by EM, and preparations containing double-shelled particles were used for immunization.

Antiserum. Antiserum to human rotavirus was produced by inoculating a rabbit intramuscularly with purified virus mixed with equal volumes of Freund complete adjuvant. Intravenous booster inoculations were given at days 28 and 42. A week after the last booster inoculation, the rabbit was bled by heart puncture. The complement fixation titer to rotavirus antigen was 1:256. The antiserum was found to react with single- as well as double-shelled rotavirus by SPIEM.

Indirect ELISA. The ELISA assay for human rotavirus was performed by adding 100 μl of bovine anti-rotavirus serum (Wellcome Vir 681) diluted 1:300 in 0.05 M sodium carbonate (pH 9.6) to each well of a polystyrene microtiter plate (M29 AR; Dynatech Ltd.) and incubating at room temperature for 16 h. The plate was then washed four times in 0.15 M saline containing 0.05% Tween 20 before the addition of 100 μl of the stool suspensions diluted 1:30 in phosphate-buffered saline (pH 7.4; 0.5% bovine serum albumin, 0.05% Tween 20). The plate was then incubated at 37°C for 2 h and subsequently washed four times before the addition of 100 μl (1:300) of rabbit anti-human rotavirus serum (kindly supplied by B. Vestergaard, Denmark). After incubation for another 2 h at 37°C, the plate was again washed four times. Enzyme-labeled swine anti-rabbit serum (Orion Diagnostica) was added, followed by incubation at 37°C for 2 h. The plate was then washed five times before 100 μl (1 mg/ml) of P-nitrophenylphosphate substrate (Sigma 104) was added. After 30 min of incubation at room temperature, the absorbance caused by the reaction of the substrate and bound enzyme was measured in a Titer-tek Multiscan spectrophotometer (Flow Ltd.) at 405 nm. Samples were considered positive if absorbance values were higher than the mean of negative control specimens plus 3 times the standard deviation. Under standard conditions, this value was calculated to be 0.070 absorbance units.

SPIEM. SPIEM was performed as previously described (16). Carbon–Formvar-coated grids (400 mesh) were floated for 20 min on 10-μl drops (50 μg/ml) of protein A (Pharmacia, Uppsala, Sweden) and drained. Thereafter the grids were floated on 20-μl drops of human rotavirus antiserum diluted 1:1,000 for 20 min. The antibody-coated grids were washed with 10 drops of phosphate-buffered saline, drained again, and finally floated on 20 μl of virus specimen for 1 h at room temperature. Before staining with 3% phosphotungstic acid (pH 7.2), the grids were washed with 10 drops of phosphate-buffered saline and 10 drops of distilled water. The specimen was screened for the presence of rotavirus particles for 5 min in a Philips 300 electron microscope at a magnification of ×28,000.

Direct EM. A drop of stool suspension was applied to a carbon–Formvar-coated electron microscope grid (400 mesh). After 30 s, the drop was removed by blotting from the edge of the grid with filter paper, and the adherent particles were negatively stained. Excess stain was removed with filter paper. The specimen was screened for the presence of rotavirus particles for 5 min in a Phillips 300 electron microscope at magnification of ×28,000.

Preparation of immunosorbent-purified rotavirus antibodies. Five stool specimens, all with a large number of rotavirus particles, were prepared as 10% suspensions and pooled.

(i) Preparation of rotavirus. A 200-ml virus suspension was clarified for 15 min at 2,000 rpm. The supernatant fluid was extracted with trichlorofluoroethane and centrifuged at 2,000 rpm for 10 min. The resulting aqueous phase was subsequently centrifuged through a 10-ml cushion of 30% sucrose at 40,000 × g for 2.5 h. The virus pellet was suspended in distilled water containing 0.001 M EDTA. After 30 min at 37°C, the suspension was frozen and thawed 10 times. NaSCN (3 M) was added for 1 h before dialysis against glycine-NaOH (0.05 M; pH 10.4) containing NaCl (0.85%) overnight, followed by dialysis against NaHCO3 buffer (0.1 M; pH 8.3) containing NaCl (0.5 M).

(ii) Immunosorbent procedure. Rotavirus treated as described above was coupled to CNBr-Sepharose 4B as described by the manufacturer (Pharmacia, Uppsala, Sweden). The immunosorbent column was loaded with 3 ml of rabbit anti-human rotavirus serum. After 1 h of binding at room temperature, the column was washed with phosphate-buffered saline, and the antibodies bound to the gel were eluted by 0.2 M glycine-hydrochloride buffer (pH 2.8). The eluate was neutralized with solid Tris and dialysed against 0.15 M NaCl. The protein content of the eluate was 50 μg/ml.

RESULTS

We have previously evaluated the conditions for the use of protein A in SPIEM (16). The optimal concentration of protein A and the rabbit anti-human rotavirus serum was determined by box titrations; the highest virus-trapping efficiency was achieved with a protein A concentration of 50 μg/ml and a 1:1,000 serum dilution.

During the period January to December 1981, 525 stool specimens were obtained from children suffering from acute gastroenteritis. All specimens were tested by SPIEM, direct EM, and an indirect ELISA for the presence of rotavirus. Rotavirus was detected in 187 of the 525 samples; SPIEM detected 183 (97.8%), and direct EM and ELISA detected 161 (86%) and 166 (88.7%), respectively (Table 1).

Four samples which were easily found to be positive by direct EM and ELISA were negative by SPIEM at the first check. When the samples were rechecked, three of the four were positive. No technical explanation could be found as to why the last one was repeatedly negative. It is possible that these four samples contained virus coated with antibodies, preventing adsorption to...
the grid. Rotavirus was detected in 15 samples only by SPIEM. None of these specimens demonstrated more than five virus particles on the grid. When SPIEM was repeated, only 11 of the 15 samples were found to be positive.

The majority of the rotavirus particles detected by SPIEM were single shelled. However, double-shelled particles were also seen, but at a lower frequency. Aggregates of rotavirus were not observed (Fig. 1). Viral capsomers were sometimes seen in the absence of complete single- or double-shelled particles. Adenovirus was occasionally seen non-specifically attached to the protein A antibody-coated grid in samples with extremely high concentrations of adenovirus.

All 166 samples scored positive by ELISA were also positive by either EM or SPIEM. Samples with absorbances close to the cut-off level were generally found to be positive by SPIEM within a few minutes of screening.

Three samples in which rotavirus was not detected by direct EM on the first examination were found to be positive when the examination was repeated. The phenomenon is probably due to a change of electrical charge of the grid. Some samples seen with direct EM contained rotavirus particles surrounded by a halo, probably caused by the presence of antibodies. These were also seen by SPIEM, but at a much lower frequency.

The sensitivity of the methods was evaluated by testing a dilution series. A fecal specimen containing rotavirus was serially diluted in negative feces at 0.5 log10 dilution steps. The results in Fig. 2 are mean values of duplicate tests and show that SPIEM was about 1.0 log10 more sensitive than ELISA and 1.5 log10 more sensitive than direct EM.

We also investigated whether the sensitivity of SPIEM could be further improved if purified rotavirus antibodies were used instead of whole serum for virus trapping. Purified antibodies against human rotavirus were obtained by loading rabbit anti-human rotavirus serum on an immunosorbent column containing immobilized human rotavirus. Antibodies with human rotavirus specificity were bound to the gel and subsequently eluted as described above. The eluate, with anti-human rotavirus specificity, had a protein content of 50 μg/ml. Optimal virus

Table 1. Comparison of SPIEM, direct EM, and ELISA for detection of human rotavirus in stool specimens

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Rotavirus detected by</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SPIEM</td>
<td>Direct EM</td>
<td>ELISA</td>
</tr>
<tr>
<td>151</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>-</td>
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<td>6</td>
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<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Results were obtained with stool suspensions diluted 1:1 for SPIEM and direct EM and 1:30 for ELISA. Rotavirus was detected in 183 samples (97.8%) by SPIEM, 161 samples (86.0%) by direct EM, and 166 samples (88.7%) by ELISA.*
trapping by SPIEM was achieved with a protein A concentration of 25 μg/ml and an eluate dilution of 1:80. The efficiency of the unfractionated rabbit antiserum and the eluate prepared from the same serum was evaluated by testing virus dilutions, as described above. The results in Fig. 3 are mean values of duplicate tests. In all virus dilutions the unfractionated serum trapped more virus particles than did the immunosorbent-purified antibodies. However, the sensitivity was equal in this test. The negative staining gave better contrast and distribution on grids coated with unfractionated antiserum.

The efficiency of electron microscope procedures is closely related to the observation time. A comparison of the time required to detect rotavirus in stools by SPIEM and direct EM was therefore done. Twenty-five randomly selected stool specimens positive by ELISA were used. The elapsed time was recorded to the first recognition of a virus particle by SPIEM and direct EM at a magnification of ×28,000. The
results in Table 2 show that all specimens were detected within the first 2 min of observation by SPIEM, whereas direct EM required up to 9 min for virus detection. However, 90% of the specimens were detected within the first 3 min of observation by this method.

**DISCUSSION**

The need for rapid and simple diagnostic procedures has led to the development of various methods for virus diagnosis. Direct EM, IEM, and solid-phase immunooassays such as ELISA and radioimmunoassays have been shown to be suitable for detection of small quantities of virus or viral antigens directly in stool specimens. Direct EM, used for the initial demonstration of rotavirus in stool, has been used to find the multitude of viruses now known to occur in human feces. However, the sensitivity of the method has been a matter of controversy, as evidenced by a number of reports. Thus, in comparison with immunoelctroosmophoresis, direct EM has been found to demonstrate lower (5), higher (8), or equal (15) sensitivity. In our study the technique was found to be less sensitive than ELISA. However, due to the absence of an immunological reaction in direct EM, other gastroenteritis viruses, such as adenovirus, calicivirus, astrovirus, etc., may be visualized. A factor which may reduce virus detection by EM is the elimination of virus aggregates by the low-speed clarification step used to remove stool debris and bacteria from the stool suspensions (9).

In the present study it was observed that some samples containing great numbers of rotavirus particles were scored negative on the first examination by direct EM. Furthermore, the grids demonstrated from time to time an uneven distribution of virus particles on their surfaces. These phenomena can probably be explained by the electrical charges of the grids.

The protein A SPIEM was shown in our study to be the most sensitive of the three methods used for detection of rotavirus. The sensitivity was higher than that of ELISA and considerably higher than that of direct EM. Similar results have been reported by others (10a). The high sensitivity of protein A SPIEM has also been reported by Nicolaeiff et al. (10), who detected rotavirus by this method in 71% of their specimens compared to only 20% by direct EM. They also reported that precoating of grids with protein A improved the virus-trapping capacity 5- to 10-fold as compared to grids coated with only antiseraum. Rubenstein and Miller (11) found that the sensitivity of the SPIEM method using antibody-coated grids without protein A did not exceed that of ELISA.

<table>
<thead>
<tr>
<th>Elapsed time (min)</th>
<th>SPIEM</th>
<th>Direct EM</th>
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<tbody>
<tr>
<td>0.25</td>
<td>28</td>
<td>8</td>
</tr>
<tr>
<td>0.5</td>
<td>76</td>
<td>52</td>
</tr>
<tr>
<td>1</td>
<td>92</td>
<td>72</td>
</tr>
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<tr>
<td>10</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

By employing a comparison similar to ours (Table 2), Brandt et al. found that the IEM clumping method occasionally required longer electron microscope reading time than direct EM (2). Our results show that rotavirus was detected more rapidly by SPIEM than by direct EM. Independent of the method used by Brandt et al. (2) and in the present study, most of the positive specimens were detected within 10 min of observation. The difference in sensitivity and the required reading time is probably explained by the use of different immune electron microscope procedures. Furthermore, the presence of preclumped virus in stool specimens can give misleading results with the IEM clumping method (9), a pitfall avoided by the use of SPIEM.

Purified rotavirus antibodies did not improve the virus-trapping efficacy. This may be explained by the lack of homology between the immobilized virus antigen, serotype of antiseraum, and serotype of virus used in the titrations. The sensitivity of the unfractionated antiseraum and the purified antibody preparation was the same, but the latter trapped fewer virus particles in each virus dilution sample. We also found that the purified antibody preparation lost titer when frozen, probably due to protein aggregation. The addition of bovine serum albumin could perhaps prevent this effect.

Rotavirus occurs in feces as single- and double-shelled particles. The latter possess type-specific antigens on the external shell, whereas antigens responsible for the group activity are located at the inner shell. In this study most rotavirus detected by SPIEM was single shielded, which indicates that the antiseraum used reacts with the group antigen. This is of great importance for a test in which rotaviruses of all serotypes are to be detected.

A suitable balance between the minimum time
and the maximum detection of rotavirus in stool was achieved by observing SPIEM preparations for about 3 to 5 min and direct EM preparations for up to 10 min.

The pretreatment of EM grids with staphylococcal protein A and the subsequent anchoring of specific antibodies increased the number of particles adsorbed to the grid. Furthermore, the coating prevented contaminating material as well as nonspecific virus adsorption. The fact that SPIEM gave a more even distribution of virus particles than direct EM made the examination easier, faster, and more reliable. The direct visualization of virus in an electron microscope is a clear advantage, since no confirmatory tests are normally necessary. A disadvantage of ELISA techniques is the possible occurrence of nonspecific reactions (13, 18). In our study no false-positive reactions were seen by ELISA; all positive stool specimens were positive by SPIEM or direct EM. During the 1-year study the ELISA used had a higher sensitivity than direct EM and was found to be the most suitable method for screening large numbers of stool specimens. By use of direct EM, a microscopist could handle approximately 40 samples a day; by SPIEM slightly fewer could be handled due to the time-consuming coating of grids.

To detect a broader spectrum of viruses in stool specimens, a pool of antisera against different viruses could be used, e.g., antisera to rotavirus and enteric adenovirus. Since by SPIEM the virus is identified by its morphology, the main function of the antisera is to increase the number of particles attached to the grid.

The present study showed that SPIEM, with protein A for anchoring of specific antibodies, is a useful tool for rotavirus detection. The high sensitivity of the method makes it well suited for identification of viruses which are cumbersome to study by conventional methods.

LITERATURE CITED


