Rapid Diagnosis of Rotavirus Infections: Comparison of Electron Microscopy and Immunoelectro-osmophoresis for the Detection of Rotavirus in Human Infantile Gastroenteritis

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SUMMARY

Eighty-seven faecal samples from infants and children suffering from acute gastroenteritis were investigated for the presence of rotavirus by immunoelectro-osmophoresis (IEOP) and electron microscopy (EM). Sixty-one % of the samples contained rotavirus antigens when examined by IEOP whereas only 50 % were diagnosed as positive by EM. However, where it was possible to perform EM within the same day that the sample was received it took 24 h to establish the diagnosis by IEOP. The high sensitivity of the IEOP method was achieved by application of antiserum produced in rabbits to rotavirus immunoprecipitates. The specificity and sensitivity of the diagnostic antiserum produced were tested by application of different immunoelectrophoretic methods.

INTRODUCTION

It is now well established that rotavirus (also named orbi-, reo-like-, duo- or infantile gastroenteritis virus) is the major cause of acute gastroenteritis in infants and young children. Since the original paper by Bishop et al. (1973) there have been reports from many parts of the world concerning this new virus (leading articles: Lancet, 1975a; British Medical Journal, 1975b; Medical Journal of Australia, 1976). In our paper we report the presence of this virus infection in Copenhagen. As it still seems difficult to culture the human rotavirus in vitro (Wyatt et al. 1974, 1976; Banatvala et al. 1975; Purdham et al. 1975; Albrey & Murphy, 1976), the most widely used diagnostic method has been electron microscopy (EM) applied to partly purified suspensions of faecal samples (Flewett, Bryden & Davies, 1973; Bishop et al. 1974; Middleton et al. 1974; Kapikian et al. 1974; Tufvesson & Johnsson, 1976a) although other methods have been used (Bishop et al. 1973; Kapikian et al. 1975; Peterson, Spendlove & Smart, 1976; Ørstavik et al. 1976). All these methods are laborious and/or expensive and some of them can hardly be named ‘rapid diagnostic procedures’. To overcome these disadvantages, inspired by the report of Spence et al. (1975) we decided to try the immunoelectro-osmophoresis (IEOP) method, previously
applied with success in our laboratory in the investigation of rubella infections (Grauballe et al. 1975). A similar work was published recently by Middleton et al. (1976), who, in contrast to the results of the present paper, found that EM was superior to IEOP in diagnosing rotavirus infection.

METHODS

Patients. Faecal samples were collected from children aged 0 to 5 years during the first five days of gastrointestinal illness. Sixty-six samples originated from patients admitted to various paediatric departments in hospitals of the Copenhagen area, while 21 samples were from infants attending day nurseries in the same area. All samples were collected during the period December 1975 to May 1976. The age distribution of the patients is shown in Fig. 1, 56% of the patients were boys.

Immunoelectrophoresis. The basic principles of these techniques have been described in detail elsewhere (Weeke, 1973; Krøll, 1973; Just Svendsen, 1973), and the application of the techniques in virology has been described in previous papers from our laboratory (Vestergaard, 1973; Grauballe et al. 1975; Vestergaard & Grauballe, 1975). Minor modifications of the exact experimental conditions of the methods used in the present paper are given in the legends to figures. A brief summary of the general principles of the methods employed is given here. Crossed immunoelectrophoresis (Weeke, 1973; see Fig. 2, 4 and 8) consists in electrophoresis of the antigens in question in the first dimension gel. Antibody containing gel is then placed in connection to the first dimension gel containing the antigens separated by the initial electrophoresis. The direction of the electrophoretic current is then turned in a right angle to the current in the first dimension. The pH of the gel buffer and the electroendosmosis of the gel should be chosen in such a way as to make the antigens migrate towards the anode and IgG antibodies migrate equally towards the anode and cathode. In IEOP (Grauballe et al. 1975; see Fig. 3, 5, 6, 9, 10, 11 and 13) wells containing antibody and antigen are cut in agarose gel in two rows. After applying the current, antigens will migrate towards the anode and antibody will migrate towards the cathode. For line immunoelectrophoresis (Krøll, 1973; see Fig. 7 and 12) antigens are embedded in the whole ‘first dimension’ gel instead of separating the antigens by electrophoresis, as described for crossed immunoelectrophoresis. The gel containing antibody is placed in the same way as described for crossed immunoelectrophoresis. To achieve a better separation of the precipitates representing the different antigens it is often convenient to insert a blank intermediate gel between those containing antibody and antigen. In fused rocket immunoelectrophoresis (Just Svendsen, 1973; see Fig. 14), two rows of closely situated wells were cut in a blank gel. Samples of fractions obtained by various separation techniques (e.g. CsCl-gradient centrifugation) were placed in these wells. The antigens were allowed to diffuse for 1 h before the gel containing antibody is placed in connection to the gel containing the wells, and the current was applied. The presence of the antigens and the amounts in which they are present in different fractions can be detected by the precipitating pattern.

Production of antiserum used for IEOP. Bovine rotavirus (Lincoln isolate, kindly supplied by Dr C. A. Mebus, University of Nebraska, Lincoln, U.S.A.) was cultured in primary bovine kidney (BK) cells. BK cells were cultured in Eagle's MEM with 10% foetal bovine serum at 37 °C. Prior to infection, the cells were washed thoroughly with Hank’s BSS. After infection, the cells were maintained in Eagle's MEM without serum. The medium without cells was harvested when c.p.e. became obvious and used for immunization of rabbits: equal volumes of medium and Freund's complete adjuvant were emulsified and
2.5 ml inoculated i.m. Five weeks later, the rabbits received 1 ml of an emulsion of medium and incomplete adjuvant weekly for five weeks, after which they were bled. The antiserum was absorbed with 10% (v/v) bovine serum, removing unwanted antibodies to bovine serum proteins. Forty-five μl of a suspension in PBS of a human faecal sample ascertained by EM to contain rotavirus was then tested in crossed immunoelectrophoresis against the absorbed antiserum. The precipitate of the most slowly migrating antigen, indicated by an arrow on Fig. 2, was cut out of similarly prepared unstained agarose plates and used for immunization of rabbits, as described previously (Vestergaard, 1975). The antiserum produced in this way were pooled and absorbed with 10% (v/v) bovine serum.

Faecal extracts. A 1:4 suspension in PBS of faecal samples, brought to the laboratory on ice, were prepared in a safety cabinet on a Whirli-mixer after adding a few glass beads. When the sample was homogeneous, one half of it was submitted to EM and the rest centrifuged at 10000 g for 10 min. Fifteen μl of the supernatant was tested for the presence of rotavirus antigen by IEOP.

Tissue culture antigen. Bovine rotavirus antigen for immunoelectrophoresis was prepared from virus infected BK cells, as described elsewhere (Vestergaard, 1973), with some modifications. Briefly, packed cells were solubilized in three volumes of a buffer solution containing 0.05 M-Tris/HC1, pH 7.5, 0.1 M-NaCl, 15 mM-NaNO3 and 5% (v/v) Triton X-100 (Serva, Heidelberg, West Germany) at 22 kHz for 90 s and submitted to 100000 g for 1 h. The supernatant fluid was used as antigen.

Human rotavirus antigen. Human faecal samples known to contain rotavirus were pooled and solubilized as described above, submitted to 10000 g for 15 min, and the supernatant fluid treated with an equal volume of Freon TF (trichloro-trifluoro-ethane) on a Whirli-mixer until a homogeneous suspension was obtained. The suspension was then centrifuged at 10000 g for 15 min and the supernatant re-centrifuged at 100000 g for 1 h. The supernatant from the final centrifugation was used as the human rotavirus antigen.

Experimental infections of calves. These experiments will be described in detail elsewhere (Meyling et al. in preparation). Briefly, five colostrum deprived calves were experimentally infected with bovine rotavirus. Faecal samples were obtained at the day of infection and then at regular intervals p.i. The first day p.i. four samples, the next day two, and then one sample every day. Extracts of the faecal samples were prepared as described above.

Determination of buoyant densities of rotavirus antigens. A 20% (w/v) suspension in PBS was made from a human faecal sample ascertained by EM and IEOP to contain abundant amounts of rotavirus. The suspension was purified with Freon TF and concentrated with polyethylene glycol (PEG) 6000 in 0.5 M-NaCl, as described by Albrey, Murphy & Path (1976). A sample (1.4 ml) of the resuspended PEG-deposit was layered on top of a two-step gradient consisting of 4.5 ml of 45% (w/w) and 4.5 ml 30% (w/w) CsCl made up in 0.01 M-tris/HCl buffer, pH 7.2, and centrifuged in a Spinco 65 rotor at 55000 rev/min for 17 h at 4 °C. The gradient was collected, and CsCl concentrations calculated from refractive indices. Antigen-containing fractions were revealed by fused rocket immunoelectrophoresis.

Electron microscopy. The above mentioned faecal suspensions were treated by differential centrifugation at low speed to remove bacteria and debris (1000 g for 1 h and 10000 g for 10 min). Four ml of the supernatant was layered on top of a continuous CsCl gradient from 60 to 10% (w/v) in PBS. After centrifugation in an SW 27.1 Spinco rotor at 25000 rev/min for 18 h at 1 °C, the gradient was photographed and CsCl concentration determined
Fig. 1. Distribution by age of 87 patients with acute gastroenteritis. In 54 (61%) patients rotavirus was detected in the faeces by IEOP. Of these, 41 were positive by EM. Faecal samples from two further patients revealed rotavirus by EM. □, Negative by EM and IEOP; ▼, positive by EM, negative by IEOP; ■, negative by EM, positive by IEOP; ▬, positive by EM and IEOP.

as described above. Fractions that were calculated to contain bands visualized on the photograph were dialysed against distilled water and negatively stained for EM with 2% (w/v) PTA, pH 6.8, or 4% (w/v) ammonium molybdate by mixing one drop of contrast stain with one drop of virus suspension directly on a carbon-formvar coated grid. After an absorption period of 1 min, excess water was sucked off with filter paper and the grid examined. At the same time, 0·5 ml of the supernatant from the first differential centrifugation was suspended in 4 ml of PBS and centrifuged at 50000 rev/min in an SW 56 Spinco rotor for 1 h at 1 °C. The sediment was resuspended in a few drops of bi-distilled water, stained and examined as in the parallel run experiment. For immuno-electron microscopy a similar technique was used, except that supernatant from the first differential centrifugation was mixed with an equal volume of the rabbit antiserum for IEOP. This mixture was reacted at room temperature for 1 h before ultracentrifugation.

RESULTS

Comparison of IEOP and EM for the detection of rotavirus

From Fig. 1 it appears that 61% (54/87) of the faecal samples was found to contain rotavirus when examined by IEOP. Only 77% (41/54) of these samples was found positive by EM. In two cases the EM diagnosis could not be confirmed by IEOP. Fig. 3 shows that a strong positive faecal sample may be diluted 128 or perhaps even 256 times and still react in IEOP. When the dilutions were tested by EM the last dilution giving positive results was 1:64. The samples giving the strongest positive reactions in IEOP were found
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Fig. 2. Crossed immunoelectrophoresis (Weeke, 1973) of rotavirus containing human faecal extract. First dimension: 45 μl of the antigenic preparation was electrophoresed in 1.5 mm thick 1% (w/v) agarose (Litex type HSB batch 0252, Glostrup, Denmark) electroendosmosis: Mr = −0.10 in tris-barbital buffer, pH 8.6, ionic strength 0.005, containing 1% Triton X-100. Current: 10 V/cm of gel for 80 min. Second dimension: 1 mm thick agarose gel as above containing 15 μl/cm² of absorbed rabbit antiserum to bovine rotavirus of tissue culture origin. Current: 2 V/cm of gel for 18 h. Electrophoretic buffer same as the gel buffer. Staining was by Coomassie brilliant blue.

Fig. 3. IEOP (Grauballe et al. 1975) of dilution from 1:2 to 1:512 of a positive human faecal sample against the diagnostic antiserum. Cathodic wells: 15 μl of the diluted faecal sample. Anodic wells: 5 μl of the diagnostic rabbit antiserum. Centre to centre distance of wells cut in 1.5 mm thick agarose gel: 1 cm. Gel and buffer same as in Fig. 2, except that Litex agarose type HSA batch 231 B electroendosmosis: Mr = −0.13 was used. Current: 2 V/cm of gel for 18 h. Staining same as in Fig. 2.

to be positive within 3 to 4 h of electrophoresis, by reading the unstained plates. To detect more weakly positive samples, and as a matter of convenience, the IEOP-plates were electrophoresed during the night (18 h) and stained the next day. This means that a diagnosis could be established within 24 h by IEOP, in contrast to EM where the diagnosis could be made the same day that the sample was received.

Specificity and sensitivity of the diagnostic rabbit antiserum

The antiserum raised against precipitates made up by antiserum to bovine rotavirus and extracts of human faeces containing rotavirus is much stronger than the original antiserum raised against bovine rotavirus (Fig. 2 and 4). In the plate shown on Fig. 4, the concentration of both antigen and antibodies was only one third of the concentrations
Fig. 4. Crossed immunoelectrophoresis (Weeke, 1973) of the same antigenic preparation as in Fig. 2. First dimension: 15 μl of antigen was electrophoresed under the same conditions as in Fig. 2. Second dimension: 5 μl/cm² of rabbit antiserum to the precipitate indicated in Fig. 2. Remaining experimental conditions the same as in Fig. 2.

Fig. 5. IEO (Grauballe et al. 1975) of different faecal extracts from patients suffering from acute gastroenteritis. Anodic wells: 5 μl of the diagnostic rabbit antiserum. Cathodic wells: 15 μl of faecal extracts. Remaining experimental conditions as in Fig. 3.

Fig. 6. Same as Fig. 5, except that the cathodic wells from the left to the right contained extracts of successively collected faecal samples from one of the experimentally infected calves (see text). Diarrhoea started at the time when the fourth sample was taken. The first sample was taken at the time of infection.

employed in the plate shown in Fig. 2. Still, the precipitate of the plate of Fig. 4 is more heavy and marked. It was also noted that the precipitate of the fast moving antigen seen in Fig. 2 was missing in Fig. 4 so that only one antigen was revealed by the diagnostic serum when it was examined by crossed immunoelectrophoresis against human faecal extracts. When the same serum was used in IEO against different human and bovine faecal extracts, up to four precipitates were detected (Fig. 5 and 6). The same result was
Fig. 7. Line immunoelectrophoresis (Kröll, 1973). 1, 1 mm thick gel containing 10 μl/cm² of solubilized human rotavirus antigen; 2, 1.5 mm thick intermediate gel; 3, 1 mm thick gel containing 3 μl/cm² of the diagnostic rabbit antiserum; 4, 1 mm thick gel containing 15 μl/cm² of human convalescent serum. Current: 2 V/cm of gel for 18 h. Remaining experimental conditions as in Fig. 2.

found in line immunoelectrophoresis against the human rotavirus antigen prepared by solubilization (Fig. 7 and 12). The specificity of the diagnostic serum is stressed by the fact that tissue culture antigen of bovine rotavirus produced precipitates of the same migration velocity as the antigen precipitated on the plate of Fig. 4, whereas control antigen failed to react with the serum (Fig. 8). Reactions in IEOP between human and bovine serum proteins were seen when 10 and 15 μl of the diagnostic antiserum were employed, whereas such reactions were absent when 5 μl was used (Fig. 9). For this reason 5 μl of the diagnostic serum was used for the detection of rotavirus in faeces by IEOP.

In attempts to further characterize the antibodies to rotavirus present in the diagnostic rabbit antiserum, a few acute and convalescent serum samples from children suffering from gastroenteritis were tested in IEOP against the solubilized human rotavirus antigen. As shown in Fig. 10, several convalescent sera reacted with the antigen. When these sera were tested by a modified IEOP technique against the same antigen preparation, three lines of identity were formed between all sera (Fig. 11). The modification of the IEOP consisted of a displacement to the left of the anodic wells, which contained alternating rabbit antiserum and different human convalescent sera, and in placing the holes close together. Furthermore, 2 h of passive diffusion were allowed before the current was applied to ensure fusion of antibodies. One of the human convalescent sera was finally tested in line immunoelectrophoresis together with the diagnostic rabbit antiserum against the solubilized human antigen mentioned above (Fig. 7). The technique was also applied to two convalescent sera from gnotobiotic calves experimentally infected with bovine rotavirus (Fig. 12). These sera were kindly supplied by Dr G. N. Woode, Institute for Research on Animal Diseases, Compton, U.K. There is one line of identity between the human convalescent serum and the diagnostic serum formed by the antigen producing the highest titre in the rabbits, i.e. the precipitate located nearest to the cathodic pole (Fig. 7). Almost the same was found in the two convalescent sera from the calves, except that the same antigen seems to be resolved into two by the calf sera (Fig. 12). The influence on the
Fig. 8. Crossed immunoelectrophoresis (Weeke, 1973). First dimension gel: 45 μl of bovine rotavirus tissue culture antigen. Second dimension gel and remaining experimental conditions as in Fig. 4.

Fig. 9. IEOP (Grauballe et al., 1975). Anodic wells A, B and C contained 5, 10 and 15 μl, respectively, of the diagnostic rabbit antiserum. Cathodic wells 1, 2 and 3 contained 15 μl of the dilutions 1:10, 1:50 and 1:100 of human serum. Remaining experimental conditions as in Fig. 3. Similar results were obtained when bovine serum was tested in the same way (not shown).

Fig. 10. IEOP (Grauballe et al., 1975). Anodic wells: 5 μl of different alternating acute and convalescent sera from patients with acute gastroenteritis. Cathodic wells: 15 μl of solubilized pooled human rotavirus antigen. Remaining experimental conditions as in Fig. 3.
Fig. 11. Modified IEOP (see text). Anodic wells: every second well contained 5 μl of the diagnostic rabbit antiserum, the rest of the wells contained 5 μl of different convalescent sera from Fig. 10. Remaining experimental conditions as in Fig. 10, except that the wells were cut close together in each row.

Fig. 12. Line immunoelectrophoresis (Kröll, 1973). 1, 1 mm thick gel containing 10 μl/cm² of solubilized human rotavirus antigen; 2, 1.5 mm thick intermediate gel; 3 and 4, 1 mm thick gel containing 15 μl/cm² of two different convalescent sera from experimentally infected gnotobiotic calves; 5, 1 mm thick gel containing 3 μl/cm² of the diagnostic rabbit serum. Remaining experimental conditions as in Fig. 7.

IEOP technique of storage and transport of stools was studied by daily repeated examinations of faecal extracts left to stand at room temperature. As shown in Fig. 13, no effect was found after seven days, which means that rotavirus antigen may still be present and native after surface mailing of stools.

**Experimental infections of calves**

The results of these experiments will be published elsewhere (A. Meyling et al. in preparation), but Fig. 6 demonstrates that the three first samples, all taken before symptoms were evident, were negative when tested by IEOP, whereas the first positive
sample coincided with the onset of diarrhoea. The samples then stayed positive for six days post infection, whereafter they became negative again (not shown on the figure). A similar pattern was found in all the experimentally infected calves.

**Buoyant density of the antigen**

Fig. 14 demonstrates that the highest concentration of antigen was found in the CsCl fraction corresponding to a density of 1.28 g/ml. But the detection of antigen starts in the fraction corresponding to a CsCl density of 1.36 g/ml.

**Electron microscopy**

Fig. 15 and 16 show the appearance and size of rotavirus found in the faecal samples. When the two figures are compared, it becomes evident that, although more laborious than the direct examination of deposit from short-term ultracentrifugation, the gradient method results in the best preserved and undamaged particles, ensuring a more certain diagnosis. The gradient centrifugation furthermore resulted in the determination of the average buoyant density of full rotavirus particles to 1.36 g/ml, although bands occurred at different densities from 1.28 to 1.37 g/ml, as reported by Rodger, Schnagl & Holmes (1975). Finally, the diagnostic antiserum failed to agglutinate human rotavirus particles.

**DISCUSSION**

The buoyant density of rotavirus found in the present work is in agreement with previous reports (Rodger et al. 1975; Kapikian et al. 1976). In these reports it is also stated that
Fig. 14. Fused rocket immunoelectrophoresis (Just Svendsen, 1973) of rotavirus antigen after isopycnic banding in a CsCl gradient (see text). Thirty µl of each fraction from the CsCl gradient were added to wells made in 1.5 mm thick agarose gel. Antibody containing gel was the same as the second dimension gel in Fig. 4. Remaining experimental conditions as in Fig. 7.
Fig. 15. CsCl fraction of a faecal sample showing a cluster of full and empty virus particles of uniform size. Only a few damaged particles are visible.
Fig. 16. Total sediment of the faecal sample with numerous damaged virus particles of different size and integrity.
the fractions of 1.28 to 1.29 g/ml of CsCl contained empty particles and particles with only one capsid layer. Flewett et al. (1974) reported that convalescent sera from calves reacted with the inner but not the outer capsid layer of human rotavirus. Considering this and the way in which the diagnostic rabbit antiserum was produced in the present work, our results, especially the fact that the diagnostic serum reacted mainly with virus antigen present in the fractions of 1.28 g/ml, seem to be consistent with these previous data.

The technique of cutting out virus precipitates from crossed immunoelectrophoretic agarose plates and the further production of antisera to the precipitated antigens have previously been used successfully at our laboratory, resulting in the achievement of monospecific and highly avid antibodies for immunoelectrophoretic analysis and diagnosis of virus antigens (Vestergaard, 1975). The reason why the diagnostic serum used in the present work, although avid and monospecific (i.e. only reacting with one antigen) when examined by crossed immunoelectrophoresis (Fig. 4), revealed up to four precipitates in IEO and line immunoelectrophoresis (Fig. 5, 6, 7 and 12), is uncertain. One explanation could be that the antigen used for immunization was contaminated with small amounts of other antigens, resulting in the production of minor amounts of antibodies to these antigens also. Such antibodies may only be detected in the more sensitive IEO or when greater amounts of antigen are used, as in the line immunoelectrophoretic investigations. The number of precipitates seems to be in agreement with the results of Middleton et al. (1976) who reported up to four precipitates in different antigenic preparations of rotavirus, using guinea pig antisera. We have demonstrated that the antigenic activity was mainly located at a density of 1.28 g/ml in CsCl gradients, and we believe that two of the four precipitates found in the present work are identical with the precipitates designated lines 3 and 4 by Middleton et al. (1976). According to these authors, lines 3 and 4 were only associated with soluble antigen. This might also explain why the antiserum used for IEO in our study failed to agglutinate virus particles when examined by immunoelectron microscopy.

The second and third samples from the left in Fig. 6 react with weak 'precipitates' near the anodic wells. Such reactions, which may confuse the reading of the IEO test, were found occasionally. To overcome this problem we found it convenient to test all specimens in parallel with 5 µl of a pool of normal rabbit serum together with the diagnostic serum.

In contrast to the results presented by Middleton et al. (1976) we found that the IEO technique was more sensitive than EM. In the dilution experiment, IEO was two to four times more sensitive than EM. We believe that the utilization of a more avid antisem is responsible for the fact that we diagnosed 13 more cases by IEO than by EM. In two cases a positive EM-diagnosis could not be confirmed by IEO.

In conclusion, we think that IEO is a convenient, rapid and reliable diagnostic method for rotavirus infections, providing that highly specific and avid antisera are available. We found that such antisera could be produced in rabbits by injections of immunoprecipitates obtained by crossed immunoelectrophoresis, thereby avoiding a number of sophisticated purification steps.

Note added in preparation. In a paper published by Tufvesson, B. & Johnsson, T. (1976b), the authors state that IEO is almost as sensitive as EM in detecting rotavirus, when guinea pig antiserum to bovine rotavirus is employed in the test. We think that the problems concerning production of antisera for IEO already discussed in the present paper may also be applied to the results given in this recent report.
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