Comparison of electron microscopy, enzyme-linked immunosorbent assay, solid-phase radioimmunoassay, and indirect immunofluorescence for detection of human rotavirus antigen in faeces

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SUMMARY Four techniques were compared for their practicability, speed, and sensitivity for the detection of human rotavirus. Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) were found to be the most sensitive means of identifying rotavirus, and, once processed, up to 40 specimens could be examined daily. Electron microscopy, although less sensitive than these techniques, had the advantage of being able to detect other viral agents present in faecal extracts. Indirect immunofluorescence failed to detect rotavirus as often as the other three methods. In laboratories where routine examination of faecal specimens from patients with gastroenteritis is required, ELISA and RIA are useful alternatives to electron microscopy.

Rotaviruses are recognised as important causative agents of acute gastroenteritis in children and the young of various other species (Flewett and Woode, 1978), and there is a special need for a sensitive and rapid method for their identification in faeces. The detection of rotavirus antigen provides evidence of infection earlier than the detection of specific immunoglobulin M or G (Yolken *et al.*, 1978) and does not require venepuncture, a practice unpopular with paediatricians and their patients. Therefore, it is important in the diagnostic situation that the method for detecting antigen is practical, sensitive, and rapid. Many techniques have been investigated in recent years in an effort to replace the electron microscope for the routine diagnosis of human rotavirus (HRV) infections, as it is generally thought that limitations on the availability of the instrument, its running costs, and the time required for the examination of each specimen make electron microscopy (EM) an unsuitable method of examining large numbers of specimens. These methods include immune electron microscopy and a fluorescent virus precipitin test (Peterson *et al.*, 1976), counter-immunoelectrophoresis (CIEP) (Middleton *et al.*, 1976; Tufvesson

and Johnsson, 1976; Birch *et al.*, 1977), indirect immunofluorescence (IIF) (Bryden *et al.*, 1976), free viral immunofluorescence (Yolken *et al.*, 1977b), a viral RNA detection technique (Espejo *et al.*, 1977), radioimmunoassay (RIA) (Kalica *et al.*, 1977), complement fixation (Zissis *et al.*, 1978), and enzyme-linked immunosorbent assay (ELISA) (Yolken *et al.*, 1977a).

Some of these tests have not provided the necessary sensitivity and reliability required for the detection of rotavirus. In this laboratory CIEP has been found to be considerably less sensitive than EM (Birch *et al.*, 1977), and many faecal extracts have been found to have anticomplementary activity, although Zissis *et al.* (1978) have reported the removal of this activity in some faecal extracts using fetal calf serum. In addition, the free viral immunofluorescence test presents considerable problems in interpretation. Detection of viral RNA as a diagnostic technique requires further evaluation but may provide a useful method for the identification of rotaviruses in faeces and their subsequent classification into subtypes. As many patients with gastroenteritis are admitted to Fairfield Hospital each winter, we have been able to compare the sensitivity of ELISA, RIA, and IIF with EM for the detection of HRV in faeces, in the hope of eliminating the need for an

electron microscope for routine examination of large numbers of specimens.

Material and methods

(a) PATIENTS AND SPECIMENS

A single faecal specimen was obtained from each of 161 patients aged 15 years and under with symptoms of gastroenteritis admitted to Fairfield Hospital between April and August 1978. Specimens were collected as soon as possible after the patient had been admitted, usually within 24 hours.

(b) PROCESSING OF SPECIMENS AND EM

Solid specimens were made to 20% (w/v) in Hanks' complete balanced salt solution and homogenised, using an MSE homogeniser (model 7700-A). Liquid specimens were processed without further dilution or homogenisation. The specimens were then centrifuged at 2000 rpm for 10 minutes, and the resulting supernate was centrifuged at 10000 g for 30 minutes; 12 ml of this clarified supernate were layered onto 1·5 ml of 45% (w/v) sucrose in 0·002 M Tris buffer, pH 7·0, and centrifuged at 35000 rpm (200000 g) for 75 minutes using an SW41 rotor in a Beckman L2 ultracentrifuge. The supernate was discarded, and the pellet was resuspended in 0·002 M Tris, pH 7·0. A drop of the resuspended pellet and a drop of the supernate obtained after centrifugation at 10000 g were separately stained with 3% phosphotungstate, pH 7, on Formvar carbon-coated copper grids and examined in a Philips 301 electron microscope.

(c) PREPARATION OF HYPERIMMUNE ANTI-
    ROTAVIRUS SERUM

Eighty millilitres of a human faecal specimen, rich in rotavirus particles, was processed as in (b) above. The pellet obtained after centrifugation at 200000 g was resuspended in 0·002 M Tris and layered onto 51% (w/v) CaCl in cellulose nitrate tubes. The specimen was spun at 200000 g for 16 hours, and the visible band containing complete particles by EM was collected and dialysed for 48 hours against three 2-litre changes of 0·01 M phosphate buffered saline, pH 7·2 (PBS). One millilitre of the so-treated specimen was mixed with an equal volume of Freund's complete adjuvant and then inoculated subcutaneously into five regions on the chest and stomach area of an adult rabbit. This inoculation procedure was repeated four times over an 11-week period, and the rabbit was exsanguinated.

(d) PREPARATION OF ANTI-HUMAN
    IgG-PEROXIDASE CONJUGATE

A pure human immunoglobulin G (IgG) immunosorbent was prepared following the method of

Avrameas and Ternynck (1969). Briefly, 2·5% aqueous glutaraldehyde was added to 250 mg human IgG (Cohn Fraction II, CSL, Melbourne, Australia) which had been further purified by ion-exchange chromatography (Purcell *et al.*, 1973). Two millilitres of the resulting polymer were incubated for 16 hours at 4°C with 8 ml of goat anti-human IgG (Hyland, Travenol Laboratories, Melbourne, Australia) followed by three washings with normal saline (0·85% NaCl) and elution of purified anti-human IgG with 0·1 M glycine-HCl buffer, pH 2·8. This volume was reduced to approximately 1·5 ml by negative-pressure dialysis (Sartorius Membrane Filter, Göttingen, Germany), followed by dialysis against three changes of 0·01 M carbonate buffer, pH 9·5, overnight at 4°C.

One millilitre of purified anti-human IgG containing approximately 5 mg per ml of protein was conjugated to 3 mg horse-radish peroxidase (HRPO type VI, Sigma Chemical Co, St. Louis, Mo, USA) following the method of Nakane and Kawaoi (1974). The conjugate was stored at −20°C in 100 μl aliquots in the presence of 10 mg bovine albumin (Cohn Fraction V, CSL, Melbourne, Australia) per ml.

(e) ELISA

The double antibody sandwich-immunoglobulin ELISA (Voller *et al.*, 1978) was used for the detection of antigen. The wells of a microtitre plate (type 1-220-24, Cooke Engineering Inc, Alexandria, Va, USA) were coated with 100 μl of a 1:1000 dilution in PBS of hyperimmune rabbit anti-rotavirus serum, and the plate was incubated at 4°C for 5 hours. The plate was then washed six times with PBS containing 0·05% (w/v) polyoxyethylene monolaurate (PBS/Tween 20). Each well was filled to capacity with 1% (w/v) bovine albumin in PBS, and the plate was incubated overnight at 4°C. Next day the plate was washed as above, and 20 μl of faecal supernate, obtained after clarifying centrifugation at 10000 g, was added to each of four separate wells, and the plate was incubated for 150 minutes at 4°C followed by 90 minutes at 37°C. (A known rotavirus-positive and rotavirus-negative faecal extract was included on every test plate.) The plate was again washed as above, and 50 μl of a 1:80 dilution in PBS of human serum negative for rotavirus antibody was added to the first two antigen-containing wells, and 50 μl of a 1:80 dilution in PBS of human serum positive for rotavirus antibody was added to the remaining two antigen-containing wells. (These human sera were acute and convalescent serum specimens obtained from a child with rotavirus gastroenteritis, documented by seroconversion and electron microscopy.) The plate was then incubated for 60 minutes at 37°C and again washed as above, and 50 μl of a 1:200 dilution in PBS of anti-human IgG-HRPO conjugate

was incubated in each well for 1 hour at 37°C. After a further six washes in PBS/Tween 20, 50 μl of *o*-phenylenediamine (*o*-pd, BDH Chemicals Ltd, Poole, UK) substrate was added to each well, and the plate was incubated at room temperature in the dark. (The substrate was prepared by adding 2 ml of 1% (w/v) *o*-pd in methanol to 98 ml distilled water containing 200 μl of 3% (v/v) H_2O_2). The reaction was stopped after 15 minutes by the addition of 50 μl of $8N$ H_2SO_4 to each well.

(*f*) RADIOLABELLING OF IgG
IgG was precipitated from hyperimmune rabbit anti-rotavirus serum at 4°C by the dropwise addition of an equal volume of saturated ammonium sulphate at 10-second intervals with continuous gentle stirring. Complete precipitation was allowed to occur over 1 hour at 4°C with continuous stirring. The precipitate was pelleted by centrifugation at 2000 rpm for 15 minutes and resuspended in distilled water to the original volume. The above procedure was then repeated. The twice-precipitated globulin was dialysed against distilled water for 1 hour at 4°C, followed by dialysis against 0·005 M phosphate buffer, pH 8·0, containing 0·1% sodium azide, for two days at 4°C. The IgG was labelled with ^{125}I using the modified method of Hunter and Greenwood described by Purcell *et al.* (1973).

(*g*) RIA
The solid-phase RIA was a modification of that described by Purcell *et al.* (1973). One hundred microlitres of hyperimmune rabbit anti-rotavirus serum, diluted 1:1000 in 0·2 M PBS, pH 7·4, containing 0·1% sodium azide, was added to each well of a polyvinyl microtitre plate (Cooke No. 1-220-24) and incubated for 4 hours at 4°C. The plate was washed twice with normal saline, 200 μl of 1% (w/v) bovine albumin in normal saline was added to each well, and the plate was incubated overnight at 4°C. After two washes with normal saline, 25 μl of faecal extract, obtained after clarification by centrifugation at 10000 *g*, was added to duplicate wells, and the plate was incubated overnight at 4°C. (Known rotavirus-positive and -negative faecal extracts were included on every plate tested.) The plate was then washed six times with normal saline, and 50 μl of iodinated anti-rotavirus

IgG was added to each well. After incubation for 4 hours at 37°C, the wells were washed six times with normal saline, and each well was separated with a hot wire using the method of Boarer *et al.* (1977). The wells were transferred to a counting tube, and the residual radioactivity was determined using an automatic gamma counter.

(*h*) RIA NEUTRALISATION TEST
Fifty microlitres of faecal extract was incubated overnight at 4°C with 50 μl each of PBS, pre-infection guinea-pig serum diluted 1:200 in PBS, and post-infection guinea-pig anti-rotavirus serum diluted 1:200 in PBS; 50 μl of each mixture was then added to duplicate wells of an antibody-coated plate and tested as above. A specimen was considered to be neutralised, and therefore positive for rotavirus, if the total counts in the post-infection serum wells were reduced by at least 50% compared with the PBS and pre-infection serum wells.

(*i*) INDIRECT IMMUNOFLUORESCENCE
The method was based on that of Bryden *et al.* (1976). Tissue culture plates (No. STC-1, Disposable Products, Adelaide, Australia) were seeded with 2×10^5 LLC-MK2 cells per well in 0·1 ml of 10% (v/v) newborn calf serum in medium 199 containing penicillin and streptomycin. When the cells had formed a monolayer, the medium was removed and the plate was washed twice in PBS; 20 μl of faecal extract, clarified by centrifugation at 10000 *g*, was added to each of three wells, and 0·2 ml of maintenance medium, consisting of 2% (v/v) fetal calf serum in BME with penicillin and streptomycin, was immediately added. The plate was sealed, placed in a microtitre plate basket (Dynatech, Alexandria, Va, USA), and spun at 1500 rpm (500 *g*) for 90 minutes. It was then removed and incubated at 37°C for 16 hours, and washed twice in PBS, and the cells were fixed at room temperature by flooding each well for 5 minutes with methanol which had been stored at −70°C. Fifty microlitres of a 1:50 dilution in PBS of hyperimmune rabbit anti-rotavirus serum was added to two of the test wells and 50 μl of PBS was added to the third well. The plate was incubated at 37°C for 45 minutes followed by two washes in PBS; 50 μl of sheep fluorescein-labelled anti-rabbit immuno-

Table *Results of comparison of ELISA, RIA, EM, and IIF for their ability to detect human rotavirus (HRV)*

Technique	RIA	ELISA	EM	IIF
No. of specimens examined	161	161	161	161*
HRV positive	89 (55·3%)	88 (54·7%)	72 (44·7%)	46 (28·6%)
HRV negative	72	73	89	107

*8 specimens were toxic.

globulin (Wellcome Reagents Ltd, Beckenham, Kent, UK) diluted 1:15 in PBS was added to each well and the plate was incubated at 37°C for 45 minutes. The plate was washed twice in PBS, and the cells were counterstained by flooding with dilute Evans Blue (1:150000) for 30 seconds and rinsed once in distilled water. The cells were examined by inverting the plate and reading under low power (10× objective, 8× eyepiece) with vertical illumination.

Results

(a) ELECTRON MICROSCOPY

Of 161 specimens studied, 72 (45·3%) contained rotavirus by EM (see Table). Virus particles had typical morphology, being about 70 nm in diameter with characteristic outer radial 'spikes'. Of the 72 EM-positive specimens, 18 were reported as negative before concentration by centrifugation at 200000 *g*. There were no specimens positive by EM that were not positive by ELISA or RIA. Adenoviruses were visualised in 10 specimens, small virus-like particles, approximately 30 nm in diameter, were seen in two specimens, one of which also contained rotavirus, and corona virus-like particles were seen in one specimen.

(b) ELISA

Of 161 specimens studied, 88 (54·7%) contained rotavirus by ELISA (see Table). Only faecal extracts clarified at 10000 *g* were tested for the presence of rotavirus antigen. All specimens containing viruses other than rotavirus by EM were negative by ELISA. The specimen containing rotavirus and 30 nm particles was positive by ELISA. The positive HRPO/*o*-pd reaction gave a characteristic orange colour, easily distinguishable from the colourless negative reaction. All plates were read by eye 15 minutes after the addition of substrate. Longer periods of incubation often resulted in the appearance of background colour, making interpretation difficult. It was found practical to perform the ELISA procedure on two plates on any one day. Allowing for the inclusion of suitable controls on each plate, this enabled 42 specimens to be examined daily if required.

(c) RIA

Of 161 specimens examined, 89 (55·3%) were positive by RIA (see Table). Only specimens clarified by centrifugation at 10000 *g* were tested. Initially, 99 of the 161 specimens gave counts per minute (cpm) greater than or equal to 2·1 times the mean cpm of the negative controls. However, the RIA neutralisation test eliminated 10 of these specimens. Each of

these RIA false-positive specimens was negative by EM, ELISA, and IIF.

(d) IIF

Of 161 specimens examined, 46 (28·6%) were positive by IIF (see Table). There were no specimens positive by IIF that were not positive by any of the other three techniques. The fluorescence seen was similar to that described by Bryden *et al.* (1976); fluorescence was always cytoplasmic and had a distinctive granular appearance. The number of fluorescing cells varied from specimen to specimen, usually from a minimum of 1% fluorescing cells to a maximum of 10%. Rotavirus fluorescence was so typical that as few as one or two fluorescing cells in the whole monolayer could be identified with confidence and the specimen considered positive. Faecal extracts were toxic to the cells in about 10-15% of specimens. Usually this toxicity resulted in altered morphology of the cells under the light microscope. Eight specimens (5%) were so toxic as to make interpretation impossible.

Discussion

Of the four tests used to detect rotavirus antigen, ELISA and RIA were almost equal in sensitivity, and both were more sensitive than EM and IIF. In addition, both RIA and ELISA were consistently able to detect viral antigen in the supernate obtained after 10000 *g* clarifying centrifugation, whereas concentration of rotavirus by ultracentrifugation was at times required before virus could be visualised by EM. IIF has been reported as being nearly as sensitive as EM for the detection of rotavirus (Bryden *et al.*, 1976), but in our hands it often failed to detect virus when EM was positive. There may be several reasons for this, namely, that damaged rotavirus particles may not be detectable by IIF owing to their inability to enter cells, that the line of LLC-MK2 cells used by both laboratories may differ in sensitivity to rotavirus due to differences in passage history, or that toxic specimens altered the sensitivity of cells to the infecting virus without significantly altering their morphology. In addition, Bryden *et al.* used centrifugation providing 1200 *g* at the plate, whereas our method utilised 500 *g*. This difference may have some effect on the sensitivity of the two systems. Bryden *et al.* (1976) reported that primary and secondary human embryonic kidney and primary chicken kidney cells could be used in the test but were not as sensitive as LLC-MK2 cells. Preliminary results in this laboratory show that CV-1 cells are also not as sensitive as LLC-MK2 cells (C. Birch, unpublished results).

Detection of antigen by ELISA was both simple and sensitive, and a result could be obtained within

one day. Plates coated with rotavirus-specific antibody in the presence of bovine albumin can be stored for several weeks at 4°C ready for use. Three important factors influence the success of the ELISA system for detecting rotavirus: firstly, the initial use of rotavirus-specific coating antibody ensures that only rotavirus antigen attaches to the solid-phase, and this, together with bovine albumin, eliminates non-specific adsorption of other agents present in the faecal extract. In our ELISA system, the coating antibody was probably directed specifically against a rabbit rotavirus because the rabbit used for production of hyperimmune serum had initial (preinoculation) low levels of serum antibody. Nevertheless an optimal dilution of 1:1000 of this serum was capable of binding rotaviruses of human origin. Although rotavirus can be adsorbed directly to the plate by incubation overnight at 4°C in pH 9·5 carbonate buffer, other agents, viral or otherwise, present in the faecal extract also adsorb under these conditions, leading to non-specific false-positive results (C. Birch, unpublished observations). Secondly, the washing procedure is important, and failure to remove even minute amounts of residual conjugate can lead to false-positive reactions on the addition of substrate. For washing, we used a semi-automatic medium dispenser/aspirator designed and made at this hospital. This allows eight wells to be aspirated and fresh medium to be added at one time, and the plate can be washed satisfactorily in about 3 minutes. Finally, the conjugate must be both sensitive and specific. The conjugate prepared in this laboratory could be used at a dilution of 1:400 although in practice it was used at 1:200. It can be stored at −20°C for long periods—there has been no reduction in activity over four months of testing—and it can also be frozen and thawed several times without loss of activity. The use of coating antibody and the incubation of faecal extracts with both acute and convalescent human sera on the same plate alleviates the need for confirmation of positive results with a neutralisation test. In no case was ELISA positive when RIA was negative.

RIA, although requiring longer to perform than ELISA, has been adapted to fit into the schedule of a busy laboratory. As with the ELISA test, plates can be coated with specific antibody and stored for several weeks in the presence of 1% bovine albumin. The plates can be washed using the medium dispenser/aspirator described above. A neutralisation test is required to eliminate potential false-positive results.

Because ELISA and RIA are similar in sensitivity and both are more sensitive than either EM or IIF for the detection of rotavirus antigen, either of these techniques is the most suitable currently available for the diagnosis of rotavirus infections. It is not surprising that ELISA and RIA are more sensitive as both should be capable of detecting both whole virus and/or disrupted viral antigen. Middleton *et al.* (1977) reported that RIA was as sensitive as EM for the detection of virus in stools, and Yolken *et al.* (1978) reported that ELISA was at least as sensitive as EM and RIA. The need for the presence of complete virions for EM and IIF is a disadvantage for their detection as often virus particles are in poor condition in specimens that have been collected several days after the onset of symptoms. Thus, they are sometimes difficult to identify positively by EM and are probably non-infectious for LLC-MK cells. The undeniable advantage of EM is that it is essential in the investigation of epidemic diarrhoea or in surveys of an aetiological nature as only a proportion of aetiological agents of gastroenteritis are known. ELISA, RIA, and IIF are specific for single agents only. The IIF technique is probably best applied in the selective identification of different rotavirus serotypes using the neutralisation technique outlined by Thouless *et al.* (1978).

The disadvantages with RIA are the hazards involved in handling radioactive materials and the short half-life of ^{125}I. Recently, Voller *et al.* (1978) have reported that *o*-phenylenediamine may have mutagenic properties, and the need for handling dangerous materials may therefore be a problem in the ELISA test. However, alternative enzyme/substrate combinations can be used. Alkaline phosphatase conjugates, with paranitrophenol phosphate as a substrate, are reputedly safe. Provided that care is taken with the handling of reagents, we believe that the ELISA test is a simple, reliable, and sensitive replacement for EM and, in laboratories where radioactive counting apparatus is not available, is also a useful alternative to RIA for the detection of HRV.

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