Enzyme-Linked Immunosorbert Assays Based on Polyclonal and Monoclonal Antibodies for Rotavirus Detection


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Received 5 July 1983/Accepted 27 October 1983

We describe two enzyme-linked immunosorbent assays for rotavirus antigen in feces, which were designed to be as sensitive and specific as possible, and easy to use anywhere. Both are indirect methods, using the antibody capture method, but the second assay utilizes a rotavirus group-specific monoclonal "detecting" antibody instead of the hyperimmune polyvalent guinea pig antisera used in the first assay. Both tests were found to be more sensitive than electron microscopy for detecting virus. To develop these tests, solid phase, antiserum production methods, treatment of the test antigen with EDTA, substrate, stability of reagents, and the need for confirmatory "blocking" tests were all examined. The first assay described is that used at present by the World Health Organization for their worldwide diarrheal disease control program.

The enzyme-linked immunosorbent assay (ELISA) technique has been adopted by the World Health Organization as the standard method for the detection of rotavirus antigen in stools. Much has been written about the role of this technique in diagnosing rotavirus diarrhea (16), and its value is now firmly established. For use in developing countries, tests for rotavirus must be highly sensitive, specific, simple to perform, inexpensive, include a confirmatory test for positive reactions, and have a reasonably long shelf life. Most tests previously described for rotavirus detection do not satisfy all of these criteria. In developing countries the main use of diagnostic tests for rotavirus is to provide information about epidemiology (in so far as the World Health Organization diarrheal disease control program is concerned), and for this reason we do not consider speed a first priority.

In this paper we describe the development of two similar ELISAs based on polyclonal and monoclonal antibodies, respectively, for rotavirus infections.

MATERIALS AND METHODS

Solid phase. Many different microtiter plates from different makers were tested for their usefulness as solid phases. These included plates made of polystyrene plastics, some of which had been irradiated by the manufacturers with gamma rays from a 60Co source, and those made of polyvinyl. Their efficiency in binding both monoclonal and polyclonal antibodies was determined by comparing optical density (OD) curves derived from ELISA titration tests for rotavirus antigen as described below.

Capture antibody. Capture antibody was, in most cases, a hyperimmune rabbit anti-rotavirus serum ("720") raised against complete and incomplete rotavirus particles of subgroups I and II (derived from the feces of infected children) by the method of Beards (2). A hyperimmune serum from guinea pigs immunized in the same way was also used. The use of the monoclonal antibody as a capture antibody was also investigated; for this, immunoglobulin G (IgG) was separated from ascitic fluid by gel filtration. With three-dimensional chessboard titration, optimum pH, the molarity of NaCl in the phosphate buffer, and globulin concentration for its attachment to the solid phase were determined by using the single-sandwich ELISA test described below.

Antigens. Antigens were 10% (vol/vol) fecal extracts in phosphate-buffered saline (PBS) containing 320 mg of crystalline and 10 mg of amphotericin B per ml, clarified by low-speed centrifugation and stored at −20°C. Immediately before use they were diluted 1:4 in PBS (pH 7.4) containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20) and 0.01 M EDTA disodium salt (PBS-T-EDTA). Specimens were obtained from the United Kingdom and other countries during 1980 to 1983, and to date more than 3,000 specimens have been tested.

"Detecting" antibody. Three sera were evaluated: (i) a hyperimmune guinea pig anti-rotavirus serum obtained from the National Institutes of Health, Bethesda, Md., produced under contract for the World Health Organization; (ii) a guinea pig serum similar in titer and specificity produced by us by the same method as that used to produce the capture antibody; (iii) a rotavirus group-specific monoclonal antibody produced from the spleen cells of BALB/c mice which had been immunized with purified complete and incomplete rotavirus particles of a stool-derived human rotavirus belonging to subgroup II. Mice were injected subcutaneously with 50 µl of purified human rotavirus in 250 µl of PBS, pH 7.6, and mixed with an equal volume of Freund incomplete adjuvant. After 2 weeks a further dose of 12.5 µl of purified human rotavirus serotype 1 was given as above. At 2 weeks after this last dose, tail vein blood was assayed for rotavirus-specific antibodies as before. A 500-µl amount of PBS containing 12.5 µl of purified human rotavirus serotype 1 was injected intravenously. After 4 days mice were killed by cervical dislocation, and the spleens were removed aseptically. The spleen cells were hybridized with P3/NS1/1-Ag 4-1 myeloma cells (kindly provided by J. S. Porterfield, Oxford University, United Kingdom), using the method described by Galfre et al. (8). Screening was performed by ELISA, and cloning was carried out by limiting dilution with thymocyte
feeder layers. The monoclonal antibody used in the test was obtained by injecting 10^7 hybridoma cells into the peritoneal cavity of pristane-primed BALB/c mice and harvesting the resultant ascitic fluid 14 to 21 days later.

**Conjugates.** For the polyclonal test an affinity-purified goat anti-guinea pig IgG (H + L)-alkaline phosphatase conjugate obtained from Kirkegaard and Perry, Gaithersburg, Md., was chosen and used at a dilution of 1:200. For the monoclonal test the conjugate was an affinity-purified goat anti-mouse IgG labeled with horseradish peroxidase (Kirkegaard and Perry) and used at a dilution of 1:1,000. In both cases the optimal dilution of the conjugates was determined by duplicate chessboard titrations, using fecal extracts containing high and low concentrations of rotavirus antigen.

**Direct conjugation of detecting monoclonal antibodies.** IgG was purified from ascitic fluid by ammonium sulfate precipitation and affinity chromatography on a protein A-Sepharose column. The IgG-containing fractions were detected by adsorption of light at 280 nm, and their identity was confirmed by precipitation with a rabbit anti-mouse globulin antiserum in a gel diffusion test (7). Three different methods of conjugating the purified IgG with alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.), horseradish peroxidase (Daiichi), or both were tried. To a mixture of enzyme and antibody 0.2% glutaraldehyde was added. After being mixed for 18 h at 4°C, it was dialyzed against phosphate buffer as previously described (1). This method was used to make alkaline phosphatase and horseradish peroxidase conjugates. The periodate method already described (11) was used for alkaline phosphatase conjugation. The succinic ester linkage method of Nilsson et al. (12) was used to conjugate peroxidase.

**Substrates.** For alkaline phosphatase conjugates the substrate was p-nitrophenyl phosphate disodium (5-mg tablets; Sigma) in diethanolamine buffer, pH 9.8, at a concentration of 1 mg/ml. For peroxidase conjugates three substrates were evaluated: (i) orthophenylamido diamide (OPD) (Miles Laboratories) at 0.4 mg/ml, (ii) 3,3',5,5'-tetramethylbenzidine (TMB) (Miles Laboratories) at 0.1 mg/ml, and 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS) (Sigma) at 0.4 mg/ml. For OPD a 0.1 M citrate phosphate substrate buffer (pH 6.0 to 6.1) was used, for TMB a 0.1 M sodium citrate-acetate buffer (pH 6.0 to 6.1) was used, and for ABTS a 0.1 M citrate-phosphate buffer (pH 4.0) was used. The pH of each buffer was critical. If, using TMB or OPD, the pH was more than 6.3 and less than 5.9, considerable loss of sensitivity resulted. At >6.6 and <5.6 pH there was no reaction. TMB is insoluble in water and so was first dissolved in dimethyl sulfoxide (2 mg in 20 ml). Hydrogen peroxide (30% [vol/vol]) or urea hydrogen peroxide (BDH Ltd., Poole, United Kingdom) was added to each buffer to a concentration of 1.3 mmol/liter. Urea hydrogen peroxide is more stable: good quality hydrogen peroxide is not always readily available in countries with tropical climates.

The effect on the sensitivity of ELISA with these substrates was determined by performing comparative titrations and plotting curves of optical density versus conjugate dilution. For this determination doubling dilutions of conjugate in PBS were placed in microtiter plates, and constant amounts of substrate were added to each well. The zero value given by the spectrophotometer was adjusted for each substrate by using a reading from a well to which no conjugate had been added.

**Polyclonal test procedure.** For the screening test irradiated polystyrene microtiter plates (Nunc 239454) were coated with hyperimmune rabbit anti-rotavirus serum 720 diluted 1:10,000 in 0.1 M carbonate-bicarbonate buffer, pH 9.8, and kept overnight at 4°C or for 2 h at 37°C. Fecal extracts (ca. 10% in PBS containing 320 mg of crystalline and 10 mg of amphotericin B per ml) were diluted 1:4 in PBS containing 0.1% Tween and 0.01 M EDTA, and 100 μl was placed in each well of the microtiter plates. (The antibiotics were not necessary for the ELISA test but were added because we were also trying to isolate virus in tissue culture.) The detecting antibody was one of the hyperimmune guinea pig antisera described above and was used diluted 1:10,000 in PBS-Tween containing 1% bovine serum albumin. The final layer was alkaline phosphatase-conjugated with anti-guinea pig antibody diluted 1:800 in PBS-T-T-bovine serum albumin. The detecting serum and conjugate incubations were each for 2 h at 37°C. p-Nitrophenyl phosphate disodium (1 mg/ml) in diethanolamine buffer, pH 9.8, was used as the substrate. Reactions were stopped by adding 50 μl of 3 M NaOH to the 100 μl of substrate in each well. Controls included known positive and negative samples, two wells containing no coating antibody, two wells to which only substrate had been added, and two wells to which substrate and conjugate had been added. ODs were read on a Dynatech MR580 Microelisa spectrophotometer set at 405 nm. The screening test was considered positive when the mean ODS in the wells were \( E_{\text{op}}^{25cm} \geq 0.1 \). The wells containing substrate only were used to adjust the zero value given by the instrument.

No samples were reported as positive until they had been tested in duplicate in a confirmatory blocking test. This test was similar to the screening test except that the samples were diluted in PBS-T-EDTA and pre- and postimmune goat anti-rotavirus serum (obtained from the National Institutes of Health, produced under contract for the World Health Organization) and used at dilutions of 1:40. This blocking test was considered positive if the ODS in the two wells containing postimmune serum were >50% lower than the wells containing preimmune goat serum. The 1:40 dilution of goat antiserum was insufficient to block some strongly positive samples, and the latter were retested at a 1:40 dilution of the 10% (vol/vol) antigen instead of the initial 1:4 dilution.

**Double-sandwich ELISA, using monoclonal detecting antibody.** The double-sandwich ELISA was performed exactly as the polyclonal test, except that the detector antibody was monoclonal antibody produced in the peritoneal cavity of a mouse as described above and had a working titer of 1:10,000; the conjugate and substrate were from the TMB-peroxidase system described above. Before the addition of substrate, the plates were washed once in substrate buffer (without substrate); this reduced the background reaction (9). The reactions were stopped with the addition of 50 μl of 1 M HCl per well.

**Single-sandwich ELISA, using monoclonal antibody only.** Microtiter plates were coated with monoclonal IgG at dilutions of 1:30,000 and 1:300,000 in 0.1 M phosphate buffer, pH 8, and containing 1.0 M NaCl. Rotavirus-positive specimens in 0.1% phosphate buffer, pH 8, of feces diluted as above in PBS-EDTA buffer from 1:10 to 1:1,000 were allowed to react overnight at 4°C. The next day after washing as above, serial dilutions of conjugated monoclonal antibody were added and allowed to react for 2 h. After the wash, substrate was added. After optimal dilutions (1:100,000 for counting serum and 1:100 for conjugate) had been thus determined, this test was performed in parallel with the polyclonal test on all specimens of feces for diagnosis in the winter of 1981 to 1982. Monoclonal antibodies conjugated...
with alkaline phosphatase by using the glutaraldehyde method and with peroxidase by using the N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and periodate methods were evaluated and compared.

Effect of EDTA. Beards and Bryden have already given a preliminary description of the effect of EDTA when incorporated into the antigen buffer for rotavirus group-specific ELISA (3). In this study the effect of 0.01 M EDTA on four samples, including one sample from a neonate which had a high calcium content (7) was examined by performing parallel titrations, with and without EDTA, and plotting OD curves.

Stability of reagents. Our objectives in this study included the development of an ELISA kit with an efficiency that was not impaired by shipment to developing countries in which adverse weather conditions, storage, and handling might present a problem. Two experiments were performed to investigate this. Complete kits were air freighted to India and Australia from which they were returned to our laboratory unopened. They were then used in parallel with kits that had been prepared at the same time but stored at 4°C. (We thank V. Mathan and I. Holmes for their kind cooperation.) Reagents considered the most delicate and critical to the test (the conjugate and detector antibody) were stored for 2.5 weeks at 40°C in the presence and absence of sodium azide 0.01% and tested in parallel with reagents that were kept frozen.

To determine the stability of the individual components for the polyclonal and monoclonal tests, trials were undertaken to determine the half-life of each reagent in the polyclonal ELISA at different temperatures. The reagents were divided into adequate volumes for use during the trial and placed in incubators at 24, 37, and 45°C. Throughout the duration of its trial each reagent was tested weekly, using standardized incubation periods, against samples of the same batch of reagents which had been stored at 4 or 20°C as appropriate. The trials were continued for 34 to 41 days or until the activity of the reagent was less than 50% of its original value.

Use of precoated and dried plates. To simplify the test procedure the stability and efficiency of precoated and dried plates was investigated. Nunc 239454 microtiter plates were coated by Seward Laboratories Ltd. with rabbit anti-rotavirus serum 720 for 2 h at 37°C as described above. They were then washed with distilled water, dried for either 1 or 2 h at 40°C, and sealed individually with aluminum foil, enclosing a desiccant. After storage for 2 weeks they were tested in parallel with freshly coated plates. They were also tested after being sent by airmail to laboratories in India, Bangladesh, and Australia from which they were returned by airmail unopened.

Subgrouping of rotaviruses. Subgrouping was performed by the ELISA method of Thouless et al. (13).

Electron microscopy. Electron microscopy was used as the reference test throughout the investigation. After clarification by low-speed centrifugation, the specimens were concentrated by using ammonium sulfate precipitation, absorbed onto carbon-Formvar-coated grids, and negatively stained with potassium phosphotungstate (pH 7.0).

RESULTS

Figure 1 illustrates the efficiency of the ELISA reaction in a number of different microtiter plates from different makers; more plates were tested than are shown here, but the results shown are representative of the different types of plates studied. Irradiated polystyrene plates had a significantly higher antibody binding capacity than the other plates tested. Not shown are results from some brands of plates, fortunately few, whose behavior was variable and unpredictable. Pretreatment of plates for 1 h with 2% glutaraldehyde, followed by washing in PBS, raised the performance of the nonirradiated polystyrene plates to about that of the polyvinyl plates (this procedure was suggested to us by the late Raoul Scherrer). It had no beneficial effect on irradiated polystyrene plates.

“Edge effects” (wells along the edge giving different results from inner wells) were sometimes observed with polyvinyl but never with irradiated polystyrene plates. One could always use the whole polystyrene plate.

Figure 2 illustrates OD versus dilution curves given by a sample of clarified fecal suspension, using a goat anti-rotavirus serum obtained from the National Institutes of Health, a hyperimmune guinea pig antiserum raised against incomplete rotavirus particles, the rabbit 720 antiserum, and the monoclonal antibody as capture antibody at dilutions of 1:10,000. The same sera (at the same dilution) used as detecting antibody gave very similar curves. It can be seen that by far the best results were given by monoclonal antibody, although the endpoint obtained with rabbit serum 720 was not greatly different. Higher concentrations of serum gave no advantage when used either as capture or detecting antibody.

Figure 3 illustrates the relative sensitivities of the polyclonal and double-sandwich monoclonal test when used to detect the same antigens. Open symbols indicate that subgroup I rotaviruses were used as antigens, and closed symbols represent subgroup II antigens. No attempt was made to standardize the concentrations of virus in the fecal samples because we had no precise non-serological method for determining content of specific virus protein. Thus, the differences obtained with each test for each subgroup do not necessarily reflect any differences in sensitivity in detecting
antigen of different subgroups. However, the greater sensitivity of the double-sandwich monoclonal test can be seen. It could detect both of these two antigens even when they were diluted 1:500; the polyclonal test, on the other hand, could not detect them beyond a 1:8 to 1:16 dilution.

Figure 4 illustrates curves of OD versus peroxidase conjugate concentration, obtained with three different substrates (OPD, TMB, and ABTS). The use of TMB gave an approximately four- to eightfold increase in the sensitivity of the test. No significant difference in sensitivity was observed between either OPD or ABTS.

No effect on the monoclonal antibody was observed when stored in the presence of 0.01% sodium azide for 2.5 weeks, but the conjugate lost some activity (0.3 OD units) when sodium azide was not included, possibly because of bacterial contamination (but this could not be confirmed).

Figure 5 illustrates the effect of adding 0.01 M EDTA to antigen buffer. This caused a reproducibly higher OD reading and antigen dilution endpoint, observed with many samples. This effect was demonstrated in both the monoclonal and polyclonal test and also with a commercially available one (3). Sample d was a neonatal specimen which had a high calcium content, demonstrating that the concentration of EDTA used was sufficient to chelate the calcium in these samples. This sample also gave a prozone reaction; this was observed with many other samples but only when EDTA was included and might perhaps be a result of the high calcium level present in the low dilutions of antigen. However, increasing the concentration of EDTA 10-fold did not remove the prozone.

Figure 6 illustrates OD versus antigen dilution curves obtained with precoated dried plates and freshly coated plates. The freshly coated plates did not give results that were significantly better. Table 1 gives the estimated half-lives of the test reagents. All were stable for at least 1 month at 37°C, apart from reconstituted conjugate which deteriorated more quickly. The kits that were sent by air freight to Australia and India and returned performed the same as the control kit.

False-positive reactions. The incidence of false-positive reactions appears to depend on the reagents used. Brandt et al. (4) reported that with their ELISA they had 73% false-positive results. Through the courtesy of A. Z. Kapikian the same reagents were made available to us, and we too found many positive reactions which could not be confirmed by electron microscopy or blocked by specific antisera or fecal specimens obtained through one winter. Such false-positive reactions occurred with both our polyclonal and monoclonal test, but they were very rare (on the order of 1%). However, few of the specimens tested came from babies, whose feces according to several personal communications, give more false-positive results than do specimens from older children. No false-positive results were obtained throughout one winter with over 300 samples of feces tested by the single-
sandwich monoclonal test. However, a false-positive result was generated when serum containing rheumatoid factor in high titer was mixed into a rotavirus-negative fecal. A blocking test was therefore always used to confirm positive results, using pre-inoculation serum from the immunized animal as the control. Although hyperimmune goat serum was not as useful as rabbit serum for coating plates, it was very effective as a blocking antibody, as was the rabbit serum. Occasionally, a sample of feces needed to be diluted 10-fold to demonstrate blocking; one extremely virus-rich specimen had to be diluted 1:100. Blocking tests could be performed in the wells of the test plate and did not have to be performed separately. No difference in OD was observed when 22 samples were tested in parallel by both methods.

Figure 7 is an electron micrograph of the reaction between the monoclonal antibody and a subgroup 1 serotype 2 rotavirus suspension. (This was a different subgroup from that used to produce the monoclonal antibody.) Antibody adhered only to incomplete particles. This and positive ELISA performed with rotaviruses from rabbits, mice, calves, lambs, pigs, monkeys (SA11), and horses demon-

![Figure 4](image_url)

**FIG. 4.** Peroxidase conjugate dilution curves using three substrates. Symbols: ●, TMB; O, OPD, □, ABTS. The use of TMB gives an approximately four to eightfold increase in sensitivity with this test.

![Figure 5](image_url)

**FIG. 5.** The effect of the inclusion of 0.01 M EDTA (disodium salt) into the buffer used to double dilute four antigens (a through d). Symbols: O, with EDTA; ●, without EDTA. Sample (d) was a neonatal sample with a high (Ca^{2+}) content, (1.65 mM/ml, direct cresolphthalein complexone procedure). For these samples greater sensitivity was reproducibly obtained after inclusion of EDTA.

![Figure 6](image_url)

**FIG. 6.** Comparison of plates freshly coated with antibody (720 at 1:10,000) and precoated dried plates when used to detect doubling dilutions of the same antigen. Symbols: ●, freshly coated, wet plates; O, dried at 40°C for 1 h; □, dried at 40°C for 2 h. Only an insignificant drop in sensitivity was observed after drying.

### Table 1. Half-life in days of polyclonal test reagents at various temperatures

<table>
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<th>24°C</th>
<th>37°C</th>
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<tr>
<td>720</td>
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<td>&gt;41</td>
<td>20</td>
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<tr>
<td>Hyperimmune guinea pig</td>
<td>&gt;34</td>
<td>&gt;41</td>
<td>&lt;20</td>
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<tr>
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<td>Substrate tablets&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> Sigma Chemical Co., St. Louis, Mo.

<sup>b</sup> NT, Not tested.
strated the wide specificity of the monoclonal antibody for the rotavirus group antigen (15). Further experiments (to be reported elsewhere) demonstrated that the monoclonal antibody is IgG of subclass 2b (as assessed by fractionation on a protein A-Sepharose column) and reacts only with the major inner capsid polypeptide of the rotavirus particle as determined by immune precipitation and polyacrylamide gel electrophoresis.

**Single-sandwich direct ELISA, using monoclonal antibody only.** The standard sodium carbonate-hydrogen carbonate buffer was found to be useless for attaching the monoclonal antibody, and 0.1 M phosphate buffer (pH 8) containing 1.0 M NaCl was eventually used instead. By chessboard titration it was found that the monoclonal antibody could be used to coat plates at maximal efficiency at a dilution of 1:10,000 in this buffer. All of the monoclonal conjugates, however, had a working titer of only 1:200.

Of the methods of direct conjugation, the SPDP method gave slightly higher titers but unstable conjugates, the glutaraldehyde method gave very variable results, and the perio- date method gave low titers but stable and reproducible conjugates.

On testing many samples by the direct tests based on these conjugates in parallel with the polyclonal and indirect monoclonal test, the direct test failed to detect antigen in a significant number of feces found to be positive by the double-sandwich methods. Most of these had a fairly low antigen content as determined by the double-sandwich test. They comprised both virus subgroups; some specimens contained an appreciable number of virus particles as determined by electron microscopy.

**DISCUSSION**

Under optimal conditions the ELISA is a very sensitive, specific, and convenient test for detection of rotavirus antigen in feces. Any serological test is, however, only as good as the antisera employed. Our experience suggests that only high-titer monoclonal or polyclonal antibodies to the rotavirus group antigen are of value in these tests. Our results indicated that for use as capture antibody, rabbit sera were better than guinea pig sera which were better than goat sera. Earlier experiments demonstrated that convalescent sera from naturally acquired infections or gnotobiotic animals were not satisfactory as regards titer or specificity. The recent advance in tissue culture propagation of rotaviruses (14) should allow such high-titer sera to be more easily produced and made available; until recently we have had to use antigen purified from feces as inoculum.

Because a group-specific antibody was required, attempts were made to raise satisfactory antisera from tissue culture-adapted bovine rotavirus (UK Compton strain). The results were discouraging, since a significant number of electron microscopy-positive samples were not detected when tested by ELISA with such sera. Furthermore, the ODs obtained with those samples giving positive reactions did not correlate with the number of particles seen under the electron microscope. It was discovered later that although the anti-bovine rotavirus sera contained group-specific antibodies, they also contained an approximately equal proportion of subgroup I-specific antibodies and were biased towards samples containing subgroup I rotaviruses. To overcome this problem, while the monoclonal reagents were being developed, we produced polyvalent hyperimmune sera by inoculating mixtures of rotavirus from a number of sources and both subgroups, which at that time could only be obtained by purification from feces.

The choice of a solid phase was very important. Irradiated polystyrene plates had the highest binding capacity. Cost is also important; unfortunately, we have not yet found a method of cleaning these plates so that they can be used again.

The effect of EDTA on rotavirus particles has been exploited not only for pretreatment of samples for testing, but also for pretreatment of inocula when raising antisera. EDTA converts complete rotavirus particles (1.36 g/ml in CsCl) to their incomplete form (1.38 g/ml in CsCl), thus exposing the rotavirus group antigen (6). Samples containing predominantly incomplete particles are not affected by EDTA when tested by ELISA, but samples containing predominantly complete particles may give false-negative reactions unless EDTA is included. This phenomenon has been previously described (3). Sufficient EDTA must be included to overcome high calcium ion levels present in many neonatal samples (7).

The main deficiency of some rotavirus tests previously described is the absence of reagents for confirming positive reactions. As already reported (4), false-positive reactions do occur. Quite often, this is due to a rheumatoid factor-like substance in fecal extracts. The blocking test described here clearly detects such false-positive results because the rheumatoid factor-like substance reactions are blocked to the same extent by pre- and postimmune antisera. The importance of confirmatory blocking tests has been emphasized by other laboratories (4). With the use of electron microscopy as a reference test, falsely negative reactions have not, to our knowledge, been a problem. However, two samples were falsely negative, possibly owing to the presence of attached antibody, presumably IgA, which was detected by electron microscopy (no antibody was observed in any ELISA-positive sample). Another sample was negative because the virus was antigenically completely distinct from
other rotaviruses. Such rotaviruses have been described by other laboratories (5). Little is known as yet about their prevalence and pathogenicity. (A separate ELISA will be necessary to detect these viruses.)

The removal of excess reagents from the plates by washing is an important part of the test. Although great care is taken at each stage, the washings are potentially infectious and simple, safe, effective, and cheap methods for doing this need to be made available.

The choice of substrate is important. Many peroxidase substrates are carcinogens; TMB used in this study has not been found to be carcinogenic on testing in rats and gave a negative result by the Ames test (10). Also, alkaline phosphatase conjugates and substrates tend to be more expensive than peroxidase ones. Other factors which influence choice of substrate include whether the test is going to be read by eye or spectrophotometrically and, if the latter is the case, which filters the spectrophotometer is fitted with.

ACKNOWLEDGMENTS

We thank J. R. Foster for animal inoculations and Pat Tatton and Kathy Rodgers for secretarial assistance.

This work was supported principally by the Medical Research Council with some financial assistance from the World Health Organization.

LITERATURE CITED


