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Comparison of Direct and Indirect Enzyme Immunoassays with Direct Ultracentrifugation Before Electron Microscopy for Detection of Rotaviruses

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A direct and an indirect enzyme immunoassay (EIA) were evaluated against a standard of electron microscopy after direct ultracentrifugation of the specimen for their performances in detecting rotaviruses. The indirect EIA had variable background activity which influenced test specificity. The indirect EIA control (test system without the detector antibody) plus a regression line (which reflected background noise) improved test specificity. However, the results of direct EIA (Rotazyme; Abbott Laboratories, North Chicago, Ill.) sensitivity (86%) and specificity (96%) were better than those of the indirect EIA in tests on 73 rotavirus-positive and 78 rotavirus-negative specimens. Endpoint titrations of purified SA-11 rotavirus showed greater sensitivity of the direct EIA test. Electron microscopy, performed after direct ultracentrifugation, and direct EIA were approximately 2 $\log_{10}$ more sensitive in the detection of purified SA-11 rotavirus than was electron microscopy with standard methods of unconcentrated specimen preparation. Direct EIA tests are potentially sensitive, specific, and practical for the rapid detection of rotaviruses from human clinical specimens. Further studies are needed before EIA methods for detection of human rotaviruses can be equated with the level of reliability of results obtainable with sensitive electron microscopy techniques.

Many laboratory tests have been developed for the detection of human rotaviruses since the discovery of their role as a cause of gastroenteritis. One of the most promising has been the enzyme immunoassay (EIA), which possesses similar sensitivity to radioimmunoassay (9, 10) but avoids the inherent decay or safety hazard of the radioactive label.

Many derivations of EIA exist, including blocking, homogeneous, and binding EIA. The binding EIA with the capture antibody on a solid-phase support and the detector antibody as the second portion of the "sandwich" (either directly attached to the enzyme [direct system] or sequentially followed by a conjugate consisting of an anti-immunoglobulin antibody linked to an enzyme [indirect system]) has been widely utilized for antigen detection in microbiological analysis. In a manner analogous to the increased sensitivity of indirect versus direct fluorescence microscopy testing (8), one might expect increased sensitivity of the indirect EIA. In addition, the commercial availability of antispecies enzyme-antibody conjugates has made the indirect EIA a more practical approach for many investigators who wish to establish this diagnostic technique.

We have developed an indirect binding EIA for rotavirus detection which we have evaluated in parallel with a recently released commercial direct EIA, the Rotazyme test (Abbott Laboratories, North Chicago, Ill.). These EIA tests were evaluated for sensitivity, specificity, and application for diagnosis of rotavirus infection in comparison with a reference standard of electron microscopy (EM) after direct ultracentrifugation of the specimen (4).

MATERIALS AND METHODS

Procedure for the establishment of indirect EIA. SA-11 rotavirus (from the supply of Mark Gurwith, University of Manitoba, Winnipeg, Manitoba, Canada, and originally courtesy of H. H. Malherbe) was propagated in primary African green monkey kidney monolayers (Connaught Laboratories, Willowdale, Ontario, Canada) in a 150-cm^2 flask until 4+ cytopathic effect had occurred. The cells were frozen and thawed three times, and the cell suspension was clarified at 1,000 × *g* (TJ-6 Centrifuge; Beckman Instruments, Fullerton, Calif.) for 15 min at 4°C. Virus in the supernatant

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fraction was pelleted by ultracentrifugation in a Beckman type 35 rotor (82,000 × *g*) for 1 h, followed by suspension of the pellet in 0.1 M Tris-hydrochloride buffer (pH 7.5). Two successive sucrose gradients were used to purify virus (12, 24, 36, 48, and 60% [wt/vol] sucrose in 0.1 M Tris-hydrochloride buffer [pH 7.5]) with rate zonal ultracentrifugation in a Beckman SW27.1 rotor (83,000 × *g*) for 1 h. The virus band, detected by electron microscopy after negative staining with 1.5% sodium phosphotungstate (pH 7.0), was pelleted in a Beckman type 50 rotor (145,000 × *g*) for 1 h and suspended in phosphate-buffered saline (PBS [pH 7.4]). Virus was titrated by hemagglutination with 0.5% human type O erythrocytes. One-milliliter volumes of virus were stored at −70°C until use.

Adult male New Zealand white rabbits and guinea pigs were immunized after determination that these animals had no antibody to SA-11 virus by the hemagglutination inhabition test (titer, <1:10). Each animal received an initial intramuscular injection in two sites of 0.5 ml of a 1:1 emulsified mixture of incomplete Freund adjuvant and SA-11 rotavirus. Booster inoculations of SA-11 virus in PBS (pH 7.4) were given intramuscularly at 2 and 4 weeks after the initial injection. These animals were bled from the central artery of the ear (rabbits) or by cardiac puncture (guinea pigs), for a total of 7 and 5 weekly bleedings, respectively, and the serum samples of each animal were individually pooled and frozen at −70°C. The pooled sera of animals which had produced high titers of antibody to SA-11 virus (≥1:640 by hemagglutination inhibition after animal sera were treated with kaolin) were precipitated twice with ammonium sulfate, utilizing a 33% suspension for precipitation of rabbit sera and a 40% suspension for precipitation of guinea pig sera. After dialysis in 0.0175 M phosphate buffer, pH 6.3 (rabbit serum), and 0.005 M phosphate buffer, pH 8.0 (guinea pig serum), overnight at 4°C, the immunoglobulin G (IgG) was collected through a DEAE 40 ion-exchange column (Schleicher & Schuell Co., Kenne, N.H.), utilizing the same buffers. The protein fractions eluted from the column were detected at a 280-nm wavelength by a UV spectrophotometer (Pye Unican SP8-100), pooled, and concentrated by a concentrator with an XM100 filter (Amicon Corp., Lexington, Mass.). The purity of the IgG of each species of animal was determined by immunoelectrophoresis with goat anti-rabbit and goat anti-guinea pig whole serum (Northeast Biomedical Laboratories, Windham, Maine). The final antibody titer to the SA-11 virus was determined by the hemagglutination inhibition test. The final amount of immunoglobulin protein was quantitated with a UV spectrophotometer.

Performance of the indirect EIA. The indirect EIA test system utilized polystyrene beads (6 mm in diameter; Precision Plastic Ball Co., Chicago, Ill.) as the solid-phase support system. The beads were held in a 20-well radioimmunoassay test system support tray (Abbott Laboratories). IgG of guinea pig and rabbit antiserum were utilized for the capture antibody and detector antibody, respectively. The conjugate was a commercial preparation of goat anti-rabbit IgG coupled to alkaline phosphatase (Northeast Biomedical Laboratories). An Abbott Pentawash washer-aspirator was used to separate the unbound from bound reagents for this test system. The substrate was disodium *p*-nitrophenylphosphate (1 mg/ml; Sigma Chemical Co., St. Louis, Mo.), and all results were read in a Stasar III spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a microcuvette.

The following test parameters were determined arbitrarily by convenience or from previously published protocols. Overnight incubation of the capture antibody was done at 4°C with the polystyrene bead, using 0.5 ml of capture antibody per bead diluted in carbonate buffer (pH 9.6). Other incubation conditions were as follows: the test specimen was incubated at 45°C for 2 h, the detector antibody and the conjugate were incubated at 37°C for 1 h, and the substrate was incubated at 37° for 30 min in a water bath; eight washings were performed with distilled water containing 0.05% Tween 20 between addition of reagents; the beads were transferred into a new support tray after the washing step which followed incubation with the conjugate; the diluent for the conjugate was PBS (pH 7.4) with 0.05% Tween 20 and 1% bovine serum albumin (BSA) fraction V (GIBCO Laboratories, Grand Island, N.Y.); reagent volumes in the 20-well dish were 0.2 ml, except for the substrate volume of 0.5 ml; and 0.05 ml of 3 M NaOH (pH 9.5) was added to the substrate to stop the reaction.

To optimize sensitivity of the test system while maintaining the intensity of background activity at approximately less than 0.1 optical density (OD) units, a checkerboard titration to determine the best conjugate concentration was performed with serial dilutions of detector antibody (passively adsorbed to the polystyrene beads) and conjugate. A subsequent checkerboard titration was performed to optimize the capture antibody by covariation of dilutions of the capture antibody and purified SA-11 virus. The control for each clinical specimen consisted of a parallel run of the specimen without detector antibody.

Factors which affected the background activity of the indirect EIA were evaluated. An approximately 5% (wt/vol) fecal suspension (from each of rotavirus-negative specimens from five children) was made in 0.01 M PBS (pH 7.4) and PBS containing 0.025 M EDTA; 0.5% gelatin; 0.5% BSA; and 2 drops of 1 N HCl. These specimens were tested in duplicate as control specimens by the indirect EIA without detector antibody.

To evaluate the effect of incubation temperature on both specific activity and background activity, a positive control of sucrose gradient-purified SA-11 rotavirus and rotavirus-positive fecal specimens from five children were tested by the indirect EIA with incubation temperatures of the fecal suspensions at 37, 45, and 56°C for 3 h.

The direct EIA consisted of the Rotazyme test, which was used as recommended by the manufacturer (Abbott Laboratories).

Clinical specimens. Specimens tested by EIAs were collected from diagnostic specimens submitted to the Cadham Provincial Laboratory, Winnipeg, Manitoba, Canada, over the winter of 1980 to 1981 from young children with gastroenteritis. The initial detection of rotavirus in clinical specimens was by electron microscopy with a 201 electron microscope (Philips Electronic Instruments, Inc., Mahwah, N.J.) after direct ultracentrifugation of virus to the specimen grid with an EM-90 rotor (Beckman Instruments) in an Airfuge

ultracentrifuge (4). The original clinical specimens (73 rotavirus positive and 78 rotavirus negative) were stored at −70°C.

For the indirect EIA test, clinical specimens were made up to a final 2 to 5% suspension in 0.01 M PBS with 0.5% BSA–0.025 M EDTA and adjusted to a final pH of approximately 7.0 (determined by a 0.02% phenol red indicator in the diluent) with the addition of 7.5% sodium bicarbonate.

Sensitivity of EIA systems by in vitro testing. A batch of SA-11 rotavirus cultured in African green monkey kidney cells was purified with a cesium chloride gradient by ultracentrifugation at 82,500 × *g* for 29.5 h, and virus was collected from gradient fractions with a density of 1.358 to 1.379. The virus suspension was dialyzed against PBS for 48 h, pelleted with a Beckman type 50 rotor at 152,000 × *g*, and resuspended in 1.0 ml of PBS. This stock virus solution was diluted in PBS or PBS with 0.025 M EDTA in serial 0.5-log dilutions from 10^{-1} to 10^{-3}. Duplicate tests of the direct EIA (Rotazyme) were performed with each dilution of virus in PBS, and our indirect EIA system and the Rotazyme test were also performed on dilution of rotavirus in PBS-EDTA on the same day. The endpoint of the Rotazyme test was the last dilution having an OD of greater than 0.050 units plus the OD of the diluent control with a bead. The endpoint titer of our indirect EIA was the last dilution having an OD of test minus control (without detector antibody) of greater than 0.025 units. The number of virus particles visualized by electron microscopy at each dilution in the PBS diluent before and after direct ultracentrifugation were counted with a hand counter over five grid squares, and the virus counts were averaged.

Statistical methods. To determine the best cutoff value, we evaluated representative statistical variability for specimens with high background "noise" as detected by the control system for that specimen. Three replicate tests were performed for each of 44 human fecal specimens which represented a variety of background noise levels of activity from preliminary control results. A simple linear regression line from these replicate data (means and standard deviations) was generated by plotting the standard deviation of the control versus the amount of background noise activity. Ninety-five percent confidence intervals for this regression line were established from the standard deviations calculated from the background values of the control system at arbitrary values of OD (0.025, 0.05, 0.075, etc.). Two times the upper 95% confidence limit was then used as a series of points at these values to generate a corrected cutoff line. The effect on the sensitivity and specificity of the indirect EIA results was evaluated by comparing a cutoff value at a variety of arbitrary OD differences between the test and the control specimen with and without this corrected cutoff line. Because differences between the test and control specimens in the range of very low OD values may reflect test variability rather than real differences, the analyses of results for clinical specimens were performed at arbitrary cutoff lines beginning at 0.025 and also at 0.030, 0.035, and 0.050 OD.

RESULTS

The hemagglutination titer of the sucrose gradient-purified SA-11 virus was 1:512 per 0.025 ml. This preparation contained approximately 20 to 50 complete and incomplete virus particles per electron microscopy grid field at magnification ×30,000 without ultracentrifugation of virus onto the specimen grid.

The hemagglutination inhibition titers of the guinea pig and rabbit antisera were 1:2,560 and 1:5,120, respectively. Immunoelectrophoresis of the purified sera showed them to be composed of IgG only.

The optimal dilution of the capture, detector, and conjugate antibody concentrations were 1:200 (1.9 μg/ml), 1:200, and 1:4,000, respectively.

The effect in the indirect EIA at 45°C of various diluents on the background activity of specimens negative for rotavirus is shown in Table 1. BSA had an effect which appeared to be superior to that of gelatin and a more closely neutral pH; EDTA in the diluent acted independently to reduce background noise. Whereas elevated temperatures of incubation of rotavirus-positive fecal suspensions usually reduced background noise, the specific signal was also reduced at 45°C and dramatically decreased at 56°C for the indirect EIA (Table 2).

Of the clinical specimens, 73 were rotavirus positive by electron microscopy, and 78 were rotavirus negative. Using the cutoff value of 0.05 OD plus the diluent OD test result, the Abbott Rotazyme direct EIA test identified 63 specimens as positive (86% sensitivity) and 74 specimens as negative (96% specificity).

Statistical analysis confirmed our hypothesis that the indirect EIA test variability was greater for specimens with a high background OD activity. This was expressed by a regression line with the equation $rd = 0.001 + 0.155\,x$, which had a correlation coefficient of 0.91 (Fig. 1). Table 3 shows the results of testing rotavirus-positive (Fig. 2) and rotavirus-negative (Fig. 3) human fecal specimens by the indirect EIA with and without the correction line through a series of arbitrary cutoff values (the OD of the test specimen minus the OD of the control specimen) of 0.025, 0.030, 0.035, and 0.050 OD units. For example (Table 3), a cutoff value of 0.025 OD units resulted in 89% sensitivity and 71% specificity versus 77% sensitivity and 86% specificity when the corrected OD cutoff value of 0.025 OD units was used.

Results of the sensitivity of the two EIA systems in comparison with quantitative particle counts are shown in Fig. 4. All three systems were similar, but the Rotazyme direct EIA was more sensitive than was our indirect EIA by approximately 0.5 to 1.0 $\log_{10}$ of virus at the endpoint of each method (Fig. 4). The addition of 0.025 M EDTA to the diluent increased the sensitivity of Rotazyme SA-11 detection by ap-

TABLE 1. Effect of type of diluent on background activity of rotavirus-negative fecal specimens from five children when tested as controls (without detector antibody) in the indirect EIA test

Diluent[a]	Sp act of following specimen no.:				
	1829	1830	1833	1837	1895
PBS (0.01 M; pH 7.2)	0.532	0.354	0.161	0.061	0.057
	0.442	0.273	0.113	0.065	0.067
EDTA (0.025 M)[b]	0.268	0.039	0.032	0.027	0.015
	0.245	0.039	0.055	0.019	0.020
BSA (0.5%)[b]	0.052	0.022	0.066	0.010	0.012
	0.066	0.035	0.069	0.012	0.014
Gelatin (0.5%)[b]	0.108	0.177	0.084	0.019	0.049
	0.108	0.213	0.069	0.025	0.056
HCl (1 N)[c]	0.854	2.113	0.139	0.130	0.642

[a] Tested in duplicate, except for HCl.
[b] Diluted in 0.01 M PBS (pH 7.2).
[c] Two drops were added to the original fecal suspension.

proximately 0.5 to 1.0 $\log_{10}$ and increased reactions of the positive specimens. Approximately 8 to 10 rotavirus particles per grid square were visualized from the stock $CsCl_2$ gradient-purified virus, but none were detected at a 10^{-1} or $10^{-1.5}$ dilution by standard unconcentrated methods. After direct ultracentrifugation of virus onto the specimen grid from the 10^{-1}, $10^{-1.5}$, 10^{-2}, $10^{-2.5}$, and 10^{-3} dilutions, the mean numbers of virus particles detected by electron microscopy were, respectively, 35.2, 5.0, 8.8, 0.6, and 0. Thus, the endpoint of electron microscopy for purified SA-11 rotavirus detection was at least 100 times more sensitive after direct ultracentrifugation of virus to the specimen grid than were standard methods without concentration.

DISCUSSION

Progress has been rapid in the development of methods to detect rotaviruses from human clinical specimens. The currently accepted standard for rotavirus diagnosis remains electron microscopy, as the virus is not routinely propagated in cell culture. We have recently shown an enhanced sensitivity of virus detection after direct ultracentrifugation of clinical specimens for rotavirus detection (4). Both a direct EIA (antiviral antibody conjugated directly to the enzyme) and an indirect EIA (antiviral antibody reacting with an antispecies antibody linked to the enzyme) were evaluated against this standard. Direct comparisons of the two EIA tests are not valid because of differences in reagents and test conditions, although antisera for both immunodiagnostic methods were produced against SA-11 rotavirus. The current study demonstrated a greater sensitivity of the direct EIA procedure by endpoint titrations of purified SA-11 virus. The endpoint of rotavirus detection by the direct EIA was within 0.5 $\log_{10}$ of results obtained after concentration by direct ultracentrifugation before electron microscopy, and the sensitivity of both methods exceeded that of the standard electron microscopy method by approximately 2.0 $\log_{10}$. A theoretical advantage of increased sensitivity of the indirect EIA was not verified in this study. A recent report by Grauballe et al. questioned the need for indirect EIA systems to obtain the necessary sensitivity

TABLE 2. Effect of incubation on specific activity and background activity of purified SA-11 rotavirus and rotavirus-positive fecal specimens from five children when tested by the rotavirus indirect EIA

Temp (°C)	Sp act of following specimen no.:											
	SA-11		5981		6033		6106		6115		6186	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
37	1.220	0.131	0.259	0.098	0.648	0.346	0.307	0.136	0.175	0.075	0.418	0.195
45	0.608	0.068	0.139	0.032	0.422	0.087	0.102	0.025	0.110	0.035	0.301	0.193
56	0.440	0.008	0.125	0.087	0.305	0.009	0.059	0.001	0.087	0.054	0.103	0.018

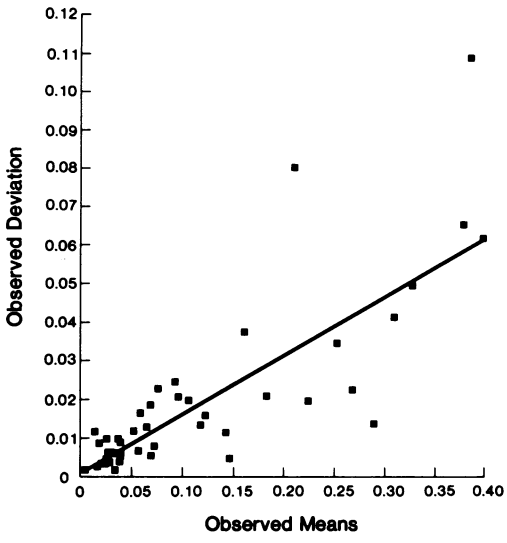

FIG. 1. Determination of the statistical variability of the background OD as a function of the mean of the background OD of three replicate tests of 44 human fecal specimens tested as controls (without detector antibody) by indirect EIA.

for rotavirus detection, although no comparison between direct and indirect EIA tests was made (3).

Possible reasons for different sensitivities of the two EIA systems include differences in the quality of the reagents, including conjugates. A recent report has shown higher ratios of conjugate activity with positive sera to conjugate activity with negative sera for horseradish peroxidase than for alkaline phosphatase conjugates and variability in the sensitivity of commercially manufactured enzyme conjugates (6). Also, the additional washing steps of our indirect system (more washes and one extra set of washes) may have allowed greater desorption or loss of the linked reagents.

The control system which we utilized in our current study differed from that of previous studies. Because the detector antibody in our indirect EIA system did not contribute to background noise interaction with the conjugate (either with or without fecal samples), our control for background activity consisted of the reaction that occurred without the detector antibody present. Others have utilized the preimmunization serum of animals to control for results obtained using postimmunization serum in the diagnostic test (12). However, published reports showed that problems of specificity with this method, such as false-positive detections of rotavirus, were noted (2). Another study in which an indirect EIA test was used showed high levels of background activity with a cutoff value of 0.5 OD units (9). In our study, we found wide variability in the background activity of human infant fecal specimens for the indirect EIA. We were able to reduce these nonspecific reactions by using EDTA in the fecal sample diluent, adjusting the suspension of the fecal specimen to a pH of approximately 7.0 (7), and using 0.5%

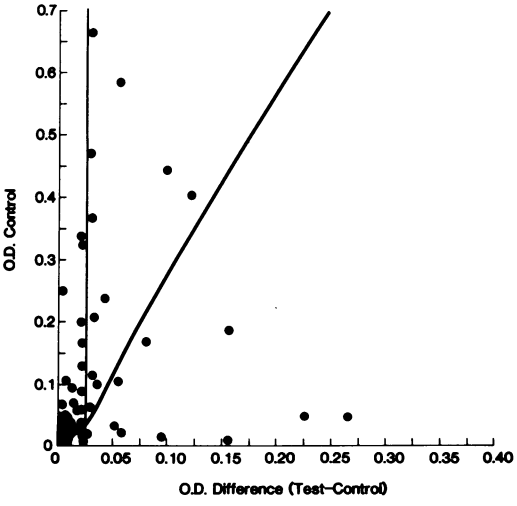

FIG. 2. Results of indirect EIA testing of 78 rotavirus-negative human fecal specimens (●). An arbitrary cutoff value of 0.025 OD difference (—) (test minus control) is compared with results when the arbitrary 0.025 cutoff value is modified (—) to include a correction factor which takes into consideration the statistical variability of control specimens that have higher OD readings.

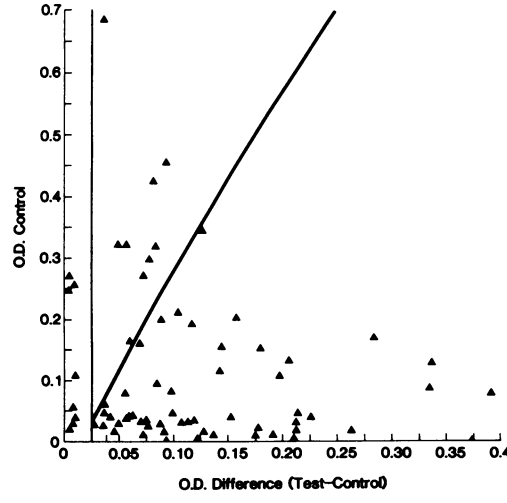

FIG. 3. Results of indirect EIA testing of 73 rotavirus-positive human fecal specimens. The cutoff values are the same as those described in the legend to Fig. 2.

TABLE 3. Results of indirect EIA detection of rotavirus from human clinical specimens by comparison of two cutoff methods

Cutoff value and reference result[a]	Cutoff value alone				Corrected cutoff value[b]			
	No. +	No. −	% Sensitivity	% Specificity	No. +	No. −	% Sensitivity	% Specificity
0.025			89	71			77	86
+	65	8			56	17		
−	23	55			11	67		
0.030			88	77			75	87
+	64	9			55	18		
−	18	60			10	68		
0.035			88	81			70	89
+	64	9			51	22		
−	15	63			9	69		
0.050			75	82			66	87
+	55	18			48	25		
−	14	64			10	68		

[a] Reference results were determined by electron microscopy after direct ultracentrifugation of virus to the specimen grid.

[b] The cutoff OD value was corrected by incorporation of two times the upper 95% confidence limit of the statistical variability of the background noise of representative control specimens.

BSA in the fecal suspension (which was superior to gelatin). (Yolken found that differences existed between proteins used for preincubation of the specimens and that 1% goat serum was superior to other animal sera [12].) In spite of these manipulations, we were unable to eliminate or predict the wide range of control background activity of our indirect EIA. The effect of this background activity on the test results showed that, as the background activity increased, the statistical variability of results increased, resulting in reduced test specificity and

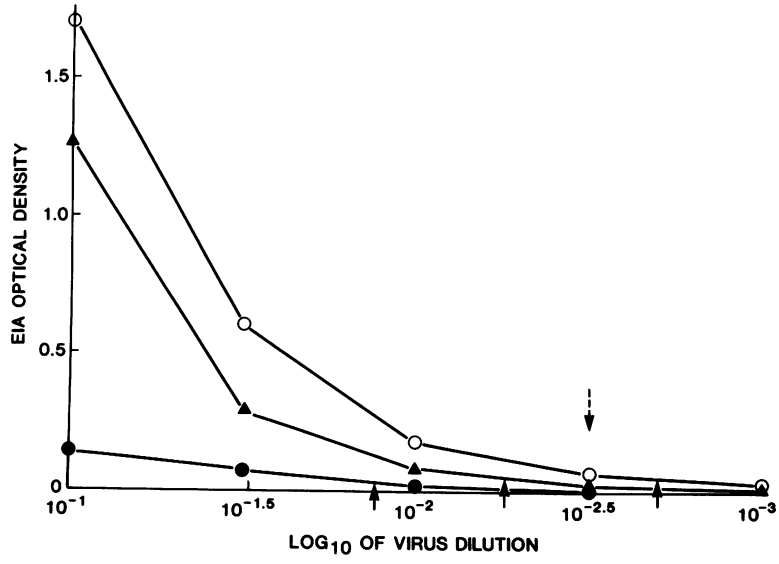

FIG. 4. Comparison of the sensitivity of detection of purified SA-11 rotavirus by a direct EIA with 0.025 M EDTA in the virus diluent (○) and no EDTA in the virus diluent (▲) versus an indirect EIA with EDTA in the diluent (●). The interpolated EIA endpoints of each EIA at its cutoff are indicated (↑). The non-interpolated endpoint determined by electron microscopy after direct ultracentrifugation of virus to the specimen grid is shown (↓).

illustrating that a single arbitrary cutoff value might not be applicable to all specimens tested by an EIA system if background activity is variable or large in relation to the cutoff value. Incorporation of the control we have described (the test system without the detector antibody), plus the use of a regression line which described representative statistical variability of specimens, did improve the specificity of the indirect EIA system. Results with such a system could be read from a nomogram. If the specificity of a test were improved to a predetermined acceptable level by such a method, this could be useful as a routine diagnostic test.

The commercially available direct EIA system for rotavirus detection (Rotazyme) was less time consuming and simpler to perform than our indirect system and gave greater sensitivity and specificity for rotavirus detection in human fecal specimens. The Rotazyme test and the indirect EIA of Grauballe had little background activity. However, the sensitivity of the Rotazyme test with our specimens (86%) was more in keeping with results (85%) of Bishai et al. (F. R. Bishai et al., Abstr. Conjoint Meet. Infect. Dis. 49th, Ottawa, Ontario, Canada, 25–27 November 1981, p. 146). Both Canadian studies showed a lower sensitivity than did the results of Yolken (97% [11]).

The addition of EDTA to the sample diluent increased the sensitivity of the direct test for purified SA-11 rotavirus, verifying the results of the developer of the commercial test (A. Rubinstein, personal communication). The mechanism of this increased sensitivity has not been elucidated, but hidden antigen may be exposed after EDTA treatment. We have not tested for an increase in sensitivity of detection of human rotaviruses with and without diluent containing EDTA, nor do we know whether human rotaviruses may possess the same hidden antigen.

The direct EIA systems are potentially sensitive, specific, and practical for the rapid detection of rotaviruses from human clinical specimens. Further data are required concerning the detection of human rotaviruses with antisera used in the EIA test when these sera have been prepared against a variety of human and animal strains of rotaviruses (1, 5). These data will be necessary before immunodiagnostic tests become as widely accepted as electron microscopy as the standard for the reliable diagnosis of human rotavirus infections.

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