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COMPARISON OF ENZYME-LINKED IMMUNOSORBENT ASSAY, ELECTRON MICROSCOPY, AND REVERSED PASSIVE HAEMAGGLUTINATION FOR DETECTION OF HUMAN ROTAVIRUS IN STOOL SPECIMENS

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SUMMARY An enzyme-linked immunosorbent assay (ELISA) using microplates as solid phase, rabbit antiserum against human rotavirus Wa strain as catching antibody, and the same reagent labeled with β-D-galactosidase as conjugate, has been developed for detection of human rotavirus antigen(s) in stool specimens from patients with acute gastroenteritis. The limit of detection of purified human rotavirus by ELISA was 15.6 ng/ml (1.56 ng/well) of viral protein. The sensitivities of ELISA, electron microscopy, and the reversed passive haemagglutination method (ROTA-CELL) were compared. ELISA was more sensitive than electron microscopy and the reversed passive haemagglutination method. The ELISA blocking assay was useful for detection of an antibody response to human rotavirus in paired sera from children in two institutions during outbreaks of rotavirus gastroenteritis.

INTRODUCTION

Rotavirus is an important cause of acute gastroenteritis in infants and young children (Bishop et al., 1974; Kapikian et al., 1976; Konno et al., 1977). Several methods have been developed for detection of rotavirus in stool specimens, such as electron microscopy (EM), complement fixation (CF), immunofluorescence (IF), radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and reversed passive haemagglutination (R-PHA) (Davidson et al., 1975; Kapikian et al., 1975; Bryden et al., 1977; Kalica et al., 1977; Yolken et al., 1977a; Cukor et al., 1978; Sanekata et al., 1979; Sarkkinen et al., 1979; Sarkkinen et al., 1980; Grauballe et al., 1981). EM is a reliable method, but it cannot be used to examine a large number of specimens at one time. IF is rather subjective and laborious. ELISA is similar to RIA except that an enzyme is used instead of a radioactive isotope as the

immunoglobulin marker. Both RIA and ELISA are objective and can be automated. RIA is subject to restrictive legislation because of possible health risks and utilizes reagents with a short shelf-life, whereas ELISA is free from restrictive legislation and the reagents have a very long shelf-life. In addition, ELISA needs only cheap, simple equipment. Because of these advantages, ELISA is suitable for use in small laboratories and in developing countries. An ELISA kit called Rotazyme (Abbott) for detecting rotavirus using beads as solid phase, guinea pig antiserum against simian rotavirus SA-11 as catching antibody, and rabbit antiserum against the same virus labeled with horseradish peroxidase as conjugate, is now marketed (Rubenstein and Miller, 1982). The present report describes an ELISA using microplates as solid phase, rabbit antiserum against human rotavirus as catching antibody, and the same reagent labeled with β-D-galactosidase as conjugate, and compares ELISA with EM and R-PHA for detection of rotavirus in stool specimens from cases of acute gastroenteritis. ELISA blocking assay was used to measure the antibody response to human rotavirus in paired sera from children in two institutions during outbreaks of rotavirus gastroenteritis.

MATERIALS AND METHODS

1. *Stool specimens*

Stool specimens were collected from 159 infants and young children in Japan from January 1980 to January 1982. Of these, 148 were obtained from patients with acute gastroenteritis and 11 from children admitted to our hospital with illnesses other than gastroenteritis.

2. *Paired sera*

Twenty-one paired sera were obtained from institutionalized children during outbreaks of rotavirus gastroenteritis.

3. *Preparation of rabbit anti-human rotavirus serum*

Human rotavirus Wa strain was propagated in MA-104 cells in the presence of trypsin, and purified by CsC1-gradient centrifugation. The protein content was determined by the method of Lowry et al. (1951). Purified human rotavirus solution was mixed with an equal volume of incomplete Freund's adjuvant, and injected intramuscularly into the back of a rabbit. Then 2 and 4 weeks later, the virus solution mixed with the adjuvant was injected intramuscularly. The animal was exsanguinated one week after the last injection.

4. *Preparation of anti-rotavirus IgG-β-D-galactosidase conjugate*

Rabbit anti-human rotavirus IgG was prepared by salting out (twice) of the serum with 50% ammonium sulfate followed by Sephadex G-25 column chromatography using 0.01 *M* phosphate buffer (pH 7.0). Rabbit anti-human rotavirus IgG was conjugated with β-D-galactosidase by the method of O'Sullivan et al. (1978).

5. *ELISA for detection of rotavirus antigen*

The wells of a microtiter plate (flat microplate, Cooke Engineering Inc.) were coated with 100 μl of rabbit anti-human rotavirus IgG (20 μg/ml) or with 100 μl of 10% normal rabbit serum diluted in 0.05 *M* bicarbonate buffer (pH 9.5). After incubation at 4 C overnight, these solutions were sucked off, and 300 μl of 1% bovine serum albumin (BSA) in 0.01 *M* phosphate buffered saline (PBS) was added to each well. After incubation at room temperature for 30 min, the wells were washed three times with 300 μl of 0.01 *M* PBS containing 0.1% Tween 20 (PBS-Tween 20). The plates were dried at room temperature and stored at 4 C until use. A 10% stool suspension was prepared in 0.01 *M* PBS and centrifuged at 3,000 rpm for 30 min. Volumes of 100 μl of the supernatant and 100 μl of 0.01 *M* PBS containing 0.1% BSA and 1 m*M* $MgCl_2$ were added to duplicate wells coated with rabbit anti-human rotavirus IgG (test wells) and to duplicate wells coated with 10% normal rabbit serum (control wells). After incubation at 4 C overnight, the stool specimens were sucked off and the wells were then washed five times with 300 μl of PBS-Tween 20. Then 150 μl of anti-human rotavirus IgG-enzyme conjugate in 0.01 *M* PBS containing 0.1% BSA, 1% normal rabbit serum and 1 m*M* $MgCl_2$ was added to each well and kept overnight at 4 C. The wells were washed three times with 300 μl of PBS-Tween 20, and then 150 μl of 0.1% o-nitrophenyl-β-D-galactopyranoside in 0.01 *M* PBS con-

taining 0.1% BSA, 5% methanol and 1 m*M* $MgCl_2$ was added. After incubation for 6 h at room temperature, the enzyme-substrate reaction was stopped by adding 150 μl of 3% Na_2CO_3 and the optical density at 405 nm (OD_{405}) was measured with a microplate photometer (Corona MTP-12).

6. *ELISA blocking assay for detection of anti-rotavirus antibody*

A volume of 100 μl of 0.01 *M* PBS containing 0.1% BSA and 1 m*M* $MgCl_2$, and an equal volume of human rotavirus antigen were added to each well of an anti-human rotavirus IgG coated microplate. Human rotavirus Wa strain grown in cell culture was titrated in ELISA antigen assay, and a dilution 8 times greater than the end point (i.e., 8 U of antigen) was used in the blocking assay. After incubation at 4 C overnight, the wells were washed three times with 300 μl of PBS-Tween 20, and 100 μl of 0.01 *M* PBS containing 0.1% BSA and 1 m*M* $MgCl_2$ and 100 μl of test sera diluted in two-fold steps starting at 1:8, were added. Then unneutralized virus was measured by the ELISA antigen assay as above.

7. *EM*

A 10% stool suspension was prepared in 0.01 *M* PBS and clarified by centrifugation at 3,000 rpm for 30 min. The supernatant was mixed with an equal volume of trifluorotrichloroethane, and centrifuged at 3,000 rpm for 30 min. The supernatant was centrifuged at 10,000 rpm for 20 min, and 4 ml of the supernatant was layered onto 1 ml of 45% (W/V) sucrose and centrifuged at 75,000 *g* for 150 min. The pellet was transferred to a 400-mesh collodion-carbon coated copper grid, stained negatively with 3% phosphotungstic acid, and examined in an electron microscope (HITACHI HU-12 A).

8. *R-PHA*

R-PHA was carried out in a ROTA-CELL (Nissui) following the manufacturer's instructions.

RESULTS

Figure 1 shows the result of titration of purified human rotavirus antigen by ELISA. The protein concentration of this viral antigen was 80 μg/ml as measured by the method of Lowry et al. A specimen with a P/N value, (i.e., the

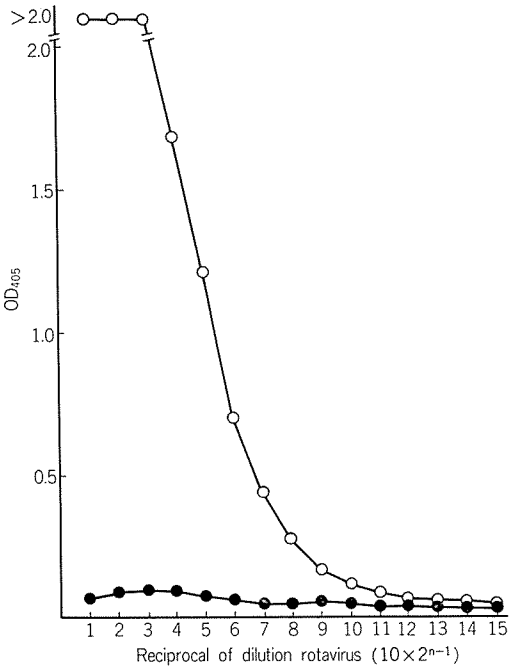

FIGURE 1. Dilution curve of purified human rotavirus by ELISA. Symbols: ○, OD of test wells; ●, OD of control wells.

average OD value of test wells divided by the average OD value of control wells) of more than 2.1 was defined as positive for rotavirus. ELISA was capable of detecting 15.6 ng/ml (1.56 ng/well) of purified viral protein.

Eleven stool specimens from children without acute gastroenteritis gave negative results by ELISA. The P/N values of these negative controls ranged from 0.8 to 1.2. Of 148 stool specimens from childrens with actue gastroenteritis, 67 (45.3%) gave a positive result by ELISA. Rotavirus was detected in many stool specimens collected between November and February.

Figure 2 shows the relation of the detection of rotavirus by ELISA from November to February to the days after the onset of illness. Stool specimens obtained within eight days after the onset of illness were positive for rotavirus at high rate, and two specimens taken 12

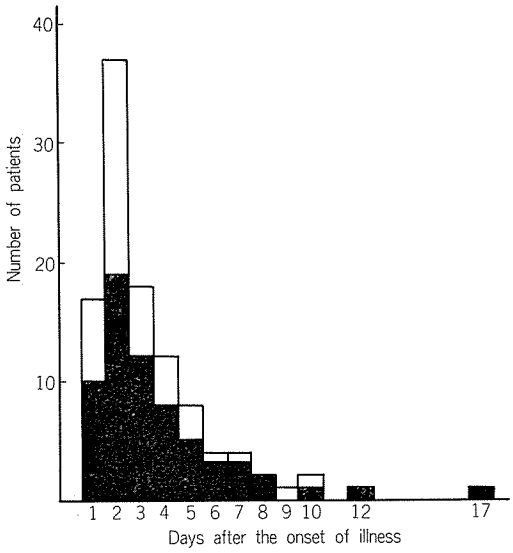

FIGURE 2. Relation between the detection of rotavirus by ELISA in stools from patients with acute gastroenteritis and the days after the onset of illness. Symbols: ■, positive for rotavirus; □, negative for rotavirus.

and 17 days after the onset of illness were also positive.

In all 38 stool specimens were tested by ELISA and EM for rotavirus or related antigens, and the results are presented in Table 1. All the EM-positive specimens were also positive by ELISA, but of 17 EM-negative specimens, 9 were positive by ELISA.

Results on 71 stool specimens tested by ELISA and R-PHA are shown in Table 2. All 57 specimens that were positive by R-PHA were also positive by ELISA. Of 15 R-PHA-negative specimens, 5 were positive by ELISA.

ELISA blocking assay was used to measure the antibody response to human rotavirus in paired sera from children in two institutions during outbreaks of rotavirus gastroenteritis.

Figure 3 shows the dilution curves of paired sera obtained from two children (A, B), and indicates that semiquantitative assay of anti-rotavirus antibody is possible by ELISA blocking assay. The percent blocking was

TABLE 1. *Comparison of ELISA and EM for detection of rotavirus in stools from acute gastroenteritis patients*

		ELISA	
		+	−
EM	+	9	0
	−	8	21

TABLE 2. *Comparison of ELISA and R-PHA for detection of rotavirus in stools from acute gastroenteritis patients*

		ELISA	
		+	−
R-PHA	+	57	0
	−	5	10

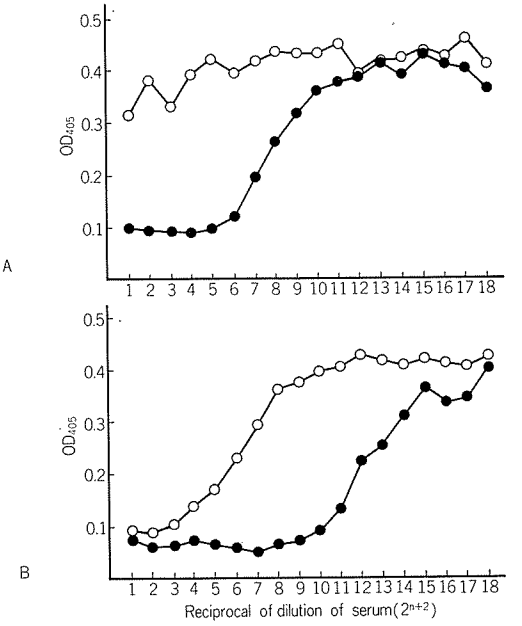

FIGURE 3. Dilution curves of paired sera obtained from two children (A, B) with gastroenteritis by the ELISA blocking assay. Symbols: ○, pre-illness sera; ●, convalescent sera.

calculated by the following equation at each dilution of the paired sera:

$$\text{percent blocking} = \frac{\text{OD (pre.)} - \text{OD (conv.)}}{\text{OD (pre.)}} \times 100$$

where OD (pre.) and OD (conv.) are the optical densities at 405 nm after incubation with sera at pre-illness and convalescent stages, respectively. When the percent blocking was more than 50%, the antibody response to human rotavirus was regarded as positive. The percent blocking observed in paired sera obtained from two patients (A, B in Fig. 3) at serum dilutions of 1: 8 and 1: 256 were A, 68.9% and 70.1%; B, 18.4% and 75.7%, respectively. From the results shown in Fig. 3, we chose these two dilutions of paired sera, i.e. 1: 8 and 1: 256, to measure the rise in antibody levels. By this procedure, 16 of 21 paired sera from children in two institutions during outbreaks of rotavirus gastroenteritis showed a sero-response to human rotavirus.

DISCUSSION

This study showed that ELISA is more sensitive than EM or R-PHA for detection of rotavirus in stool specimens, and that ELISA blocking assay is useful for detection of antibody responses to human rotavirus. With ELISA it was possible to detect rotavirus antigen(s) in the supernatant of a 10% stool suspension centrifuged at 3,000 rpm for 30 min, whereas with EM ultracentrifugation of the supernatant was required in most cases. In addition, EM requires the identification of intact virus particles, and may not be effective for stool specimens containing only a small amount of rotavirus obtained some time after the onset of illness. However, ELISA can detect damaged as well as complete virus particles. Davidson et al. could not detect rotavirus particles by EM in any stool specimens collected from patients 8 days after the onset of illness (Davidson, 1975). In contrast, as shown in Fig. 3, with ELISA a positive reaction was observed with stool specimens obtained within 12 days after the onset of illness, and even with one specimen obtained 17 day after the onset of illness.

In the present study, the limit of detection of purified human rotavirus by ELISA was 15.6 ng/ml (1.56 ng/well) of viral protein. In 10% suspensions of 64 rotavirus-positive stool specimens measured by ELISA the maximum rotavirus content was equivalent to about 500 ng/ml of purified human rotavirus.

Non-specific binding due to an antiglobulin in stool specimens is reported to occur in ELISA (Yolken, 1977b). Although the exact nature of the material causing non-specific reaction is unknown, the reaction of this material is reduced by pretreatment of the specimens with mild reducing agent such as N-acetylcysteine (Yolken, 1979). The presence of non-specific activity is detected by adding the specimens to wells coated with normal rabbit serum, and specific activity is measured as the difference between the activities in wells with and without a coat of normal rabbit serum. In this study, the non-specific reaction was not a problem, possibly because we used anti-rotavirus serum prepared by inoculation of purified human rotavirus grown in cell cultures.

ELISA blocking assay was used to examine the antibody response to human rotavirus at 1: 8 and 1: 256 dilutions of paired sera. A sero-response to human rotavirus was detected in 16 paired sera obtained from 21 children in two institutions during outbreaks of rotavirus gastroenteritis.

We conclude from this work that ELISA is a simple technique, and that it is more sensitive than EM or R-PHA for detection of rotavirus. Moreover, ELISA blocking assay is simple and efficient for detecting the seroresponse to human rotavirus in paired sera.

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