

## Comparison of an Enzyme-Linked Immunosorbent Assay for Quantitation of Rotavirus Antibodies with Complement Fixation in an Epidemiological Survey

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The development of a micro-scale enzyme-linked immunosorbent assay (ELISA) with horseradish peroxidase as the marker enzyme for the detection and measurement of human rotavirus antibodies is described. A semipurified preparation of the serologically related simian agent, SA-11 virus, was used as the antigen. Test sera were reacted with antigen-sensitized wells in disposable polyvinyl microplates. Any attached antibody was detected by the addition of peroxidase-labeled anti-species immunoglobulin (conjugate) followed by assay of the enzyme reaction with its substrate, hydrogen peroxide plus 5-aminosalicylic acid. This micro-ELISA was compared with complement fixation in a seroepidemiological study of the age prevalence of rotavirus antibody in Aboriginal and European populations living in the same outback area in Australia. The ELISA (results read with the naked eye) proved to be approximately 16 times more sensitive than complement fixation. Of Aborigines, 71% had rotavirus complement-fixing antibody, as compared to 45% of Europeans. By ELISA 100% of both populations had rotavirus antibodies. Mean antibody titers in the different age groups were higher in Aborigines than in Europeans. Antibody levels rose steeply throughout the first 20 years of life, remained high during the next 20 years, then increased again at least up to the age of 60 years. The micro-ELISA was practical, simple to perform, and more suitable than complement fixation for large seroepidemiological rotavirus studies. It also has potential for serodiagnosis of the disease, both in the laboratory and in the field.

It has now been well established that a reovirus-like agent variously designated as rotavirus (14), duovirus (9), orbivirus (4), and infantile gastroenteritis virus (26) is an important cause of acute enteritis not only in Aboriginal infants and young children (34), but also in children in many other parts of the world (9, 12, 14, 20, 22, 23, 27, 38). Rotaviruses have also been found to be associated with acute enteritis in calves, mice, piglets, foals, lambs, and rabbits (8, 37, 42).

Serological evidence of rotavirus infection has been obtained by immunoelectron microscopy (21, 22), indirect immunofluorescence (10, 21, 27), neutralization in cell culture (13, 25, 37), complement fixation (CF) (15, 19, 21, 22, 26, 38), and counter-immuno-osmophoresis (26). Some of these assays are too cumbersome for large-scale seroepidemiological studies, and human rotavirus for use as antigen is variable and often difficult to obtain. The "O" (offal) agent has been claimed to be the most efficient substitute for human rotavirus as CF antigen, followed by simian rotavirus (SA-11), epizootic diarrhea of infant mice virus, and Nebraska calf diarrhea virus (18).

So far the most widely used technique in surveys for detection of rotavirus antibody has been CF, using as antigen either virus-positive extracts of human stools (5, 15, 21) or concentrates of the antigenically related Nebraska calf diarrhea virus grown in cell culture (5, 19, 36). The homologous system using human stool extract has been shown to be more sensitive than the heterologous Nebraska calf diarrhea virus for detecting antibodies in humans (5, 19). In these surveys, the community levels of rotavirus CF antibody showed that most children in Melbourne (15), as in Washington, D.C. (19), Boston (5), Toronto (26), and Marburg (36), had experienced infection by the age of 3 years. A high percentage of adult sera also showed detectable antibody.

The CF test has several deficiencies with regard to detection of rotavirus antibodies. Some subclasses of immunoglobulin G (IgG) (17) as well as IgA do not fix complement, and fecal extracts are frequently highly anticomplementary. In human enteric infections with poliovirus, much of the antibody activity has been found in the IgA class, especially in the gut (29). Woode

(41) has shown that neutralizing antibody in the gut of animals is important for protection against further rotavirus infection, whereas circulating antibody does not protect. In infant humans and rhesus monkeys, rotavirus infections can occur despite the presence of CF antibodies in serum (20, 43).

Recently solid-phase radioimmunoassays have been described for the detection of rotavirus antibody (2, 40). Although they are sensitive and offer the advantage of being suitable for the study of a large number of samples within a short period of time, the need for radioactive reagents and radiation counting equipment makes this technique impractical for many laboratories and unsuitable for field studies.

A serological test that has been developed in recent years and that appears to offer a combination of the best qualities of all is the enzyme-linked immunosorbent assay (ELISA). Described first by Engvall and Perlmann (11), ELISA is a modification of the radioimmunosorbent technique, in which an enzyme is substituted for the radiolabel of the anti-immunoglobulin.

Although originally the assay was performed in polystyrene tubes and was photometrically quantitated, ELISA has been readily adapted to microtitration, visual reading, and automation. In a semi-automated system, Ruitenberg et al. (31) demonstrated that on a routine basis as many as 4,000 sera could be tested daily. It is thus apparent that ELISA is most suitable for application on a large scale such as seroepidemiological surveys. Scherrer and Bernard (33) have recently reported an ELISA for detection of calf rotavirus which can also be used for detection of bovine rotavirus antibody, and Yolken et al. (44) have described an ELISA system for measuring IgG and IgM rotavirus antibodies in human sera.

This report describes the development of a microtitration ELISA procedure with horseradish peroxidase as marker enzyme for the detection of rotavirus antibodies, using as antigen SA-11 virus grown in cell culture (24). This procedure was applied to a serological survey of the prevalence of serum antibodies in various groups in Aboriginal and European populations from the same area. Results thus obtained are compared with those derived from the same survey carried out by the CF method, also using SA-11 virus as antigen, on the same populations.

## MATERIALS AND METHODS

**Test sera.** Samples of frozen/refrigerated sera collected during 1975 to 1977 for diagnostic purposes unrelated to this study were kindly provided by D. Graham, University of Melbourne, and by Alice

Springs Hospital, Alice Springs. All sera were stored at  $-70^{\circ}\text{C}$  before testing.

The specimens that were tested satisfactorily on patients of varying ages are as follows: Aborigines from 2 months to 80 years of age, (i) 191 by CF and (ii) 197 by ELISA; Europeans from 2 years to 70 years, (i) 233 by CF and (ii) 234 by ELISA. Almost all the patients were residents of Wave Hill, Hooker Creek, Santa Teresa Mission, Tennant Creek, Alice Springs, and their surrounding areas. All sera were coded to exclude biased interpretation of results.

**Control sera.** A pair of acute-phase (no. 243791) and convalescent-phase (no. 244393) control sera of known rotavirus CF antibody content were kindly supplied by R. Pringle, Fairfield Hospital for Communicable Diseases, Melbourne. These were initially used to standardize sera of laboratory staff members, of which one positive (1:128) and one negative (1:4) serum were selected by CF and used as CF controls throughout the year.

The positive ELISA control serum used was the same as the positive CF control. This had an ELISA titer (reciprocal) greater than 512. The negative CF control had a positive titer by ELISA. A limited survey of 44 neonatal (cord) sera by B. McLean, Royal Women's Hospital, Melbourne, yielded four which gave titers  $\leq 1:2$  in the ELISA system; one of these was used as the negative reference for ELISA tests.

**ELISA conjugates.** Globulin obtained by  $(\text{NH}_4)_2\text{SO}_4$  precipitation of goat antiserum to human immunoglobulins (IgG, IgM, IgA) (Hyland Div. Trav-enol Laboratories, Costa Mesa, Calif.) was conjugated with horseradish peroxidase (Type VI; Sigma Chemical Co., St. Louis, Mo.) by the method of Nakane and Kawaoi (28). Sodium *m*-periodate was used as a concentration of 0.04 M. The conjugates were stored in 1-ml volumes at  $-70^{\circ}\text{C}$  and at  $4^{\circ}\text{C}$  during use. No loss of activity has been noted over a 6-month period.

**Preparation of ELISA antigen.** SA-11 virus was kindly supplied by H. H. Malherbe, and has now been passaged several times in our laboratory in primary Cynomolgus monkey (*Macaca fascicularis*) kidney epithelial cells (PMK) according to the method of Rodger et al. (30). Infected (PMK) cells frozen at  $-70^{\circ}\text{C}$  were kindly supplied by S. Rodger. The virus was extracted by homogenizing twice for 30 s with 0.5 volume of fluorocarbon (Arklone). The homogenate was centrifuged at  $1,600 \times g$  for 10 min at  $4^{\circ}\text{C}$  to separate the phases. The fluorocarbon phase was reextracted with 2 volumes of 0.002 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5). The supernatant fluids containing the virus from both extractions were pooled and centrifuged at  $90,000 \times g$  for 1 h. The pellets containing the virus and some cellular debris were resuspended in phosphate-buffered saline (pH 7.4) containing 0.2% bovine serum albumin to about  $\frac{1}{2}$  the original volume. Aliquots were initially stored at  $-70^{\circ}\text{C}$  and then at  $4^{\circ}\text{C}$  during use as ELISA antigen. No loss of antigenicity was observed over a 2-month period. The number of particles in the preparation was rated as  $10^7$  to  $10^8$ /ml by electron microscopy. Control antigen was prepared by the same method from uninfected PMK cells.

**Preparation of plates for ELISA.** Volumes of 0.05 ml of antigen dilutions in 0.06 M sodium carbonate buffer (pH 9.6) were incubated at  $37^{\circ}\text{C}$  for 3 h and

then at 4°C overnight in flat-bottomed wells of polyvinyl plates (model FB48; Linbro Chemical Co., New Haven, Conn.). The optimal dilution of the antigen was found to be 1:640 by checkerboard titration (3). Control antigen was used at the same dilution.

**Micro-ELISA.** The procedure followed was based on that of Bidwell et al. (3). Before the assay, the antigen-coated plates were given three washes each of at least 3 min by flooding the wells with wash fluid (phosphate-buffered saline [pH 7.4] containing 0.05% [vol/vol] Tween 20), and were shaken dry. Samples of 0.025 ml of test sera diluted 1:4 in phosphate-buffered saline plus 1% bovine serum albumin, fraction V (Commonwealth Serum Laboratories, Melbourne, Australia), were serially twofold diluted in the wells of the sensitized plates and incubated at 37°C for 30 min. The plates were washed as before with wash fluid and then shaken dry. A 0.05-ml sample of antiglobulin conjugate optimally diluted in phosphate-buffered saline-1% bovine serum albumin (1:600 in this case, again determined by checkerboard titration) was added to each well and incubated at 37°C for 1 h, after which the plates were again washed and shaken dry. Finally, 0.10 ml of the enzyme substrate was added to each well. To prepare the substrate, 5-aminosalicylic acid (Aldrich Chemical Co. Inc., Milwaukee, Wis.) was prepared fresh daily by dissolving 80 mg of the acid in 100 ml of hot distilled water (80°C). This solution was cooled and stored at 4°C. Immediately before use, a portion of the solution was warmed to room temperature and the pH was brought to 6.0 with 1 M NaOH. To 9 parts of 5-aminosalicylic acid solution, 1 part of 0.05% (wt/vol) hydrogen peroxide was added. The enzymic action on substrate was stopped after 90 min at room temperature by the addition of 0.025 ml of 1 M NaOH. The brown reaction product was evaluated visually. For comparison purposes, some results were measured by absorbance at 403 nm, which was determined in a Zeiss spectrophotometer with a 1-cm light path. The last serum dilution that showed a darker color than the control which contained no test serum (conjugate control) was regarded as the end point.

**Control reactions for ELISA.** The following control reactions were routinely performed: (i) conjugate control (i.e., antigen-coated well to which conjugate was added and, after proper incubation and washing, the substrate was then added); (ii) positive human serum control (i.e., using the positive reference serum of known titer).

One hundred sera (50 Aboriginal and 50 European, but otherwise randomly selected) were tested against control antigen. Four sera (all European) reacted to titers comparable to those obtained against SA-11 antigen, and two others to much lower titers. For reasons of economy and because Aboriginal sera were our primary interest, we did not test all sera against control antigen, but it would be preferable to do so. The suggested procedure is to screen sera at a 1:8 dilution against control antigen, then titrate out the sera of reactors to determine whether the test results can be accepted.

**Preparation of CF antigen.** SA-11 virus was grown in PMK cells and harvested according to the method described above. The culture was frozen and thawed three times to release the virus particles and

then centrifuged at  $750 \times g$  for 20 min to remove the large cell debris. The supernatant fluid containing the virus was concentrated 10-fold by pelleting at  $90,000 \times g$  for 1 h and suspending the pellet in  $\frac{1}{10}$  volume of maintenance medium. Checkerboard titration of this preparation against the positive reference serum showed that it contained 16 U of CF antigen and that it was not anticomplementary. The infectivity titer of the SA-11 virus was approximately  $5 \times 10^6$  fluorescent foci per ml (determined in cover-slip cultures of PMK cells), and the number of particles was rated as  $10^7$  to  $10^8$ /ml by electron microscopy. Antigen was then stored in working volumes at  $-70^\circ\text{C}$ , thawed once prior to use, and diluted 1:8 for use as CF antigen. With preliminary batches of CF antigen, it was observed that every additional freezing and thawing of samples resulted in further loss of antigenicity.

**CF control antigen.** Control antigen was prepared from uninfected PMK cells by the procedure described above and used unconcentrated. It was found not to be anticomplementary.

**CF test.** The CF test was a microtitration method based on that of the U.S. Public Health Service (39). Serum dilutions commenced at 1:4, and two 50% hemolytic complement doses of complement (stabilized, from guinea pig; Commonwealth Serum Laboratories, Melbourne, Australia) were used in each test. Sera which gave a reciprocal titer of less than or equal to 4 were recorded as negative, and those which gave a reciprocal titer of greater than or equal to 8 were recorded as positive. Disposable polystyrene plates (model M-24A; Dynatech Laboratories Inc., Alexandria, Va.) were used and recycled by the washing procedure of Bradstreet and Taylor (7).

## RESULTS

**Comparison of ELISA with CF test for quantitation of rotavirus antibodies in sera.** In all, 430 sera were tested for rotavirus antibodies by each test. At first, all sera tested by ELISA were found to have titers of 1:16 or greater, but eventually negative (titer 1:2) sera were obtained from neonates so that the titer of 1:16 could be assessed as genuinely positive. ELISA titers were higher than CF titers on the same sera in 97.5% of cases. The average ratio (in logs to base 2) between the CF and ELISA titers was calculated to be  $4.2 \pm 2.1$  (standard deviation), i.e., an actual ratio of approximately 16. The modal number of the ratio distribution was 4 (Table 1). The results showed that ELISA was, on an average, 16 times more sensitive than CF. Figure 1 shows the relation between rotavirus antibody titers obtained by ELISA and by CF on 200 of the sera. The group of sera with titers relatively low by CF and high by ELISA are believed to be those in which IgA makes a significant contribution to the overall titer (L. H. Ghose, R. D. Schnagl, and I. H. Holmes, manuscript in preparation).

**Reproducibility of ELISA.** The reproducibility of the ELISA test was determined by

titration of 63 different sera in two separate trials. Analysis of the data (Table 2) showed that in 86% of cases either the titers were the same or there was a twofold difference. A fourfold or greater discrepancy was observed in 14%. Repeated tests on the reference positive serum over a 3-month period gave results which did not vary more than twofold.

**Application of ELISA and CF tests to a seroepidemiological survey among Aborigines and non-Aboriginal Australians. (i) Prevalence of rotavirus antibodies.** The percentage of sera in which titers of greater than or equal to 1:16 were detected by ELISA among different age groups of an Aboriginal and a European population are shown in Fig. 2. Antibodies were detected in 100% of all sera tested in both populations, by this test.

The percentage of sera in which titers of greater than or equal to 1:8 were detected by CF among the different age groups of the same Aboriginal and European populations is shown

TABLE 1. *Reproducibility of ELISA titer for human rotavirus on 63 sera tested on 2 separate days*

Change in titer <sup>a</sup>	Frequency	% Frequency
0	40	63.5
1	14	22
2	7	11
3	2	3

<sup>a</sup> Expressed in logs to base 2.

in Fig. 3. In the Aboriginal population, the proportion of each age group with detectable antibody remained relatively high, with a minimum of 53% positive (0 to 12 months) and a maximum of 94% positive (50 to 59 years). The prevalence of detectable antibody in the European population was generally lower for each age group, with a minimum of 33% positive (50 to 59 years) and reaching a maximum of 71% positive (10 to 19 years). The calculated average incidence of CF antibody in the Aboriginal population was 71%, as compared with 45% in the European population.

**(ii) Mean titers of rotavirus antibodies.** Geometric means of titers by ELISA for each age group in both populations are shown in Fig. 4. The average level of antibody in each age

TABLE 2. *Distribution of sensitivity ratios between CF and ELISA titers on 430 sera tested for human rotavirus antibody*

Ratios <sup>a</sup>	Frequency	% Frequency
0	10	2.5
1	28	7
2	42	10.5
3	68	17
4	112	28
5	67	17
6	39	10
7	32	8

<sup>a</sup> Difference between CF and ELISA titers in logs to base 2.

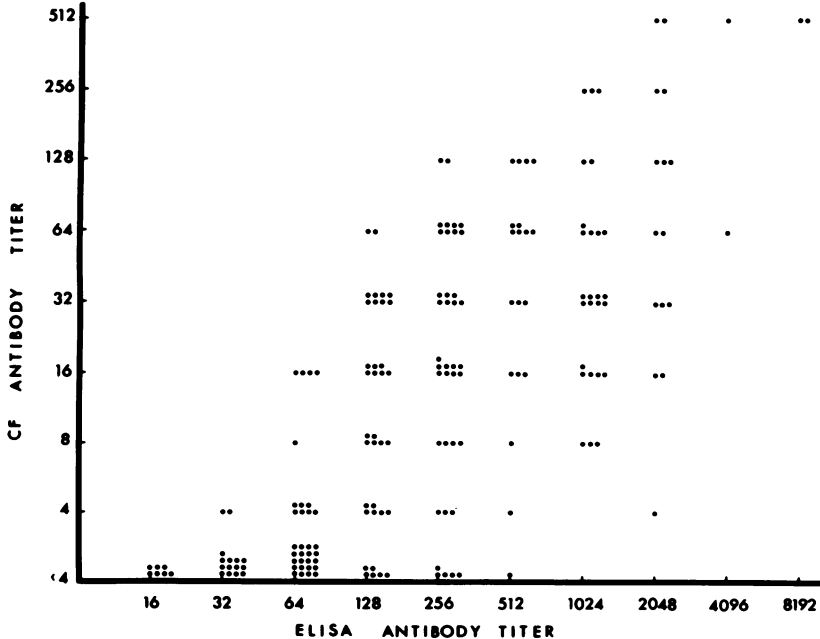


FIG. 1. *Relationship between rotavirus antibody titers obtained by ELISA and CF.*

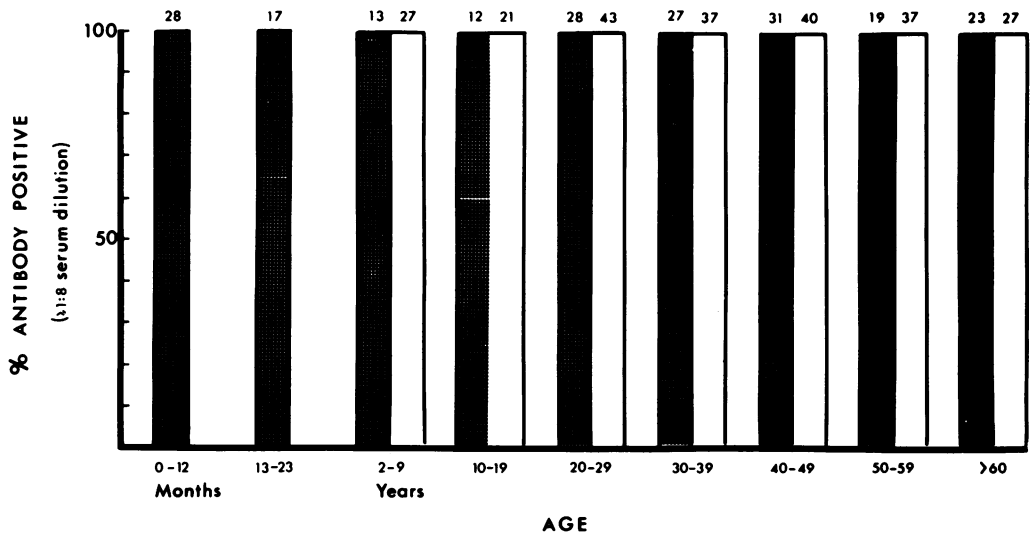


FIG. 2. Prevalence of rotavirus ELISA antibodies in different age groups in Aboriginal and European populations in Australia. (Number above column indicates total number of sera tested.) Blank column, European; hatched column, Aboriginal.

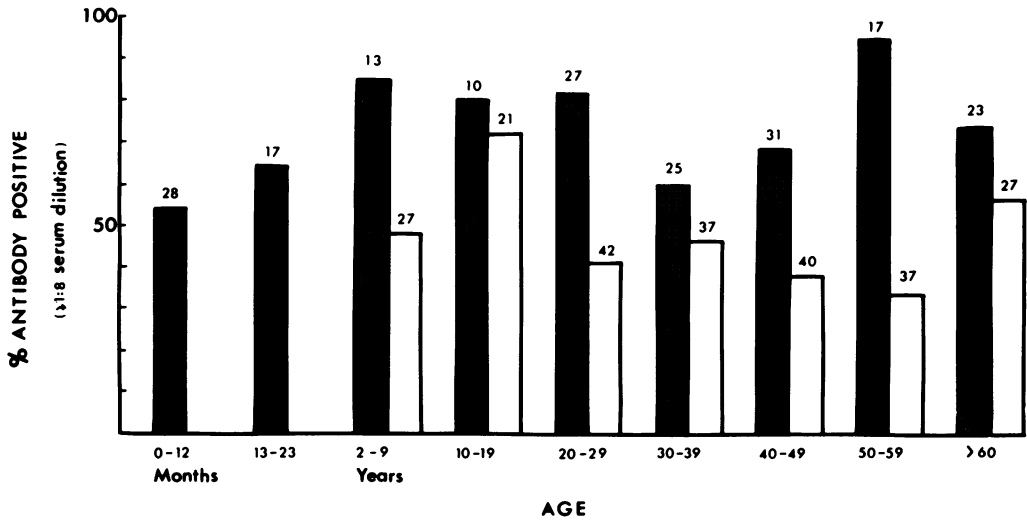


FIG. 3. Prevalence of rotavirus CF antibodies in different age groups in Aboriginal and European populations in Australia. (Number above column indicates total number of sera tested.) Blank column, European; hatched column, Aboriginal.

group remained higher among the Aboriginal population.

In the Aboriginal population, the level of antibody increased rapidly with age to a peak (geometric mean titer, 290) in the 10- to 19-year-old group. The level remained high over the next 20 years, then increased again to 373.5 in the 40- to 49-year group and to 385 in the 50- to 59-year group.

In the European population the titers were lower, generally about 50 to 60% of the levels

found in Aborigines, but the trends with age were comparable. In both populations it was clear that rotavirus antibody titers increased during adult life.

(ii) **Comparative geometric mean titers of ELISA and CF in the seroepidemiological survey of the Aboriginal population.** Figure 5 shows geometric mean of titers, both ELISA and CF, for each age group in the Aboriginal population. Similar patterns were observed in both the serological tests. ELISA titers were

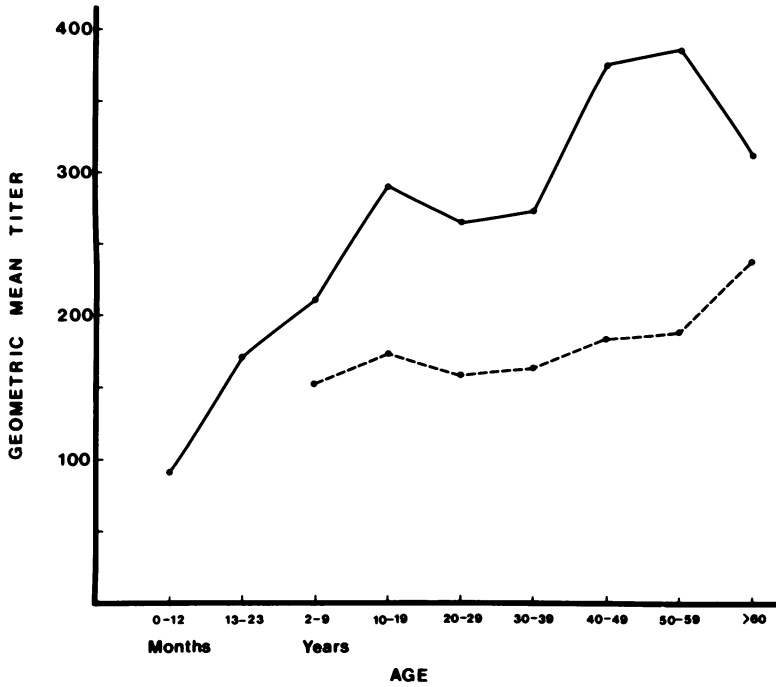


FIG. 4. Geometric means of rotavirus ELISA titers in the different age groups of the Aboriginal and European populations. (---) European; (—) Aboriginal.

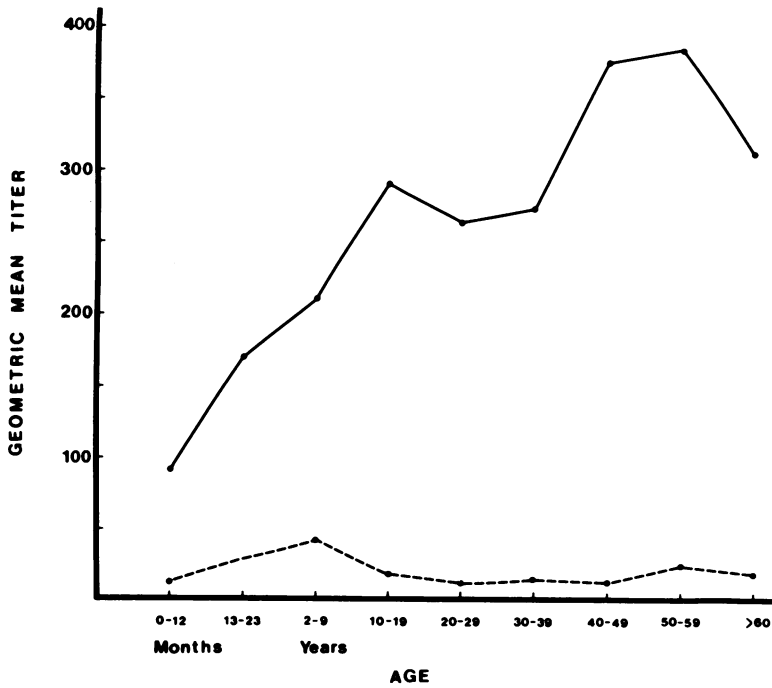


FIG. 5. Geometric means of rotavirus ELISA and CF titers in the different age groups of the Aboriginal population. (---) CF; (—) ELISA.

higher than CF titers for all the age groups, but the increased sensitivity of the ELISA test over that of CF was most marked with sera from subjects over 10 years of age.

### DISCUSSION

The micro-ELISA is a useful addition to the range of serological tests applied to epidemiological surveys. Compared with CF, which has been used for rotavirus epidemiological studies, ELISA is not only more sensitive but also capable of detecting IgA antibody, which is likely to be particularly important since rotavirus infections are limited to the gut (16). With minor modifications, the assay should be capable of quantitation of antibodies of each immunoglobulin class in sera and secretions (L. H. Ghose, R. D. Schnagl, and I. H. Holmes, manuscript in preparation).

The results show that even when the antigen is SA-11 virus, as in this study, the test appears to be adequately sensitive in detecting antibodies to human rotavirus. SA-11 virus has been shown to be an efficient substitute for human rotavirus in CF tests, but to a lesser degree than the O agent (18). Quarantine regulations in Australia and elsewhere may hinder the use of the O agent. There are difficulties in obtaining sufficient quantities of human rotavirus, and the presence of tightly bound antibodies in "purified" human fecal virus preparations (10) is likely to give rise to background reactions in conjugate controls if such preparations are used as antigens in the present type of ELISA. Although human rotavirus grown in gnotobiotic calves is probably the ideal (44), it is both costly and difficult to produce.

Schoub et al. (35) have also concluded that SA-11 virus is a very useful agent for use in the serodiagnosis of human rotavirus infection.

A number of alternatives were tested during development of the ELISA described here, and although detailed results have not been given they are worth recording in this discussion. First, with SA-11 virus as antigen, polyvinyl plates absorbed antigen much more efficiently than did polystyrene ones. Pretreatment of plates with poly-L-lysine, glutaraldehyde-fixed bovine serum albumin, or rabbit anti-rotavirus serum decreased, rather than increased, the effective binding of the antigen.

The periodate method of Nakane and Kawaoi (28) for conjugation of horseradish peroxidase with the anti-human globulin produced a conjugate which was comparable to or slightly more active than a conjugate prepared by the two-step glutaraldehyde method of Avrameas and Ternynck (1) with the same batch of globulin,

and relatively less enzyme was used in the former method. Of the substrates tested, *o*-dianisidine hydrochloride formed an insoluble product which was unsuitable for use in this system, whereas *o*-phenylenediamine produced a yellow/orange soluble product but was less sensitive than 5-aminosalicylic acid for detecting low concentrations of horseradish peroxidase. In addition, the relatively deeper color of the purplish-brown product formed with 5-aminosalicylic acid was particularly preferable for visual reading of the test. When visual readings were compared with those made by spectrophotometer (403 nm) on 1:5 dilutions of well contents, 9 out of 12 sera gave the same reading and the remaining 3 sera gave a titer of one twofold dilution higher when read spectrophotometrically. The criterion for the spectrophotometric end point was an absorbance reading two to three times that obtained with the conjugate control.

With micro-ELISA techniques in particular, reagents are inexpensive and stable. The conjugate prepared by the periodate method was diluted 1:600 just before use; it was used for 3 months without any detectable deterioration. Others (32) have reported as much as a year-long storage period without any adverse effects. The problem of the sterility of the conjugate does, however, arise. Sodium azide cannot be used since it is an inhibitor of peroxidase. Other possible ways to ensure sterility are to filter the conjugate or add 0.05% phenol (final concentration) as reported by Saunders and Clinard (32). The antimicrobial agent Merthiolate ( $0.5 \times 10^{-4}$  M Thimerosal) has been reported not to interfere with horseradish peroxidase activity (6) and will be tested in the future.

The results of this ELISA survey indicate that 100% of the age groups tested in both Aboriginal and non-Aboriginal populations had anti-rotavirus antibody. The relatively low ELISA antibody levels in Aboriginal babies up to 12 months of age explains the failure of the less-sensitive CF test to detect antibody in almost half of them. Since almost all sera contain antibody as detected by the ELISA system, the actual titers become most important in interpreting results. The comparison of ELISA and CF titers in Aborigines of different ages, as shown in Fig. 5, suggests that for some reason the relative sensitivity of ELISA increases over the first few years of life, and it remains to be determined whether this reflects a change in the quality or the immunoglobulin class of the antibody.

Previous surveys have indicated an increased prevalence of antibody in adults of child-bearing age (15, 26), and evidence of rotavirus infection

in parents of children with enteritis has been obtained (20, 45). The continuing rise in antibody titers in later life, which is most evident in the Aboriginal population in this study (Fig. 4), suggests that intermittent rotavirus infections may continue to boost the serum antibody levels of individuals throughout life. It is not known whether such infections produce symptoms, but these results confirm the previous finding that serum antibody is not necessarily protective against rotavirus infection (20, 43).

In the rotavirus system, the development of such a versatile serological tool opens up possibilities for a great number of new studies. For example, it should facilitate studies of the immune response after acute rotavirus infection. However, additional studies on paired sera from cases of rotavirus gastroenteritis and cases of gastroenteritis from other causes will be required to establish the reliability of ELISA for serodiagnosis of current infections. It remains to be shown whether the presence of high levels of IgM or IgA class rotavirus antibodies in sera or mucosal secretions indicate recent infection. Finally, although the detection and measurement of human antibodies to rotavirus have been described, by using appropriate anti-species immunoglobulins, ELISA can be used for similar studies in other animals.

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