Rotavirus Epidemiology in Vellore, South India: Group, Subgroup, Serotype, and Electrophoretype

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Rotaviruses were detected in 163 of 916 (17.8%) specimens collected from children under 3 years of age with gastroenteritis in Vellore, South India, between August 1983 and July 1985. Rotaviruses were detected throughout the study period, with a peak prevalence in December to February (winter) and June to August (southwest monsoon season). A total of 117 rotavirus strains were tested for subgroup, serotype, and rotavirus double-stranded RNA electrophoretic migration pattern; 24.8% of the strains tested were subgroup I, 69.2% were subgroup II, and 6.0% were neither subgroup I nor subgroup II. Subgroup I and II strains were circulating concurrently throughout the study. Of the 117 rotavirus strains, 32 (27.4%) were serotyped; 15 were serotype 1, 3 were serotype 2, 2 were serotype 3, and 12 were serotype 4. Three serotypes were circulating concurrently during the periods of peak rotavirus prevalence. In 100 of the 117 strains (85.4%) an RNA pattern was detected. One unusual subgroup I group A rotavirus with a long migration pattern and four atypical rotaviruses serologically related to group C were also detected.

Rotavirus is the most common pathogen detected in cases of infantile diarrhea in many parts of the world (15). It has been estimated that 5,000,000 deaths each year are caused by diarrhea in children under 5 years of age and that rotavirus may be associated with 20% of them (9). The World Health Organization is actively encouraging the development of vaccines to control rotavirus infections, and two candidate vaccines have already undergone clinical trials (11, 14). To develop the best strategy for using these vaccines it is necessary to understand the antigenic diversity of rotaviruses in different communities.

Three important rotavirus antigens have been described: the group antigen associated with the major inner capsid protein, designated VP6 (molecular size, 45 kilodaltons), the subgroup antigen also associated with the VP6 protein, and the serotype antigen associated with the two major outer capsid proteins VP7 (molecular size, 35 kilodaltons) and VP3 (molecular size, 80 to 88 kilodaltons). The contribution of these antigens to immunity to rotavirus infection is not yet completely clear, but experiments with animals suggest that serotype specificity is important (12, 24, 25).

Most epidemiological studies of rotavirus have been based on the detection of the common group antigen by an enzyme-linked immunosorbent assay (ELISA) or on the observation of rotavirus particles by electron microscopy (EM) (15). More recent epidemiological studies have been based on variations in the pattern of rotavirus double-stranded RNA genome segments in polyacrylamide gel electrophoresis (PAGE) (10) and on subgroup variations. However, there have been very few studies of serotype variations in different communities, owing to the lack of a suitable assay.

We report here an analysis of the rotavirus strains detected in Vellore, South India, over 2 years (1983 to 1985) in cases of diarrhea in children under 3 years of age. Vellore is a town of 300,000 inhabitants, but the hospital draws patients from a larger surrounding community. Vellore is situated 140

km inland from Madras at a height of 900 ft (274.32 m) above sea level; the climate is notable for its extreme dry heat in summer and two unreliable monsoons. Samples were tested for group, subgroup, and serotype antigens by ELISA, examined by EM, and analyzed by PAGE.

MATERIALS AND METHODS

Fecal specimens. Specimens were collected from children between 1 and 35 months old at the Child Health Department, Christian Medical College Hospital, Vellore, South India. Case patients were selected by proportionate, random sampling from all patients who had diarrhea lasting less than 72 h, who had not received any antibiotic or antiparasitic treatment in the 10 days before sample collection, and who had no complicating illness. Case patients were selected at the rate of 8 to 12 per week, evenly distributed through the age range, and the collections took place from August 1983 to July 1985. A total of 916 case patients were included in the study from the over 2,000 patients who fulfilled the study criteria. The prevalence of other viral and bacterial pathogens and the clinical aspects of this study will be described in detail elsewhere (M. Mathan, submitted for publication).

Detection of rotavirus by EM. An approximately 10% suspension of each fecal specimen was made in phosphate-buffered saline by vigorous homogenization in a vortex mixer for 2 to 3 min. The suspension was then clarified (10,000 × g for 30 min), and the supernatant was pelleted at 50,000 × g for 90 min. The pellet was suspended in 1 or 2 drops of distilled water, placed on a Parlodion-coated grid, and negatively stained with 3% ammonium molybdate. The grids were examined with a Philips EM 200. Immune EM studies for the confirmation of group C rotavirus were performed by the method of Cubitt et al. (8). Guinea pig serum 444 used in the immune EM studies was raised against a group C human rotavirus (5066) and had previously been shown to be group C specific (5).

Detection of rotavirus by ELISA. An approximately 10% suspension of each fecal specimen was made in phosphate-
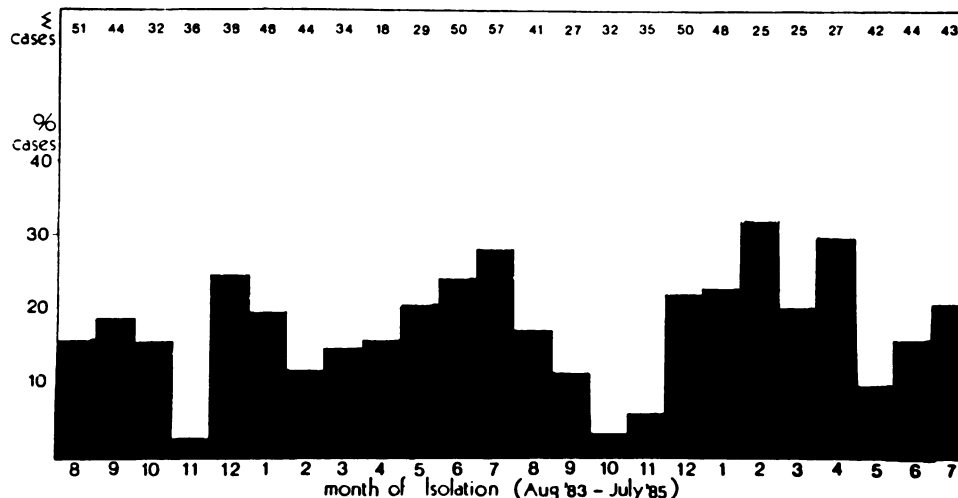
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FIG. 1. Monthly prevalence of rotavirus in case patients with diarrhea in Vellore, South India. The total number of samples tested in each month is shown at the top.

buffered saline and clarified by low-speed centrifugation ($2,000 \times g$ for 10 min). Rotaviruses were detected with a group A-specific ELISA based on polyclonal sera (3) provided by the World Health Organization Reference Laboratory, East Birmingham Hospital, Birmingham, United Kingdom.

Detection of rotavirus double-stranded RNA by PAGE. Rotavirus double-stranded RNA was extracted from 0.5 ml of 10% fecal suspensions, and PAGE was performed as previously described (22).

Subgrouping and serotyping of rotavirus strains. For both subgrouping and serotyping, an NADP-enhanced enzyme immunoassay was used (2). Briefly, polystyrene microdilution plates (Nunc) were coated with a 1/10,000 dilution of serum from a rabbit which had been hyperimmunized with a mixture of rotavirus isolates representing serotypes 1 to 4. The plates were kept overnight at 4°C, and the wells were emptied.

Stool samples (100 µl; 10 to 20% [vol/vol]) extracted in 0.1 M Tris-buffered saline (pH 7.5) containing 0.1% (vol/vol) Tween and 3% (wt/vol) bovine serum albumin (TBS/T/BSA) were added to each of 16 wells in pairs across plates. The plates were kept at 4°C overnight and washed six times with TBS/T.

Monoclonal antibodies as ascitic fluids were diluted 1/10,000 in TBS/T/BSA, and 100 µl was added to two wells for each antibody; eight different monoclonal antibodies were used on each plate (A, SGI, SGII, 60, RV 4:2, RV 5:3, RV 3:1, and ST3). (Monoclonal antisera RV 4:2, RV 5:3, RV 3:1, and ST3 were generous gifts from B. Coulson. These monoclonal antibodies are serotype specific and represent serotypes 1 to 4, respectively [7]. Monoclonal antisera 60, SGI, and SGII were generous gifts from H. Greenberg and R. D. Shaw [21]. Antibody 60 is cross-reactive with VP7 of different serotypes and was used as a control for complete particles.) The plates were incubated for 2 h at 37°C and washed.

Anti-mouse polyvalent gamma globulin-alkaline phosphatase conjugate (Sigma Chemical Co.) was diluted 1/2,000 in TBS/T/BSA, and 100 µl was added to each well. The plates were incubated for 1.5 h at 37°C and washed six times with TBS/T.

NADP-substrate (100 µl; IQ Bio Ltd., Cambridge, United Kingdom) was added to each well, and the plates were left at room temperature (approximately 22 to 24°C) for 15 min. The plates were not washed. Ethanol-INT violet amplifier solution (200 µl; IQ Bio) was added to the 100 µl of NADP-substrate. Great care was taken not to contaminate adjacent wells. The amplifier was added in the same order as the substrate, and approximately the same time was taken to do this. The reaction was stopped with 3 M H_2SO_4 after 15 min. Optical densities were read at 492 nm.

Samples were considered to give a positive result with any serotype-specific monoclonal antibody if the optical density obtained was at least 2.5 times the value of the average optical densities obtained with the other antibodies.

RESULTS

Seasonal variation of rotavirus. Rotavirus was identified in 163 (17.8%) of the 916 case patients with diarrhea examined over 2 years (Fig. 1). The prevalence varied between 2.7% in November 1983 and 32% in February 1985. Two peaks of rotavirus prevalence were detected in both years of the study: (i) in December to February, the coolest period of the year, following the northeast monsoon in October and November, and (ii) in June to August, the season of the southwest monsoon and a hot and humid time of the year.

Detection of rotavirus by EM and ELISA. A total of 163 rotavirus-positive samples were detected by either EM or ELISA for the group antigen. Rotavirus was detected in 130 (79.8%) of the specimens by both methods, in 27 specimens (16.5%) by EM alone, and in 6 specimens (3.6%) by ELISA alone. The six ELISA-positive, EM-negative specimens were all confirmed as rotavirus by the subgrouping ELISA (two subgroup I and four subgroup II strains) and by PAGE (two short and four long electrophoretic migration patterns). Of the 27 specimens positive for rotavirus by EM but negative by ELISA, sufficient material was available from 10 specimens for detailed analysis at a second laboratory (World Health Organization Reference Laboratory, East Birmingham Hospital). Of these 10 specimens, 4 gave positive group and subgroup results on testing by the NADP-amplified ELISA and therefore represent false-negative ELISA results in Vellore; of the 10 specimens, 2 were negative by EM, ELISA, and PAGE, and 4 were confirmed as EM positive, PAGE positive, and ELISA negative. The electrophoretic patterns of two of these EM-positive,
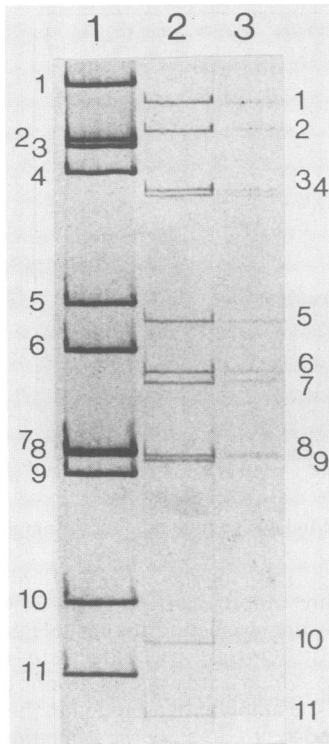
FIG. 2. Electrophoretic migration patterns of rotavirus double-stranded RNA. Lanes: 1, group A subgroup II rotavirus control; 2 and 3, atypical rotavirus strains detected in Vellore, showing the loss of the closely migrating triplet (segments 7, 8, and 9) which is characteristic of group A rotaviruses.

ELISA-negative strains are shown in Fig. 2; all 4 strains showed similar but not identical patterns. The two strains in Fig. 2 (lanes 2 and 3) were different from "typical" group A rotavirus strains in two ways: the tight triplet was broken (segments 7, 8, and 9), with segment 7 migrating more closely to segment 6, and the genome profile was longer than that of typical group A subgroup II strains. These two rotavirus strains were shown to be agglutinated by an antiserum to group C rotavirus when tested by immune EM. Of the 17 specimens which were EM positive and ELISA negative in Vellore but which were not tested further, only one rotavirus particle was seen in 6 by EM, and in four others the rotaviruses seen were aggregated, indicating that host antibody might be blocking the ELISA reaction.

Subgrouping, serotyping, and electropherotyping of rotavirus isolates. A total of 117 of the rotavirus-containing specimens identified in this study were examined in detail. These were all selected from the EM-positive, ELISA-positive group. The resulting monthly distribution of these 117 strains by subgroup and serotype is shown in Fig. 3. A total of 29 strains were subgroup I (24.8%), 81 were subgroup II (69.2%), and 7 (6.0%) were neither subgroup I nor subgroup II. Both subgroup I and subgroup II strains were found to be circulating concurrently throughout the study. Only 32 of 117 strains (27.4%) could be serotyped, and of these, 15 (46.9%) were serotype 1, 3 (9.3%) were serotype 2, 2 (6.3%) were serotype 3, and 12 (37.5%) were serotype 4. A further 14 samples (11.9%) reacted positively with a VP7-specific monoclonal antibody but not with the four serotype-specific monoclonal antibodies. Of these 14 specimens, 12 contained detectable amounts of RNA (10 short and 2 long patterns). None of them showed an unusual distribution of RNA segments. A total of 11 were typed as subgroup I, and 3 were typed as subgroup II. Five subgroup I strains were identified in August 1984, and the other nine strains were distributed evenly throughout the period of the study. A total of 71 strains showed no reaction with any of the monoclonal antibodies used.

Of the 117 specimens, 100 (85.4%) contained sufficient RNA for PAGE; 75 had long patterns, and 25 had short patterns. Of the 81 subgroup II rotaviruses, 71 (87.7%) had detectable RNA, and all of these had long patterns. Of the 29 subgroup I rotaviruses examined, 25 (86.2%) contained detectable RNA; 24 had short patterns and 1 had a long pattern. Of the seven rotaviruses that could not be subgrouped, three had long patterns and 1 had a short pattern.

DISCUSSION

Several different epidemiological patterns of rotavirus infection have been described, and these vary with the
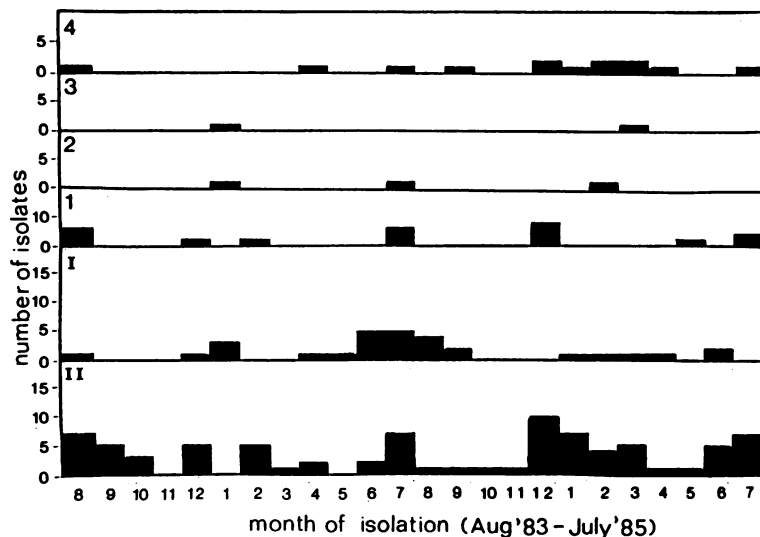

FIG. 3. Monthly distribution of rotaviruses in Vellore, South India, by subgroup (I and II) and serotype (1, 2, 3, and 4).

nature of the community and the climate of the area studied. In general, studies in temperate climates have described a winter peak, whereas those in tropical areas have described a more uniform distribution throughout the year (15, 19). Vellore is situated on latitude 13° N, and although rotaviruses were detected throughout the year, two peaks of rotavirus prevalence were detected, one in June to August, coinciding with the southwest monsoon, and one in December to February, associated with the cooler temperatures of winter. These findings confirm and extend earlier observations (16), although the overall prevalence of rotavirus (17.8%) was lower in this study. Reports of the prevalence of rotavirus in different hospital-based studies have revealed a median rate of 34% (range, 12 to 71%) (9), and the low prevalence reported here probably reflects the environment and population studied.

Previous studies of subgroup distributions have revealed a predominance of subgroup II rotaviruses similar to that described here (15). However, because of the difficulties of growing and typing rotaviruses by neutralization, there have been very few published studies investigating the distribution of rotavirus serotypes in different epidemiological settings (2, 21). In a previous study with the NADP-enhanced ELISA, 75% of the fresh rotavirus samples tested could be serotyped (2). In contrast, only 27.4% of the stored rotavirus samples tested could be serotyped in this study, although the sensitivity of the assay, as demonstrated by the use of the same controls, was similar in both studies. All of the samples were stored as neat feces at −70°C for the 2 to 4 years until testing, and they were then transported to the United Kingdom as 10% suspensions in Tris-CaCl$_2$ buffer. This retrospective study represents the first full evaluation of the NADP-enhanced serotyping ELISA. The likely explanation of the low success rate in serotyping is the finding that most of the nonserotypable specimens (71 of 85, or 83.5%) did not react with monoclonal antibody 60, a VP7-cross-reactive antibody. This finding suggests that the virus particles had degraded under the conditions of storage and transport used, and we suggest that for serotyping, specimens of feces should be transported without dilution. Alternatively, it may be necessary to do the serotyping assay rapidly after detecting the rotavirus, preferably at the site of primary detection.

There is much interest at present in the application of a rotavirus vaccine to the prevention of diarrhea resulting from rotavirus infection. Evidence from animals suggests that immunity is predominantly serotype specific (12, 24, 25). This suggestion is in contrast to the observation in infants that a primary rotavirus infection, either symptomatic or asymptomatic, may prevent illness caused by, if not infection with, different rotavirus serotypes (4). We were able to demonstrate three rotavirus serotypes cocirculating in each of the peak seasons, a result which may reflect the normal situation in a large, high-density population. Thus, it would appear that any vaccine must be cross-protective to have any impact on rotavirus diarrhea in communities like Vellore, and because of the low prevalence of rotavirus (17.8%), the findings of this study suggest that even with an effective rotavirus vaccine the perceived impact on diarrheal disease of a vaccination campaign in this population would be limited.

Fourteen rotavirus samples examined here were shown to react with a VP7-specific monoclonal antibody (23) but not with the serotype-specific monoclonal antibodies. This result suggests that intact virus particles which did not belong to serotypes 1 to 4 were present. Alternatively, these strains may have been "monotypes" (6); that is, they were serotype

1 to 4 rotaviruses, but they did not react with the particular monoclonal antibodies used in this study. The resolution of these alternative explanations awaits the more extensive application of monoclonal antibody-based serotyping assays and of sequencing (13) to rotavirus strains. However, the high number of nonreactive strains detected in this study suggests that a wider range of monoclonal antibodies needs to be used in these assays.

The distribution of rotavirus electrophorotypes has been extensively investigated, and it has been found that the technique of electropherotyping has limited epidemiological significance because the individual patterns cannot be related to antigenic changes and also because it is difficult to relate changes in electropherotype to the relatedness of different rotaviruses (1, 10). However, as we have shown, when electropherotyping is used in conjunction with serological techniques, it can help to reveal atypical and unusual rotaviruses, and it is a valuable confirmatory diagnostic test. One rotavirus strain that was detected in a child and that belonged to subgroup I had a long pattern in PAGE. Only one similar strain has been reported previously (17, 18). These strains are clearly uncommon, and they might represent rare zoonotic infections, since this type of pattern is commonly seen among animal rotaviruses.

The classification of non-group A rotaviruses is based on serological differences and terminal oligonucleotide mapping (20). However, gross perturbation of the electrophoretic pattern has been accepted as a useful indicator of rotavirus group. It is probable that the four atypical strains detected here are group C rotaviruses on the basis of their electropherotypes and serological reactions detected by immune EM with a group C-specific serum. There have been several reports of atypical rotaviruses in humans, and in one, group C rotaviruses were confirmed (5). There was no evidence of human-to-human spread of the Vellore atypical rotavirus isolates. All four atypical rotaviruses were detected in children under 1 year of age, and all of these children had mild diarrhea with only slight dehydration. It remains unclear if these strains represent zoonotic infections or if group C rotaviruses are circulating in humans in Vellore. The finding of four atypical strains over a 2-year period may reflect an increased awareness of these strains or an increasing prevalence of these atypical viruses. However, as this study has shown, they do not presently cause a significant proportion of diarrhea cases in Vellore.

ACKNOWLEDGMENTS

LITERATURE CITED

1. **Beards, G. M.** 1982. Polymorphism of genomic RNAs within rotavirus serotypes and subgroups. Arch. Virol. **74:**65–70.
2. **Beards, G. M.** 1987. Serotyping of rotavirus by NADP-enhanced enzyme-immunoassay. J. Virol. Methods **18:**77–85.
3. **Beards, G. M., A. D. Campbell, N. R. Cottrell, J. S. M. Peiris, N. Rees, R. C. Sanders, J. A. Shirley, H. A. Wood, and T. H. Flewett.** 1984. Enzyme-linked immunosorbent assays based on polyclonal and monoclonal antibodies for rotavirus detection. J. Clin. Microbiol. **19:**248–254.
4. **Bishop, R. F., D. J. S. Cameron, A. A. Veenstra, and G. L. Barnes.** 1983. Clinical immunity after neonatal rotavirus infec-

tion, a prospective longitudinal study in young children. N. Engl. J. Med. **309:**72–76.

5. **Bridger, J. C., S. Pedley, and M. A. McCrae.** 1986. Group C rotaviruses in humans. J. Clin. Microbiol. **23:**760–763.

6. **Coulson, B. S.** 1987. Variation in neutralising epitopes of human rotaviruses in relation to genomic RNA polymorphism. Virology **159:**209–216.

7. **Coulson, B. S., J. M. Tursi, W. J. McAdam, and R. F. Bishop.** 1986. Derivation of neutralising monoclonal antibodies to human rotaviruses and evidence that an immunodominant site is shared between serotypes 1 and 3. Virology **154:**302–312.

8. **Cubitt, W. D., D. A. McSwiggan, and W. Moore.** 1979. Winter vomiting disease caused by calicivirus. J. Clin. Pathol. **32:**786–793.

9. **De Zoysa, I., and R. G. Feachem.** 1985. Interventions for the control of diarrhoeal diseases among young children: rotavirus and cholera immunization. Bull. W.H.O. **63:**569–583.

10. **Estes, M. K., D. Y. Graham, and D. H. Dimitrov.** 1984. The molecular epidemiology of rotavirus gastroenteritis. Prog. Med. Virol. **29:**1–22.

11. **Flores, J., I. Peres-Schael, M. Gonzales, D. Garcia, M. Perez, N. Daoua, W. Cunto, R. M. Chanock, and A. Z. Kapikian.** 1987. Protection against severe rotavirus diarrhoea by rhesus rotavirus vaccine in Venezuelan infants. Lancet i:882–884.

12. **Gaul, S. K., T. F. Simpson, G. N. Woode, and R. W. Fulton.** 1982. Antigenic relationships among some animal rotaviruses: virus neutralization in vitro and cross-protection in piglets. J. Clin. Microbiol. **16:**495–503.

13. **Green, K. Y., J. F. Sears, K. Taniguchi, K. Midthun, Y. Hoshino, M. Gorziglia, K. Nishikawa, S. Urasawa, A. J. Kapikian, R. M. Chanock, and J. Flores.** 1988. Prediction of human rotavirus serotype by nucleotide sequence analysis of the VP7 protein gene. J. Virol. **62:**1819–1823.

14. **Hanlon, P., L. Hanlon, K. Marsh, P. Bypass, F. Shenton, M. Hassan-King, O. Jobe, H. Sillah, R. Hayes, B. M. M'Boge, H. C. Wittle, and B. M. Greenwood.** 1987. Trials of an attenuated bovine rotavirus vaccine (RIT 4237) in Gambian infants. Lancet i:1342–1345.

15. **Kapikian, A. Z., and R. M. Chanock.** 1985. Rotaviruses, p.863–906. *In* B. N. Fields (ed.), Virology. Raven Press, Publishers, New York.

16. **Maiya, P. P., S. M. Pereira, M. Mathan, P. Bhat, M. J. Albert, and S. J. Baker.** 1977. Etiology of acute gastroenteritis in infancy and early childhood in southern India. Arch. Dis. Child. **52:**482–485.

17. **Nakagomi, O., T. Nakagomi, Y. Hoshino, J. Flores, and A. Z. Kapikian.** 1987. Genetic analysis of a human rotavirus that belongs to subgroup I but has an RNA pattern typical of subgroup II human rotaviruses. J. Clin. Microbiol. **25:**1159–1164.

18. **Nakagomi, O., T. Nakagomi, H. Oyamada, and T. Suto.** 1985. Relative frequency of human rotavirus subgroups 1 and 2 in Japanese children with acute gastroenteritis. J. Med. Virol. **17:**29–34.

19. **Paniker, C. K. J., S. Mathew, and M. Mathan.** 1982. Rotavirus and acute diarrhoeal disease in children in a southern Indian coastal town. Bull. W.H.O. **60:**123–127.

20. **Pedley, S., J. C. Bridger, J. F. Brown, and M. A. McCrae.** 1983. Molecular characterisation of rotaviruses with distinct group antigens. J. Gen. Virol. **64:**2093–2101.

21. **Rowlands, M. G. M., S. G. J. Goh, K. Williams, A. D. Campbell, G. M. Beards, R. C. Sanders, and T. H. Flewett.** 1985. Epidemiological aspects of rotavirus infection in young Gambian children. Ann. Trop. Paediatr. **5:**23–28.

22. **Sanders, R. C., A. D. Campbell, and M. F. Jenkins.** 1986. Routine detection of human rotavirus by latex agglutination: comparison with enzyme-linked immunosorbent assay, electron microscopy and polyacrylamide gel electrophoresis. J. Virol. Methods **13:**285–290.

23. **Shaw, R. D., D. L. Stoner-Ma, M. K. Estes, and H. B. Greenberg.** 1985. Specific enzyme-linked immunoassay for rotavirus serotypes 1 and 3. J. Clin. Microbiol. **22:**286–291.

24. **Woode, G. N., N. E. Kelso, T. F. Simpson, S. K. Gaul, L. E. Evans, and L. Babiuk.** 1983. Antigenic relationships among some bovine rotaviruses: serum neutralization and cross-protection in gnotobiotic calves. J. Clin. Microbiol. **18:**358–364.

25. **Woode, G. N., S. Zheng, B. I. Rosen, N. Knight, N. E. Kelso Gourley, and R. F. Ramig.** 1987. Protection between different serotypes of bovine rotavirus in gnotobiotic calves: specificity of serum antibody and coproantibody responses. J. Clin. Microbiol. **25:**1052–1058.