

## Anti-VP6 IgG antibodies against group A and group C rotaviruses in South India

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### SUMMARY

In an epidemiological survey from South India, 936 serum samples were tested for IgG against recombinant baculovirus-expressed VP6 proteins from human group A and group C rotaviruses. The overall seroprevalence for group A was 100% and for group C was 25·32% (95% CI 22·64–28·21). The lowest seroprevalence for group C was in children aged <10 years (16·79%). An age-related rise in seroprevalence in group C, but not group A, suggests different patterns of exposure. Seroprevalence was similar in rural and urban subjects, unlike the higher prevalence in rural subjects in studies elsewhere.

**Key words:** Gastrointestinal infections, infectious disease epidemiology, rotavirus, serology.

### INTRODUCTION

Rotaviruses have been known to cause diarrhoeal illness in humans since they were first identified in the duodenal mucosa of children afflicted with acute gastroenteritis [1]. The viruses are typically icosahedral non-enveloped 65–75 nm particles, with a triple-layered protein capsid enclosing an 11-segment double-stranded RNA genome that codes for six structural (VP1–4, VP6, VP7) and six non-structural (NSP 1–6) proteins [2]. Rotaviruses are classified into seven groups (A–G) on the basis of reactivity to the epitopes of the VP6 protein which comprises the middle coat of the virus [3]. Only groups A, B and C are known to cause disease in humans, whereas the remaining four groups are comprised of animal rotaviruses [3]. The group A rotaviruses are the major cause of severe

gastroenteritis in children aged <5 years, resulting in an estimated 611 000 deaths annually [4]. Group B rotaviruses have been reported to cause epidemics of waterborne diarrhoea in China and also cause sporadic disease in adults in India [5, 6].

Group C rotaviruses were initially reported in the USA from young pigs with diarrhoea [7]. Similar porcine viruses were then identified in Europe and Australia [8, 9]. Group C rotaviruses were also reported to cause diarrhoea in dogs [10]. Sporadic diarrhoeal illness caused by group C rotavirus in humans has been reported from all continents, in both developing and developed countries [9, 11–16].

In order to measure exposure to this group of viruses, early seroepidemiological studies to detect antibodies to group C rotavirus used reagents derived from the prototype porcine group C rotavirus ‘Cowden strain’ [15, 17, 18]. However, studies on human group C rotavirus strains have shown that they are antigenically distinct from their porcine counterparts [19]. Recent seroepidemiological studies

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have utilized recombinant VP6 protein utilizing genes from human group C rotavirus strains, produced in the baculovirus protein expression system [20]. The prevalence of antibodies against group C rotaviruses from reported studies have ranged from 3% to 66% [15, 17, 20–22]. Interestingly, most recent studies have shown that the prevalence of antibody positivity increase with increasing age with the maximum prevalence in samples from septuagenarians [20, 22]. This study compared seroprevalence of antibodies to group A and group C rotaviruses in India utilizing recombinant VP6 proteins.

## MATERIALS AND METHODS

### Serum samples

Serum samples were collected in a probability proportional to size cluster survey conducted in the Kaniyambadi block of Vellore district and in the urban wards of Vellore town from August 1999 to February 2000 [23]. Briefly, samples from a rural population comprising of 78 villages in the Kaniyambadi block of Vellore district in South India and from an urban population in Vellore municipality were collected after the selection of households using cluster sampling. The compliance with sample collection was 94.8% for the rural population and 87.7% for the urban population. Serum samples were collected from those aged between 1 and 40 years. To accommodate the rest of the age strata, samples were collected from the Biochemistry laboratory at the Christian Medical College, Vellore, from individuals aged >40 years who were resident in the geographic area of the serosurvey. The studies were approved by the Research Committee of the Christian Medical College, Vellore.

A separate panel of 20 sera from children known to have been infected with different rotavirus genotypes was used to assess three different group A rotavirus VP6 recombinant antigens prepared as described below. The sera were collected 3–6 weeks post-discharge from children hospitalized with dehydrating gastroenteritis with G1P[8] ( $n=8$ ), G2P[4] ( $n=10$ ) and G10P[11] ( $n=2$ ).

### ELISA

#### *Rotavirus group C VP6 IgG ELISA*

All the serum samples were screened for antigroup C rotavirus IgG antibodies in an ELISA format as

previously described using baculovirus-expressed recombinant group C VP6 as antigen [22]. Briefly, 96-well microtitre plates (Falcon, USA) were coated with 100  $\mu$ l/well of a 1:1000 dilution of rabbit polyclonal anti-group C rotavirus VP6 IgG antibodies and incubated overnight at 4 °C. Wells were washed with 0.01 M phosphate buffered saline–Tween-20 (PBS-T) and blocked with 200  $\mu$ l of 5% non-fat milk in PBS for 1 h at 37 °C. After the blocking step, 100 ng of recombinant group C rotavirus VP6 protein in 100  $\mu$ l of 1% non-fat milk in PBS were added to each well and incubated for 1 h at 37 °C. The plates were washed and serum samples diluted to 1:100 in 1% non-fat milk with PBS-T were added to each well and incubated for 1 h at 37 °C. The positive control was a positively tested sample from a previous study [22]. After washing, 100  $\mu$ l of horseradish peroxidase-conjugated anti-human IgG goat antibody (Sigma, India) diluted 1:10 000 in PBS-T was added to the wells. The plates were incubated for 1 h at 37 °C, washed with PBS-T, then 100  $\mu$ l TMB substrate (Sigma) was added to each well. Finally the reaction was stopped using 50  $\mu$ l of 2 M H<sub>2</sub>SO<sub>4</sub> and the optical density (OD) was read at 450 nm. A test sample was considered positive when the OD was higher than three standard deviations of the mean OD of the reagent blanks.

#### *Expression of recombinant group A VP6 and ELISA standardization*

The expression and ELISA standardization in a well-characterized specimen collection is described in detail in a separate study (Kavanagh *et al.*, unpublished observations). Briefly, the full-length VP6 genes from a G1P[8], a G2P[4] and a G10P[11] strain from Vellore were PCR amplified, then cloned into Topo (Invitrogen, USA). The Gateway system (Invitrogen) using a BP recombination reaction generated an entry clone, followed by LR recombination to generate an expression clone in a pDEST10vector in DH5 alpha-max efficiency cells which was transformed into DH10 Bac competent cells. Bacmid purification was done using a Power Prep kit (Marligen Biosciences, USA) to isolate the recombinant bacmid DNA which was then transfected into Sf-9 cells using lipofectamine (Invitrogen). Plaque purification was carried to generate P1 stock and the P2 stock was titrated. A time-course experiment was performed, and 96 h was found to yield the best expression. The assay was optimized using a checkerboard titration of all reagents and standardized by

testing a panel of sera in parallel using IgG estimation in a modification of a widely used, previously published VP6 IgA ELISA, which uses a polyclonal capture antibody and a virus lysate [24]. The positive control used in the comparison of the two assays, and included on each plate with the recombinant protein assay, was a commercial immunoglobulin preparation (Iviglob, Vhb Lifesciences Inc., India).

#### *Rotavirus group A VP6 IgG ELISA*

Ninety-six-well microtitre plates (Immulon 1B, Thermo Electron Corporation, USA) were coated with 1 µg/well of purified group A rotavirus VP6 protein produced by recombinant baculoviruses and incubated overnight at 4 °C. Wells were washed with Tris-buffered saline–Tween-20 (TBS-T) and then blocked with 200 µl of 10% non-fat milk in TBS for 2 h at 37 °C. Serum samples were diluted to 1:200 in TBS-T with 10% non-fat milk added to the plate and incubated for 2 h at 37 °C. After washing, horseradish peroxidase-conjugated anti-human IgG goat antibody (Southern Biotech, UK) diluted 1:10 000 in TBS-T was added. The plates were incubated 1 h at 37 °C, washed with TBS-T and TMB substrate was added. The reaction was stopped using 1 M H<sub>3</sub>PO<sub>4</sub> and the OD was read at 450 nm. If the OD of the test sample was three standard deviations above the mean OD of the blanks it was considered positive.

#### Statistical analysis

Data were entered in Microsoft<sup>®</sup> Access and analysed using Graphpad Prism version 4.  $\chi^2$  test was performed to determine the significance of differences observed between two groups of subjects.

## RESULTS

### Prevalence of anti-group C rotavirus VP6 antibody

Of the 936 samples available for analysis, 237 were positive for anti-group C rotavirus VP6 antibodies resulting in an overall prevalence of 25.32% (95% CI 22.64–28.21). The prevalence rose from 16.79% in children aged <10 years to 100% in adults aged >80 years (Table 1), although smaller numbers were tested in the older age groups. There was a statistically significant increase in prevalence of anti-group C rotavirus VP6 antibodies in the 21–30 years age group compared to the previous age group of 11–20 years

Table 1. Seroprevalence by age group of antibodies to group C rotavirus in South Indian populations using a recombinant VP6 ELISA

Age group (yr)	No. of samples tested	No of samples seropositive	Percentage
1–10	417	70	16.79
11–20	190	50	26.32*
21–30	106	37	34.91
31–40	102	30	29.41
41–50	31	11	35.48
51–60	47	14	29.79
61–70	33	19	57.58†
71–85	10	6	60.0
Total	936	237	25.32

\* Values significantly different between the previous group ( $P < 0.01$ ).

† Values significantly different between the previous group ( $P < 0.02$ ).

and between the 61–70 years and the 51–60 years age groups. Overall, there were no differences by gender.

Rural and urban classification of samples were available for the 786 samples collected as part of the population sampling of subjects aged between 1 and 40 years. The results are summarized in Table 2. The prevalence of anti-group C rotavirus VP6 antibodies in the rural population aged <40 years was 23.92% (95% CI 20.38–27.46) and was not different from the prevalence in the urban population aged <40 years, which was 21.12% (95% CI 17.73–24.51). The only age group where the difference was significant between the two populations was the 1–5 years age group, where the rural children had higher levels of antibodies, but this difference was not carried to the older age groups.

### Prevalence of anti-group A rotavirus VP6 antibody

Evaluation of 20 post-infection sera from children infected with G1P[8], G2P[4] and G10P[11] strains showed that there was cross-reactivity between the three antigens, with all 20 samples positive in all three assays. There was some difference in OD values, with specific reactivity somewhat higher than cross-reactive antibodies, but this was not set up as a quantitative assay.

All 936 samples from the rural and urban collections were also tested for anti-group A rotavirus VP6 antibody from the G1P[8] strain. Contrary to rotavirus C, all samples (100%) were positive across all age groups. The youngest age groups consisting of

Table 2. Seroprevalence of antibodies to group C rotavirus in urban and rural South Indian populations aged between 1 and 40 years

Age group (yr)	Rural population			Urban population		
	No. of samples tested	No. of samples seropositive	Percentage	No. of samples tested	No. of samples seropositive	Percentage
1–5	165	32	19.39	136	12	8.82*
6–10	46	8	17.39	47	10	21.28
11–15	43	6	13.95	51	13	25.49
16–20	47	14	29.79	49	17	34.69
21–25	23	9	39.13	27	10	37.04
26–30	24	9	37.50	32	9	28.13
31–35	25	7	28.00	28	7	25.00
36–40	20	9	45.00	23	5	21.74
Total	393	94	23.92	393	83	21.12

\* Values significantly different between rural and urban population in the same age group ( $P < 0.05$ ).

children between 1 and 10 years in both the rural and urban populations were universally positive for antibodies against group A rotavirus VP6, suggesting early exposure to this pathogen.

## DISCUSSION

This population-based study from South India found a prevalence of anti-group C VP6 IgG of 25.32% and anti-group A VP6 IgG of 100%. A study conducted in the 1980s found a lower prevalence of anti-group C rotavirus VP6 antibodies (3%) [15]. The earlier study used antigen from the prototype porcine 'Cowden strain', unlike the recombinant human rotavirus antigens used in our study and may have missed infections. Given that in most enteric infections, exposure in developing countries is higher than in the more industrialized nations, it is of interest to note that the prevalence in our study is lower than the prevalences of 39% and 43% noted in two epidemiological surveys in the UK that also utilized recombinant VP6 antigen [20, 22]. Studies utilizing a similar methodology from South Africa and Brazil recorded prevalences of 33% and 36%, respectively [25, 26]. A study from Japan utilized a blocking enzyme immunoassay and reported a prevalence of 30% [27].

Our study is in accord with the findings of previous epidemiological surveys that documented an age-related rise in prevalence of antibodies against group C rotaviruses [20, 22]. The increase in antibodies with age suggests a pathogen to which exposure is limited, unlike group A rotavirus where by the age of 1 year both urban and rural children demonstrated a history

of rotavirus infection. A serosurvey does not allow determination of the exact time of exposure, but age-specific rates permit inference of the degree of exposure over time. Antibody avidity measurements may also permit estimation of the time since infection; however, repeated exposure to this virus may also result in boosting of the immune response resulting in higher prevalence of antibodies in older individuals.

The possibility of zoonotic spread of rotavirus group C has been considered based on the results of an epidemiological survey from the UK in which the prevalence of antibodies was higher in the rural population than in the urban populace [22]. This could be evidence of contact with farm animals where group C rotaviruses are found. In our study population, a higher prevalence was seen in rural children aged 1–5 years, but not in older children and adults, suggesting either that transmission is not zoonotic or that in South India, exposure to potentially infectious animals is high even in urban populations. The former is more likely, since human group C rotaviruses are more conserved and antigenically distinct from animal strains [19]. Although there were differences between rural and urban antibody prevalence in the 11–15 and 36–40 years age groups, they did not reach statistical significance (Table 2), possibly because of the limited numbers of samples tested in each age stratum. Antibodies to group A rotavirus were seen in 100% of our population compared to 85.4% of children and 97% of adults in a study from the UK [20]. The lack of correlation of antibodies against group A and group C rotaviruses suggests that their transmission patterns are independent of each other,

although it is possible that the age-dependent increase in reactivity against group C rotavirus VP6 may be the result of repeated infections with group A rotaviruses, with re-infecting group A rotavirus strains eliciting group C cross-reacting antibodies, which have been described in a previous study [28]. It is noteworthy that the antibodies against recombinant proteins produced by cloning and expression of different human (G1P[8], G2P[4]) and animal (G10P[11]) subgroup VP6 appeared to be cross-reactive. However, an assay based on recombinant proteins, and one that is set up as a qualitative assay, may also have other limitations, i.e. the conformation of epitopes and functionality have not been evaluated.

Group C rotaviruses have been reported as the cause of diarrhoea epidemics [29–31], and seroepidemiological studies have documented exposure [15, 22, 26, 27]. Lack of robust testing methods has precluded diagnosis of this virus as a cause of diarrhoea in older children and adults. Systematic prospective studies are required to document the global distribution of group C rotavirus infection and its role in causing diarrhoea in humans.

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#### DECLARATION OF INTEREST

None.

#### REFERENCES

1. Bishop RF, *et al.* Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. *Lancet* 1973; **2**: 1281–1283.
2. Estes MK. Rotaviruses and their replication. In: Knipe DM, Howley PM, eds. *Fields Virology*, 4th edn. Philadelphia: Lippincott Williams & Wilkins, 2001, pp. 1747–1786.
3. Kapikian AZ, Hoshino Y, Chanock RM. Rotaviruses. In: Knipe DM, Howley PM, eds. *Fields Virology*, 4th edn. Philadelphia: Lippincott-Raven Publishers, 2001, pp. 1787–1833.
4. Parashar UD, *et al.* Rotavirus and severe childhood diarrhoea. *Emerging Infectious Diseases* 2006; **12**: 304–306.
5. Hung T, *et al.* Waterborne outbreak of rotavirus diarrhoea in adults in China caused by a novel rotavirus. *Lancet* 1984; **1**: 1139–1142.
6. Krishnan T, *et al.* Emergence of adult diarrhoea rotavirus in Calcutta, India. *Lancet* 1999; **353**: 380–381.
7. Saif LJ, *et al.* Rotavirus-like, calicivirus-like, and 23-nm virus-like particles associated with diarrhoea in young pigs. *Journal of Clinical Microbiology* 1980; **12**: 105–111.
8. Bridger JC, Clarke IN, McCrae MA. Characterization of an antigenically distinct porcine rotavirus. *Infection and Immunity* 1982; **35**: 1058–1062.
9. Von Bonsdorff CH, Svensson L. Human serogroup C rotavirus in Finland. *Scandinavian Journal of Infectious Diseases* 1988; **20**: 475–478.
10. Otto P, Schulze P, Herbst W. Demonstration of group C rotaviruses in fecal samples of diarrheic dogs in Germany. *Archives of Virology* 1999; **144**: 2467–2473.
11. Adah MI, *et al.* Detection of human group C rotaviruses in Nigeria and sequence analysis of their genes encoding VP4, VP6, and VP7 proteins. *Journal of Medical Virology* 2002; **66**: 269–275.
12. Castello AA, *et al.* Characterization of human group C rotavirus in Argentina. *Journal of Medical Virology* 2000; **62**: 199–207.
13. Penaranda ME, *et al.* Group C rotavirus infections in patients with diarrhoea in Thailand, Nepal, and England. *Journal of Infectious Diseases* 1989; **160**: 392–397.
14. Rodger SM, Bishop RF, Holmes IH. Detection of a rotavirus-like agent associated with diarrhoea in an infant. *Journal of Clinical Microbiology* 1982; **16**: 724–726.
15. Brown DW, *et al.* Rotavirus epidemiology in Vellore, south India: group, subgroup, serotype, and electrophoretotype. *Journal of Clinical Microbiology* 1988; **26**: 2410–2414.
16. Rasool NB, *et al.* Identification of a human group C rotavirus in Malaysia. *Journal of Medical Virology* 1994; **43**: 209–211.
17. Tsunemitsu H, Jiang B, Saif LJ. Detection of group C rotavirus antigens and antibodies in animals and humans by enzyme-linked immunosorbent assays. *Journal of Clinical Microbiology* 1992; **30**: 2129–2134.
18. Nilsson M, Sigstam G, Svensson L. Antibody prevalence and specificity to group C rotavirus in Swedish sera. *Journal of Medical Virology* 2000; **60**: 210–215.
19. Fielding PA, *et al.* Molecular characterization of the outer capsid spike protein (VP4) gene from human group C rotavirus. *Virology* 1994; **204**: 442–446.
20. James VL, *et al.* Seroepidemiology of human group C rotavirus in the UK. *Journal of Medical Virology* 1997; **52**: 86–91.
21. Riepenhoff-Talty M, *et al.* Epidemiology of group C rotavirus infection in Western New York women of childbearing age. *Journal of Clinical Microbiology* 1997; **35**: 486–488.
22. Iturriza-Gomara M, *et al.* Seroepidemiology of group C rotavirus infection in England and Wales. *European Journal of Epidemiology* 2004; **19**: 589–595.
23. Kang G, *et al.* Community prevalence of antibodies to human immunodeficiency virus in rural and urban

- Vellore, Tamil Nadu. *National Medical Journal of India* 2005; **18**: 15–17.
24. **Bernstein DI, et al.** Safety and immunogenicity of live attenuated human rotavirus vaccine 89-12. *Vaccine* 1998; **16**: 381–387.
  25. **Steele AD, James VL.** Seroepidemiology of human group C rotavirus in South Africa. *Journal of Clinical Microbiology* 1999; **37**: 4142–4144.
  26. **Cox MJ, et al.** Infection with group C rotavirus in a suburban community in Brazil. *Tropical Medicine & International Health* 1998; **3**: 891–895.
  27. **Kuzuya M, et al.** Seroepidemiology of human group C rotavirus in Japan based on a blocking enzyme-linked immunosorbent assay. *Clinical and Diagnostic Laboratory Immunology* 2001; **8**: 161–165.
  28. **James VL, et al.** Enzyme-linked immunosorbent assay based on recombinant human group C rotavirus inner capsid protein (VP6) to detect human group C rotaviruses in fecal samples. *Journal of Clinical Microbiology* 1998; **36**: 3178–3181.
  29. **Oishi I, Yamazaki K, Minekawa Y.** An occurrence of diarrheal cases associated with group C rotavirus in adults. *Microbiology and Immunology* 1993; **37**: 505–509.
  30. **Matsumoto K, et al.** An outbreak of gastroenteritis associated with acute rotaviral infection in schoolchildren. *Journal of Infectious Diseases* 1989; **160**: 611–615.
  31. **Kuzuya M, et al.** Survey of human group C rotaviruses in Japan during the winter of 1992 to 1993. *Journal of Clinical Microbiology* 1998; **36**: 6–10.