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## Serological survey of anti-group A rotavirus IgM in UK adults

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### SUMMARY

Rotaviral associated disease of infants in the UK is seasonal and infection in adults not uncommon but the relationship between these has been little explored. Adult sera collected monthly for one year from routine hospital samples were screened for the presence of anti-group A rotavirus immunoglobulin M class antibodies as a marker of recent infection. Anti-rotavirus IgM was seen in all age groups throughout the year with little obvious seasonal variation in the distribution of antibody levels. IgM concentrations and the proportion seropositive above a threshold both increased with age with high concentrations consistently observed in the elderly. Results suggest either high infection rates of rotavirus in adults, irrespective of seasonal disease incidence in infants, IgM persistence or IgM cross-reactivity. These results support recent evidence of differences between infant and adult rotavirus epidemiology and highlight the need for more extensive surveys to investigate age and time related infection and transmission of rotavirus.

### INTRODUCTION

Rotaviruses are responsible for 30–40% of reported infant diarrhoea worldwide with high mortality in developing countries and significant morbidity and hospitalizations in industrialized nations [1]. Typically, most severe rotaviral disease occurs upon primary infection, usually in infants less than 5 years old, although this does not induce complete protective immunity. Repeat infections are common but generally less severe in outcome [2, 3] due to development of a partially effective immune response. Rotaviral disease is also common amongst the elderly, particularly through outbreaks in residential care [4–8].

Although there have been many studies investigating the epidemiology of disease and repeat infection (symptomatic and asymptomatic) in children [2, 3, 9–13] less attention has focused on infection

and disease in other age groups. Studies which have investigated rotaviral infection in older age groups have long suggested that infection rates could be high, especially in case contacts [14, 15]. Adult rotaviral infection has been measured using viral antigen in stools [16, 17] and through serology using IgM as a marker of recent or current infection [18–21]. However, these studies did not consider seasonal variation which is normally considerable in most geographical settings except the tropics [22].

In the UK, rotaviral disease in infants is highly seasonal with incidence peaking, currently, around April although cases occur year round [23]. The mechanisms generating seasonality are not fully understood and are the subject of debate [24, 25]. A number of factors may be involved, e.g. climatic factors [26], age-specific mixing patterns, rotaviral strain diversity and the dynamics of host susceptibility [27]. A recent study of clinical diarrhoea in adults in Japan [17] found that patterns of disease did not mirror the winter seasonality seen in infants and that peaks

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of infection occurred at different time points from year to year. These results suggest that the factors driving seasonality in infants are not having the same influence on adults where infection is clearly common and appears more temporally unpredictable. This study has investigated age-specific and seasonal levels of anti-group A rotaviral IgM in sera from a predominantly adult population in the UK.

## METHODS

### Sample collection

Sera were collected from the Department of Clinical Chemistry, Birmingham Heartlands Hospital, between September 1997 and October 1998, from those collected routinely for diagnostic screening from in-patients and patients of local GPs. Around 150 samples were collected at the end of each calendar month and selected randomly from samples about to be discarded (normally around 800 per day). Samples were taken anonymously with only sex, year of birth and reason for sample collection being recorded (when completed). It was noted when a sample had been collected from a patient with symptoms which could possibly be attributed to rotavirus infection, i.e. diarrhoea and/or abdominal pain, or respiratory infection. Ethical permission to conduct the study was obtained from the Heartlands Hospital Ethics Committee. Sera were pre-diluted 1 in 10 into 50:50 PBS/glycerol on the day of collection to avoid the detrimental effects of freeze-thawing on IgM antibody. Prediluted sera were then stored at  $-25^{\circ}\text{C}$  until use but were initially screened within 1 week.

### Antigen

Rotavirus strains UKtc (bovine, serotype 6) and SA11 (simian, serotype 3) were grown in BSC-1 cells until maximum CPE. Virus was harvested and freeze/thawed three times before centrifugation to remove cell debris. The supernatant was used as antigen and uninfected cells, similarly treated, were used as control antigen. Purified UKtc was produced using a caesium chloride gradient centrifugation based on published methods [28].

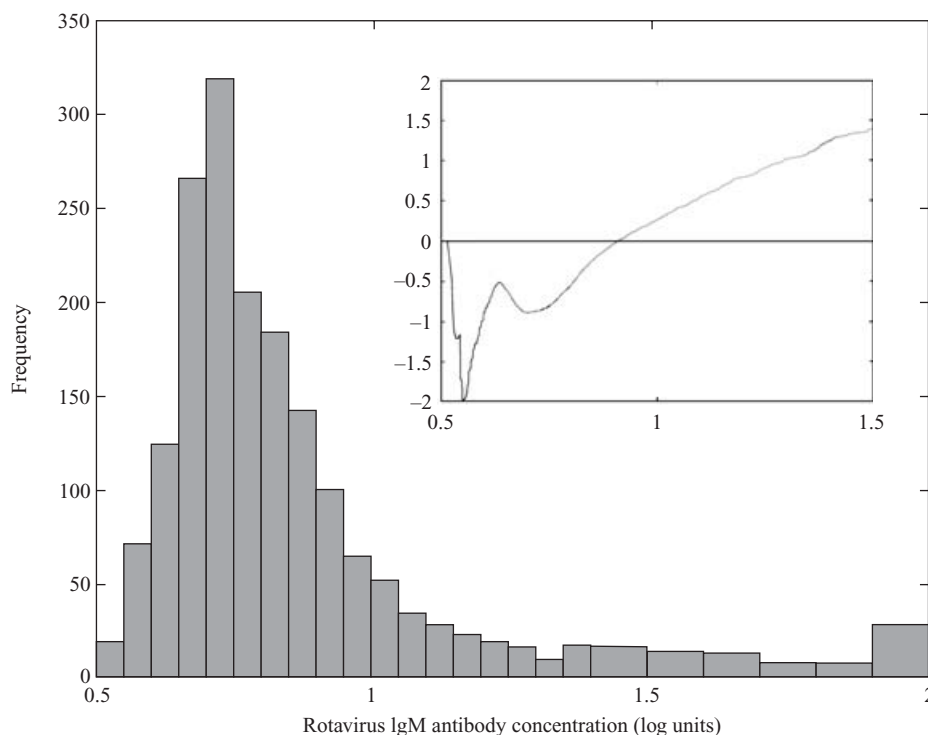
### Rotavirus IgM capture-immunoassay

Anti-rotavirus IgM was detected using a capture EIA technique based on those previously described

[21, 29]. Briefly, 96 well microtitre plates (Immunolon II, Dynatech) were coated with  $50\ \mu\text{l}$  of optimally diluted rabbit polyclonal anti-human IgM (Dako) in carbonate buffer (pH 9.6) overnight at  $4^{\circ}\text{C}$ . Plates were then blocked with  $100\ \mu\text{l}$  4% skim milk powder (SMP) in phosphate-buffered saline (PBS, pH 7.2) for 1 h at  $37^{\circ}\text{C}$ . Plates were washed three times with PBS plus 0.05% Tween 20 (Tw) (Sigma). Fifty microliters of sera, diluted to 1 in 200 in PBS-SMP-Tw, were added for 2 h at  $37^{\circ}\text{C}$  followed, after washing, by  $50\ \mu\text{l}$  rotaviral antigen (or control antigen), in PBS-SMP-Tw (2 h  $37^{\circ}\text{C}$ ). After three washes,  $50\ \mu\text{l}$  anti-rotavirus group A horseradish peroxidase conjugate (Cambridge Biotech) was added for 1 h at  $37^{\circ}\text{C}$ . Colour was developed, after further washing, using OPD/ $\text{H}_2\text{O}_2$  substrate (Dako, UK) followed by 2 M  $\text{H}_2\text{SO}_4$  and read at 492 nm (630 nm reference). Each plate contained serial dilutions of a high titre anti-rotavirus IgM positive of pooled adult (UK) sera to allow for plate to plate variation and to standardize test samples using linear regression. The standard was given an arbitrary antibody unitage and the results obtained with test sera are presented as log antibody units.

A number of controls were performed to investigate possible non-specific reactions and variability within the assay. A number of sera ( $n=360$ ) were re-screened on different plates on different days to investigate individual and day to day test variation. Sera were also screened, as above, on plates with no capture antibody or no antigen (carbonate buffer and PBS-SMP-Tw20 only) or using control antigen. Sera were also screened using two other capture antibodies (affinity-purified goat polyclonal anti-human IgM and mouse monoclonal anti-human IgM (Sigma, UK)) as well as comparing UKtc antigen with SA11 and purified UKtc antigen (pUKtc) under the same assay conditions as above. All reagent concentrations were optimized by checkerboard assay before use.

Sera ( $n=48$ ) were also screened by capture assay for IgM following depletion of IgG using protein A sepharose (Sigma, UK). Protein A sepharose was reconstituted in 50 mM Tris buffer (pH 8). Sera were pre-incubated with four times the volume of sepharose agar in Tris buffer to give a final serum dilution of 1 in 200 and incubated for 2 h at room temperature on a blood rotor then centrifuged for 5 min at 1000 rpm. Sera with and without IgG depletion were treated identically (Tris buffer alone replacing sepharose) and screened in adjacent wells on the same plate. Sera ( $n=100$ ) of all ages (18–93 years,  $x=65.5$ )



**Fig. 1.** Frequency distribution of anti-group A rotavirus IgM antibody concentrations for the total population ( $n=1768$ ). The inset graph depicts the changing value of the skewness of the distribution used to estimate the cut-off for seropositive samples.

and with a variety of rotaviral IgM concentrations (49% > 1.5 log units; 18% > 2 log units) were also screened for rheumatoid factor (Serodia-RA, Fuji-ribo Inc, Japan). Samples positive or indeterminate for rheumatoid factor were re-screened.

Results were analysed using QuattroPro (Corel, 1997) and MatLab (v6, The MathWorks, Inc). Given that IgM is generally short-lived it would be expected that the distribution of anti-rotaviral IgM levels within the population would follow a highly skewed distribution with the majority of those seropositive having very low concentrations and only a few (most recently infected) having higher concentrations. Thus, the use of known IgM seropositives from clinical samples (likely to be high titre) would not be of value in community-based studies for evaluation of a threshold for IgM detection. Therefore, the true IgM-positive prevalence cannot be calculated. The empirical cut-off chosen to classify seropositive and seronegative sera in this study was based on the assumption that seronegative samples would be normally distributed and that the distribution of seropositive samples would be skewed with the majority having low concentrations and merging with the distribution of seronegatives. A cut-off estimate was

made by taking a step-wise approach to calculating the skewness of the total frequency distribution (Fig. 1). The IgM antibody concentration where the skewness equalled zero (0.91 log antibody) was assumed to represent the upper limit of the distribution of seronegatives. The mean log antibody concentration of this assumed seronegative population plus three standard deviations was used as the cut-off for seropositivity (equal to 0.9968 log units) and for comparison of age and time-related trends. This cut-off translated into an optical density of around 0.15–0.18 with the absorbance range of seropositives increasing to around 1.0 maximally.

## RESULTS

In total, 1768 sera were collected between November 1997 and October 1998 (Table 1). It was noted, where possible, if the sample had been collected from a patient showing any symptoms of possible rotavirus infection, i.e. abdominal pain or diarrhoea (2.1%). It was assumed that this percentage was reflected amongst those samples where no reason for collection was given (14.9%) resulting in an adjusted value of 2.4%. The majority of samples (97.6%), therefore,

Table 1. *Monthly variation in rotavirus IgM prevalence*

Month 1997-8	Sample size	Sex*		Mean age (range)	% reporting symptoms†	Rotavirus IgM prevalence‡	Mean rotavirus IgM concentration (s.e.)§
		Male	Female				
November	149	78	65	56.8 (2-98)	2.5	0.154	1.38 (0.084)
December	148	63	74	58.9 (1-95)	2.9	0.189	1.40 (0.085)
January	149	64	74	61.6 (12-98)	2.8	0.188	1.22 (0.044)
February	148	73	71	63.4 (12-95)	2.5	0.162	1.38 (0.122)
March	142	62	69	55.9 (13-88)	1.6	0.204	1.41 (0.097)
April	147	58	82	57.4 (12-95)	0.9	0.136	1.36 (0.106)
May	148	54	86	57.9 (8-96)	3.2	0.176	1.53 (0.111)
June	147	65	77	60.3 (16-97)	2.6	0.177	1.35 (0.080)
July	144	65	76	60.9 (1-97)	0.7	0.111	1.31 (0.056)
August	150	64	86	60.4 (3-97)	1.6	0.100	1.44 (0.099)
September	149	73	71	56.6 (13-97)	3.9	0.161	1.24 (0.049)
October	147	81	62	57.8 (14-98)	3.7	0.136	1.27 (0.067)
Total	1768	800	893	59.0	2.4	0.158	1.36 (0.026)

\* Sex not recorded for 4.2% of samples.

† Recorded as abdominal pain or diarrhoea.

‡ Seropositive defined as > 1.0 log antibody units.

§ Mean concentrations of only those defined as seropositive (as above).

were collected for reasons other than from possible gastrointestinal infectious disease. Of 100 samples screened for rheumatoid factor, 6 were positive and 3 indeterminate upon re-screening.

Repeat screening of serum samples for anti-rotaviral IgM showed significant correlation ( $n=360$ , Pearson correlation=0.943,  $P<0.001$ ). Significant correlations were also seen when alternative capture antibodies were used with crude Uktc antigen (rabbit versus goat polyclonal anti-IgM,  $n=88$ ,  $r=0.98$ ,  $P<0.001$ ; rabbit polyclonal versus mouse monoclonal anti-IgM,  $n=88$ ,  $r=0.901$ ,  $P<0.001$ ). Comparison of antigens also gave significant correlations (rabbit polyclonal+SA11 vs. pUktc  $n=48$ ,  $r=0.878$ ,  $P<0.01$ ; mouse monoclonal+Uktc vs. pUKtc  $n=96$ ,  $r=0.977$ ,  $P<0.01$ ). No non-specific reaction occurred when capture antibody or antigen was omitted nor when control antigen was used. Significant correlations in rotaviral IgM levels were seen between samples with or without prior removal of IgG (mean depletion of IgG 82%) ( $n=48$ ,  $r=0.969$ ,  $P<0.01$ ).

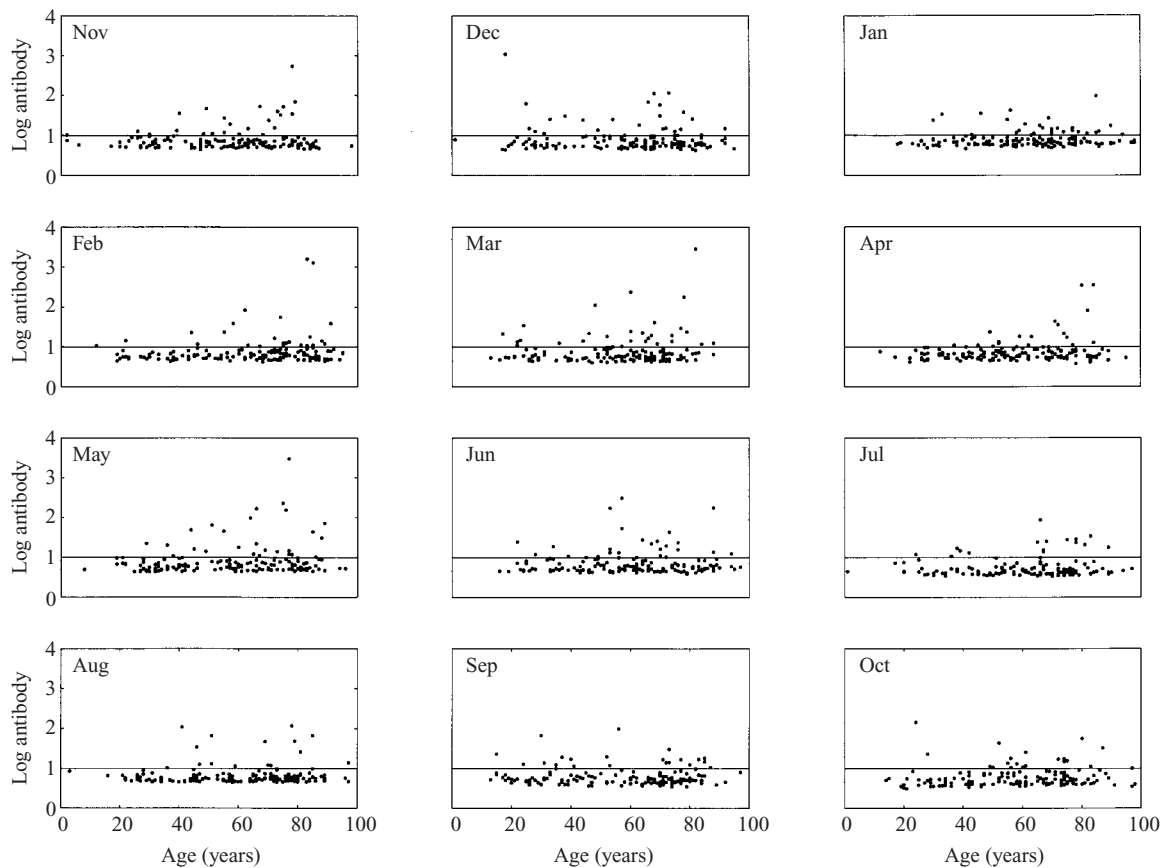
Using the estimated cut-off of 0.9968 log units, prevalence was seen to vary between months from 20.4% in March to 11% and 10% in July and August respectively. No seasonal trend in mean antibody levels was observed (Table 1). Prevalence appeared to increase with age overall and this was significant (test for trend,  $\chi^2=6.93$ ,  $P<0.01$ ) for all months

Table 2. *Age-specific rotavirus IgM seroprevalence*

Age group (years)	Sample size (mean age)	Rotavirus IgM prevalence	Mean rotavirus IgM concentration (s.e.)*
0-9	7 (3.3)	0.14	—
10-19	34 (16.8)	0.117	1.35 (0.104)
20-29	151 (25.1)	0.105	
30-39	168 (34.4)	0.137	1.21 (0.043)
40-49	197 (44.7)	0.132	1.30 (0.059)
50-59	244 (54.9)	0.159	1.33 (0.057)
60-69	317 (64.8)	0.145	1.36 (0.051)
70-79	378 (74.3)	0.195	1.37 (0.050)
80-89	226 (83.9)	0.176	1.47 (0.089)
90-99	46 (93.7)	0.152	
Total	1768 (59.0)	0.156	1.36 (0.025)

\* Single seropositive for 0-9 age group; 10-29 and 80-99 year age groups pooled. Variances for these age groups were 0.215; 0.041; 0.088; 0.127; 0.117; 0.184; 0.367 respectively. Also see footnotes in Table 1.

combined (Table 2). Mean antibody concentrations, in those over 30 years of age, were also seen to increase with age. The variance in antibody concentration in those individuals under 30 years and, more particularly, in those over 80 years, was high (Table 2). IgM levels for individuals of all ages in each month (Fig. 2) revealed that high antibody levels were commonly seen in the very elderly.



**Fig. 2.** Scatter plots of anti-rotavirus IgM concentrations by age for each month showing the estimated cut-off for seropositivity (0.9968 log units).

Increasing the cut-off for seropositivity to a highly conservative value of 1.5 log units did not change the seasonal nor age-specific seroprevalence trends.

## DISCUSSION

Primary infection with rotavirus often leads to severe disease and incomplete immunity to re-infection but subsequent infections are usually less clinically severe [1]. Re-infection in adults can still result in clinical disease, perhaps due to waning immunity or some complex immune-dynamics of heterotypic infection, but infection can often be asymptomatic [30]. The objective of this study, using IgM as a marker of current or recent infection, was to estimate how much infection may be occurring in adults and whether this correlates with seasonal patterns of clinical disease seen in younger age groups.

The assay used in this study to measure IgM was sensitive and robust and most sources of possible non-specific reactions were eliminated. A serum

dilution of 1 in 200, although high for an IgM assay, gave reduced background absorbances than lower dilutions. There was no relationship between adults designated IgM seropositive and those reporting possible symptoms of rotavirus infection. The presence of RF or IgG did not appear to affect results. Although purified rotaviral antigen was used, cross-reaction with other antigens cannot be ruled out and may require further investigation. The criteria with which to characterize individual sera as positive or negative for anti-rotaviral IgM will have a profound influence on the resulting seroprevalence. The cut-off chosen in this study relies on the fact that negative samples will be normally distributed and that, statistically, any sample with an antibody concentration which is greater than the mean of negative samples plus three standard deviations could be defined as seropositive with 99.7% certainty. Further studies should be conducted to improve the sensitivity and specificity of the capture assay and investigate the dynamics of IgM responses and their correlation

with virus shedding in semi-immune individuals. Such studies have been done in infants [31] but not, to our knowledge, in adults. The use of IgM for population-based seroepidemiological studies would be an invaluable tool for understanding transmission dynamics of viral diseases where repeat, mild or sub-clinical infection is common. Such data could be used to estimate the rate of infection ( $\lambda$ ), an important epidemiological parameter for the theoretical design of control programmes [32], by using IgM titre as a marker of time since infection.

A high prevalence of specific IgM was seen in all months (ranging from 11–20%). Although the lowest prevalences were seen in July and August there was no clear seasonal seroprevalence patterns which would correlate with known patterns of disease in infants (peaking around April). The dynamics of IgM responses to rotavirus infection in sera from adults following repeat infection are not well understood and are likely to be influenced by a number of factors such as age, the number of previous infections, time since last infection and history of serotype-specific infection (homotypic or heterotypic infection). Persistence of IgM has been observed for a number of viral infections not only where virus persists, e.g. CMV [33] but also where infection is cleared e.g. rubella [34]. The high prevalence of rotavirus IgM seen in a previous study [19] was also explained by possible persistence of antibody although this was not apparent in other studies of infants [31] or adults [20].

It may be that low levels of chronic infection persist in partially immune individuals and such infections are tolerated. Persistence of infection has been investigated in infants where shedding of rotavirus following severe disease was shown to have a broad distribution [35] with 30% shedding for up to 57 days. There was no correlation with age but the age range was limited and sample sizes small. It is not known for how long adults might shed virus following infection but asymptomatic rates of 5% have been reported [17], which may be a result of persistence.

The observation of increased antibody levels with age, with the highest concentrations commonly seen in the very elderly may simply reflect the known increased susceptibility of this age group and who, in this study, may largely come from residential care. A recent study in Sweden reported 7–13% of clinical rotavirus infection occurred in the elderly [36] and the elderly have been suggested as a reservoir for infection [37]. It should be noted that our samples

were collected from a biased section of the normal adult community, i.e. hospital in-patients or those reporting to local GPs with a variety of ailments. Nosocomial infection with rotavirus has been well documented and is known to be a major route of transmission. Therefore, care has to be taken in extrapolating these results to the normal healthy adult community. However, the high prevalence of antibody seen in all months suggests that a larger community-based study is required to investigate how much infection (as opposed to just disease) maybe occurring in different age groups throughout the year. However, the collection of large numbers of serum samples from infants, where seasonality in IgM prevalence should be observed, is extremely difficult. These studies should not only be investigated serologically but also through the detection of the viral genome using RT-PCR which could also be genotype-specific [38–40]. Significant differences in the distribution of serotypes causing clinical disease in adults versus children have been observed in the UK [40] possibly reflecting different epidemiological transmission processes.

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