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# Seroepidemiology of cryptosporidiosis in children in Papua New Guinea and Australia

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## SUMMARY

Enzyme immunoassays (EIA) were used to measure serum antibodies to *Cryptosporidium* in four immunocompetent adults with recent proven cryptosporidial infection, 379 healthy children and 73 adult volunteers in Melbourne, Australia, and 205 children in Papua New Guinea (PNG) (47 healthy children; 158 with pneumonia). Antibodies peaked 3–6 weeks after infection and fell to baseline within a few months. A high level (5000 EIA units/ml) or a significant change between paired sera, of IgG or IgM, were taken as evidence of recent infection and found in 24% of PNG children and in 8% of children and 5% of adults in Melbourne. Among PNG children with pneumonia who had high cryptosporidial antibody levels, those with measles (6/8) were significantly more likely ($P = 0{\cdot}002$) to have diarrhoea than the remainder (4/28). Symptomatic cryptosporidiosis may be associated with transient immune suppression due to viral infection. This study indicates that serological surveys can contribute to an understanding of the epidemiology of cryptosporidosis.

## INTRODUCTION

The protozoan, *Cryptosporidium* is an important cause of diarrhoea, especially in young children in developing countries and in people with immune deficiencies [1,2] but its overall prevalence varies in different populations. Enzyme immunoassays (EIA), using a human isolate of *Cryptosporidium* as antigen, were developed to measure serum IgG and IgM antibody in several otherwise healthy individuals with proven cryptosporidial infection and, in a seroepidemiological study among children and adults in Melbourne, Australia, and children in Goroka, Papua New Guinea (PNG).

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## MATERIALS AND METHODS

### *Preparation of* Cryptosporidium *antigen*

A recent human isolate of *Cryptosporidium*, was used. Newborn colostrum-deprived Friesan calves were inoculated orally with $10^7$ oocysts and killed when oocysts were detected in faecal smears. Gut scrapings were stored in potassium dichromate to prevent bacterial overgrowth. Oocysts were concentrated by sedimentation with ether-phosphate buffered saline (PBS) and purified by Percoll discontinuous density gradient centrifugation as previously described [3], but with the addition of a third layer of Percoll (Pharmacia; Uppsala, Sweden), density 1·02 g/l, to improve separation. Oocyst suspensions were examined by phase contrast microscopy and cultured on chocolate agar to exclude bacterial contamination. The antigen preparation was also examined by sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) to exclude contamination with foreign protein. The method used to prepare antigen from purified oocysts, by sonication, has also been described previously [4]. The antigen preparation was aliquoted and stored at −70 °C. Protein concentration was determined by Hartree's modification of the Lowry method [5].

### *EIA procedure*

EIA reagents at varying concentrations were assayed by chequerboard titrations to determine optimal concentrations. 100 μl of *Cryptosporidium* antigen containing 1 μg/ml of protein in 50 mM bicarbonate/carbonate buffer (pH 9·6) was added to each well of a 96-well polystyrene ELISA microtitre plate (Maxisorp, Nunc; Copenhagen, Denmark) and incubated at 4 °C overnight. The antigen solution was aspirated and excess binding sites were blocked with 20 mg/ml sodium casein (US Biochemical Corporation, Ohio, USA) in 10 mM-PBS. Plates were washed 6 times with 50 mM-Tris buffer (Boehringer Mannheim, Germany) containing 0·5% Tween 20 at pH 7·6. Comparison of various proteins, during development of the test, had shown casein to be the most effective blocking agent [6].

Test sera were diluted 1/100 in 10 mM-PBS, containing 10 mg/ml casein and 0·05% Tween 20; 100 μl of each was added to duplicate wells and incubated for 2 h at 37 °C. Plates were washed as before. Sheep anti-human IgM or IgG horseradish peroxidase conjugate (Silenus; Melbourne, Australia) diluted 1/2000 (100 μl) was added to each well and incubated for 1 h at 37 °C. Plates were washed and 100 μl of tetra methyl benzidine (TMB)/$H_2O_2$ substrate buffer was added to each well. The buffer, containing 5 ml distilled water, 5 ml 0·2 M sodium acetate (pH 6·0), 0·1 ml TMB (10 mg/ml) in dimethyl sulphoxide (Sigma Chemical Company; St Louis, Missouri, USA), 0·1 ml 0·2 M citric acid and 2·4 μl $H_2O_2$; it was prepared immediately before use and protected from light. The colour was allowed to develop for 5 min before the addition of 25 μl of 4 M-$H_2SO_4$. The optical density (OD) at 450 nm was read in a Titertek multiscanner.

Controls included in every assay, as a check for inter-assay reliability, were acute and convalescent sera from two patients with recent cryptosporidiosis (see below), a negative serum from an individual with no history of cryptosporidiosis, and dilutions of a high-titer positive serum, to allow construction of a standard

curve. Blank wells, to which all reagents except serum were added, were included in each tray as controls for non-specific reactivity (OD typically < 0·06).

*Preparation of standard curves and estimation of anti-*Cryptosporidium *EIA IgM and IgG levels*

Standard curves for IgG and IgM assays were constructed using two high titre sera, obtained during convalescence from an immunocompetent adult with cryptosporidiosis and arbitrarily deemed to contain $10^4$ EIA units/ml of anti-*Cryptosporidium* IgM or IgG (standard positive control sera). Serial dilutions of both sera, from 1/40 to 1/5120, containing from 250–1·95 (OD range 1·5–1·1), EIA units/ml anti-*Cryptosporidium*, were included in every assay and the OD values plotted against antibody units. OD values for test sera were converted into EIA units by reference to this standard curve [7]. Test sera containing higher antibody levels than standard sera were diluted further and reassayed. Assays resulting in an inter-assay correlation coefficient less than 0·85 were rejected. To determine whether changes in antibody levels between paired sera were statistically significant, residual errors were estimated for a random sample of five assays and the estimated standard error of prediction calculated for each assay. A change in OD value of 0·4 or more corresponded with a change of approximately one log in the number of *Cryptosporidium* antibody units per ml and was significant at the 5% level [8].

*Serum samples*

A total of 19 specimens of serum were collected at varying intervals from four previously healthy individuals with naturally-acquired cryptosporidiosis confirmed by positive microscopic examination of faeces for *Cryptosporidium*. 234 serum samples from 205 children aged 1–84 months (average 11 months) were collected during a study of acute respiratory infection at Goroka Base Hospital. These included 47 sera from healthy children aged 0–84 months (mean 19) and 158 'acute' sera from children with pneumonia aged 0–73 months (mean 11); convalescent sera were collected 2–3 weeks after the first from 29 of the latter (mean age 8 months). Of children with pneumonia, 32 also had diarrhoea, 28 had measles and 14 had both.

Sera were obtained from 379 children, aged 0–198 (mean 64 months), attending the Royal Children's Hospital (RCH), Melbourne, Australia. Of these, 80 were from children attending outpatient clinics during 1985 and 299 were selected at random from sera submitted for biochemical tests during the summers of 1989–90 and 1990–1. Clinical histories were not available but any who had been admitted with a diagnosis of gastroenteritis were excluded. They were assumed to be representative of the general population. Sera collected (for measurement of hepatitis B antibody) from 73 hospital staff during the summer of 1990–1 and six sera from adult volunteers were included.

## RESULTS

*Anti-*Cryptosporidium *antibody levels in naturally-acquired cryptosporidiosis*

The IgM and IgG titres were calculated in EIA units for 19 serum samples collected from the four individuals with proven *Cryptosporidium* infection; one

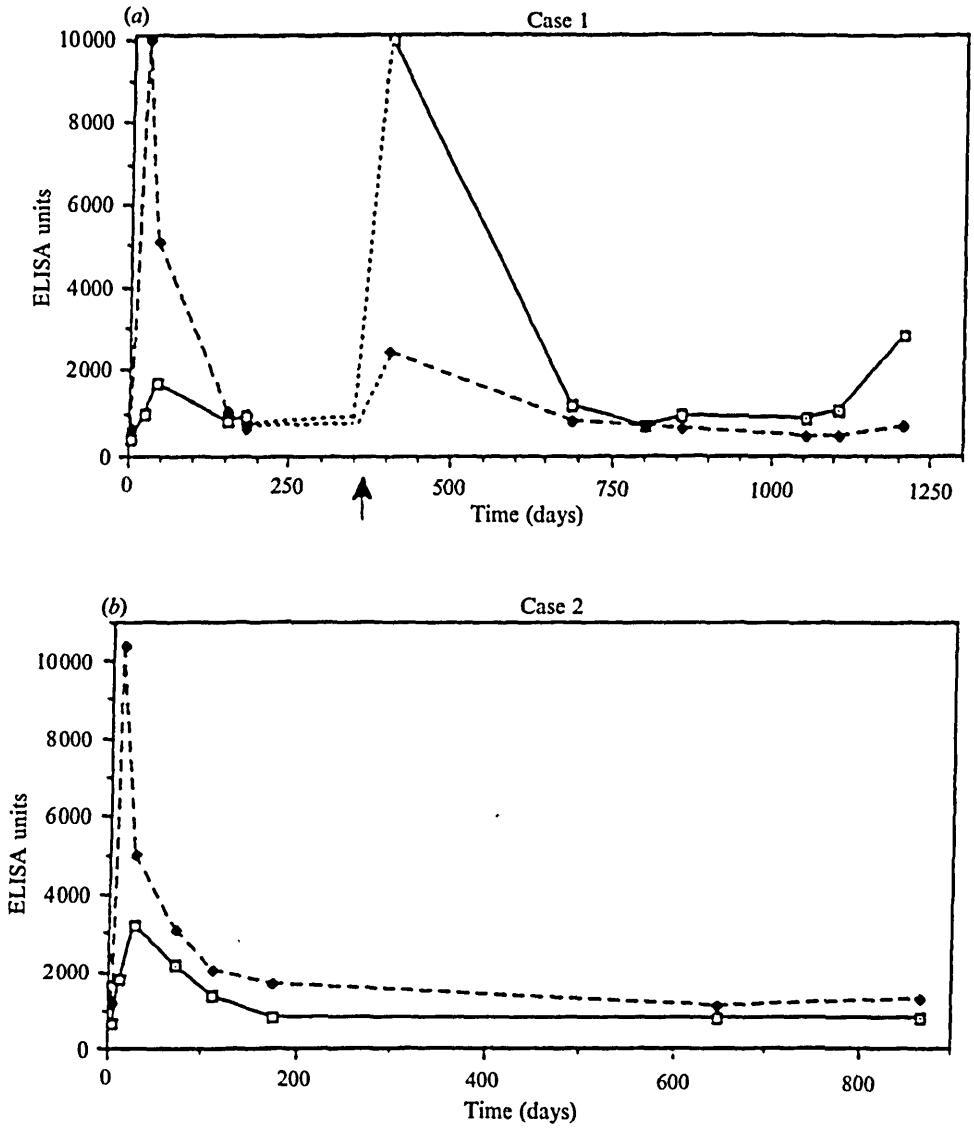


Fig. 1. IgG (⊡) and IgM (◆) antibody levels over time in immunocomponent subjects with proven cryptosporidiosis. EIA units were calculated by reference to standard sera (from case 1) assigned to an arbitrary 10000 EIA units/ml. Arrow indicates time of second exposure to *Cryptosporidium*-infected calves. Zero time is the day of onset of symptoms; in both cases cryptosporidia were detected in faeces collected during the first few days of illness.

specimen was collected from each subject within 8 days of onset (acute sera) and 1–5 from each at varying intervals thereafter (convalescent sera). Results for cases 1 and 2, plotted against time from onset of symptoms are shown in Figure 1. Case 1 was a researcher who developed diarrhoea after exposure to experimentally infected calves. Antibody levels peaked between 3–6 weeks and fell to near baseline after about 6 months. Antibody levels were again elevated within a few days of two further discrete episodes of exposure to infected animals, although no symptoms occurred. In case 3, where was a significant change in OD values

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Table 1. *Anti*-Cryptosporidium *IgM and IgG antibody levels in Papua New Guinea and Australian children and Australian adults*

| Antibody group* | Total no. | No. with antibody levels in EIA units/ml | | |
|---|---|---|---|---|
| | | 0–1999 (%, 95% CI)†) | 2000–4999 | > 5000 |
| IgM | | | | |
| RCH 1985 | 80 | 77 (96·3±4·3) | 2 (2·5) | 1 (1·3) |
| RCH 1989/91 | 299 | 235 (78·6±4·7) | 40 (13·4) | 24 (8·0) |
| PNG Children | 205 | 123 (60·0±6·8) | 32 (15·6) | 50 (24·4) |
| Adults (Melbourne) | 79 | 63 (79·8±9·0) | 12 (15·2) | 4 (5·1) |
| IgG | | | | |
| RCH 1985 | 80 | 71 (88·8±7·0) | 6 (7·5) | 3 (3·8) |
| RCH 1989/91*¶ | 289 | 229 (79·2±4·8) | 16 (11·8) | 13 (9·0) |
| PNG children† | 205 | 167 (81·5±5·4) | 16 (7·8) | 22 (10·7) |
| Adults (Melbourne) | 79 | 73 (92·4±6·0) | 5 (6·3) | 1 (1·3) |

* PNG, Papua New Guinea; RCH, Royal Childrens Hospital patients.
† Figures in parentheses are percentages, with or without 95% confidence intervals [CI].
‡ Only acute or single sera from PNG children are included.
¶ 10 RCH sera were not tested for IgG.

between sera collected on the day of onset and 95 days later. In case 4 IgG levels fell significantly between early (8 days after onset) and late (32–67 days after onset) sera. With one exception (case 4), antibody levels in acute sera (collected 0–8 days after the onset of symptoms) were less than 20000 EIA units/ml. In case 4, an early high IgG level (5000 EIA units/ml on day 8) and the absence of an IgM response, suggested reinfection.

*Serum anti*-Cryptosporidium *IgG and IgM in children from Papua New Guinea and Australia*

The distributions of cryptosporidial antibody levels in 205 PNG children and 379 Melbourne children are shown in Table 1. In acute sera from PNG children there were no significant differences in mean antibody levels or in the proportions with high level antibody between controls and children with pneumonia, with or without diarrhoea. These results were combined and compared with those obtained for Melbourne children. Most sera in all groups (80% or more in Melbourne) had antibody levels less than 2000 IgG EIA units/ml, which was taken as the arbitrary cut-off value for a negative result. High IgM levels (> 5000 EIA units/ml) were detected more commonly than high IgG and in a greater proportion (24%) of children in PNG than in Melbourne (1–8% in different years). In all groups of children there was an increase in the proportion with high antibody levels with increasing age. This was most marked in PNG children in whom the proportion rose from 15% (13/86) in children under 6 months to 64% (16/25) in those over 2 years of age. The corresponding proportions for Melbourne children were 3% (2/78) and 11% (18/168).

There was no significant difference in the proportion of children with IgG levels above 5000 units/ml between the two groups, 11% (22/205) of PNG and 8% (29/369) of Melbourne children, except in the subgroup of children over 2 years, 28% (7/25) *vs.* 12% (19/159). In both groups, high IgG levels were uncommon in infants less than 6 months old; 2·6% (2/78) in Melbourne and 3·5% (3/86) in PNG. (Although there were some statistically significant differences in proportions of

Melbourne and PNG children with high antibody levels, the groups were otherwise different, particularly with respect to methods of selection; direct comparison is therefore probably not valid.)

Year-to-year variation in the proportions of sera having high antibody levels (4% in 1985; 7% in 1989/90 and 13% in 1990/1 for IgG) in Melbourne children apparently correlated with the prevalence of infection in the community, as reflected by the number of cases diagnosed at RCH namely: 1 case in 1985, 4 in 1989/90 and 26 in 1990/1 (faecal specimens from children with diarrhoea were examined routinely for *Cryptosporidium* only during the summer months, unpublished data).

Of 36 PNG children with pneumonia whose sera contained more than 5000 IgM EIA units/ml, 8 had measles and 6 also had diarrhoea; only 4 of the remaining 28 patients with pneumonia and high level *Cryptosporium* antibody had diarrhoea ($P = 0{\cdot}002$, Fisher's exact test). None of 14 controls with high antibody levels had diarrhoea. There was no significant difference in the proportions of sera with high levels of anti-*Cryptosporidium* antibodies from children with and without measles (8/28 *vs.* 42/130).

### *Anti-cryptosporidial IgM and IgG in paired sera from Papua New Guinea children with pneumonia*

Acute and convalescent sera were available from 29 PNG children with pneumonia; 14 (48%) had significant changes in IgM or IgG levels or high antibody levels (about 5000 EIA units/ml) in one or both sera. All 3 children less than 6 months old in this group had changes in or high levels of IgM antibody only compared with 6 of 11 older children; in the remainder, both IgG and IgM levels were high or rising. The proportion and age distribution of subjects with high cryptosporidial antibody in this small group of children with pneumonia did not differ significantly from those in PNG children overall.

### *Serum anti-*Cryptosporidium *IgG and IgM in Australia adults*

Most adult sera contained less than 2000 IgM units and 1000 IgG EIA units/ml. The proportions of sera which contained more than 5000 units/ml of IgG (1·3%) or IgM (5·0%) were lower than in sera from children (13% and 8%, respectively) collected during the same period (summer of 1990/1). The geometric mean IgG level was lower in adult than children (691 *vs.* 2190 EIA units/ml) but IgM levels were similar between the two groups (1702 *vs.* 1642 EIA units/ml).

## DISCUSSION

Acute infection with *Cryptosporidium* in four immunocompetent subjects lead to the development of specific IgG and IgM antibodies in serum within a few days, reaching a peak 3–6 weeks after the onset of symptoms and declining to near baseline levels within 1–6 months. Re-exposure of one individual provoked an anamnestic IgG response. Similar findings have been reported by others [9, 10]. In one study IgG was still detectable in some immunocompetent subjects 4 months after infection but all were seronegative after 12 months. However, 10 of 26 patients with AIDS had detectable IgG for up to 10 months, possibly because of

persistent infection [9]. These studies also showed some overlap in antibody levels between patients with proven cryptosporidiosis and controls, suggesting asymptomatic infection and/or persistence of antibody and failure of some infected individuals to produce antibody [9, 10].

In this study, IgG and/or IgM levels of 5000 EIA units/ml or greater in a single serum or a significant change between paired sera, tested in parallel, were arbitrarily taken as evidence of recent infection. These criteria were based on the distribution of antibody levels in the four subjects with proven cryptosporidiosis and in PNG children with definite changes between paired sera. The number of subjects was too few to determine the average period for which detectable antibody persists but on the basis of our limited data and that of others, 'recent' was taken to mean within 6–12 months of a single episode of self-limited infection. Using these criteria, there was evidence of recent infection in 4–13% of Melbourne children in different years and 5% of adults in 1990/1 (when 13% of children were affected). By contrast, evidence of recent infection was found in 24% (single sera) to 38% (paired sera) of PNG children.

High IgM levels were found more than twice as commonly as high IgG levels in PNG but not in Melbourne children. It is not clear whether this was due to some false positive IgM results or an age-related, predominantly IgM response to primary infection in younger PNG infants. The latter is supported by the observation that several children who had significant changes in IgM between paired sera had persistently low IgG levels. Others have described a relatively poor IgG response in primary infection in adults and children [10] and this is likely to be more marked in infants. However, the possibility of a nonspecific IgM response to other infections cannot be excluded.

The proportion of children with high anti-*Cryptosporidium* IgM antibody levels was similar for PNG children with and without measles. However, children with measles were significantly more likely to have diarrhoea associated with serological evidence of recent cryptosporidial infection. An association between measles and diarrhoea due to *Cryptosporidium*, has been reported previously, in Rwandan children [11], and could be related to the immunosuppressive effect of measles virus infection. Our findings suggest that infection occurs at a similar rate in children without measles but is more likely to be transient and asymptomatic.

Most studies of cryptosporidiosis have been in patients with diarrhoea and little information is available about asymptomatic infection. The contention that some infections are asymptomatic, is supported by anecdotal data from other studies. Antibody was found in some asymptomatic household contacts of subjects with cryptosporidiosis during an outbreak in the United Kingdom [10]. In a survey of enteric protozoa in 205 children in a Thai orphanage, *Cryptosporidium* was detected in 17 (8%) the majority of whom had no diarrhoea at the time or within a month of fecal examination [12]. Cryptosporidial antibody was detected in 8 of 18 hospital staff who had had close contact with a patient with chronic cryptosporidiosis, but none of 8 with limited exposure; only two of the seropositive staff members had diarrhoea [13]. The Australian children and adults tested in the present study, including those with serological evidence of 'recent' cryptosporidial infection were asymptomatic (although the possibility that they had a history of diarrhoea within the preceding 12 months was not excluded). The development of

diarrhoea in only a proportion of individuals infected may be due to exposure to a relatively high inoculum of *Cryptosporidium* in susceptible individuals, transient immunosuppression during a coincidental viral infection or other immunosuppressive disease.

The reported prevalence of cryptosporidiosis among patients with diarrhoea is 1–3% in adults and 5–10% in children in industrialized countries [14, 15] and 10–15% in developing countries [1, 15, 16]. Laboratory-confirmed cryptosporidial infections are a small minority because symptoms are often mild (or absent) and faecal examination not performed. The prevalence clearly varies in different populations, age groups, seasons and from year to year. Depending on the sensitivity and specificity of the tests used, serological surveys should reflect the prevalence and distribution of infection more accurately than selective fecal examination. However, antibody tests are not suitable for diagnosis of infection in individual cases unless paired sera demonstrate significant changes in levels.

Previous seroepidemiological studies have demonstrated differences in the proportions of seropositive subjects in unselected populations. High levels of *Cryptosporidium*-specific antibodies were detected by EIA in the majority of institutionalised Thai children, whether or not *Cryptosporidium* was detected in faeces (93% and 89% respectively) [12]. However, the antibody response was probably specific since much lower levels were detected and in only a small proportion (6%) of age-matched USA children, presumably reflecting differences in exposure to *Cryptosporidium* between locations. A study in two Latin American populations showed a high seroprevalence overall, with significant increases in IgG seroprevalence in the third to fifth years [17], as in the present study.

Predictably, our results indicate a higher seroprevalence among children in PNG than in Melbourne. This continued beyond 2 years of age in PNG but in Melbourne the peak age of infection was between 2–4 years of age (i.e. when most children are toilet-trained). The increased frequency of high antibody levels in children, compared with adults, presumably reflect a greater risk of exposure and hence of recent infection. Our findings suggest that the criteria used to define recent infection reflect the prevalence of infection in the population.

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