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Age-specific seroprevalence to an immunodominant *Cryptosporidium* sporozoite antigen in a Brazilian population

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SUMMARY

The seroepidemiology of *Cryptosporidium* infection was investigated in a representative sample of a normal population in the State of Sao Paulo, Brazil using a recombinant form of the immunodominant 27-kDa sporozoite antigen. IgG seropositivity was low in infants following loss of maternal antibody but quickly increased to ~60% by 5 years, then 80% by the age of 10 years, after which prevalence remained constant. The broad range of antibody concentrations is consistent with previous reports that the IgG response to *C. parvum* is short-lived. There is also evidence that average antibody concentrations increase with age. Results suggest that the recombinant antigen may be a more sensitive method of measuring seroprevalence than the native antigen in Western blot. Although cross-sectional studies can provide an insight into the epidemiology of *C. parvum* in normal populations, further studies investigating the dynamics of the humoral immune responses to *Cryptosporidium* and the use of serology in epidemiological studies are required.

INTRODUCTION

Cryptosporidium is an important protozoal pathogen responsible for outbreaks of diarrhoeal disease [1]. In immunocompromised hosts it poses a serious health threat [2] but in otherwise healthy hosts it is normally self-limiting and often asymptomatic, especially upon re-exposure to the organism [3, 4]. The development of protective immunity is not well understood but studies suggest that CD4(+) cells and IFN-γ play an important role [5]. The role of the humoral immune response is less clear but responses against two immunodominant sporozoite surface proteins, at ~15/17 kDa and 27 kDa have been associated with protection [6]. These two families of antigens have been used in many serological studies, mostly in Western blot analysis, to investigate the epidemiology of *Cryptosporidium parvum* infection, largely in community outbreaks or selected populations [7–13]. There have been relatively few studies reported on normal populations to estimate age-specific rates of acquisition of infection and none, to our knowledge, on a randomly selected age-structured population. Previously reported age-seroprevalence studies [14–21] have used either whole oocyst or sporozoite preparations as antigen but the sensitivity of using crude antigens in ELISAs has been questioned [6, 22], making comparisons difficult. However, these studies have reported seroprevalence levels which vary greatly between populations and which may suggest different epidemiological transmission patterns.

A recombinant form of the 27-kDa antigen was recently developed [9] and has been used in previous studies [23–28]. The antigen was shown to be specific

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and sensitive when compared with Western blot using native antigen [9]. Here we use the antigen to investigate age-specific seroprevalence in randomly collected sera from a suburban population in Brazil.

METHODS

Serum samples

Detailed description and methods of collection of blood samples from the town of Caieiras, northern Sao Paulo, have been described elsewhere [29]. Briefly, a random sampling strategy was used to select families from selected administrative regions. A total of 512 samples including 39 pairs of maternal-cord sera were collected from Caieiras Regional Hospital. Informed consent was obtained prior to collection. The age structure of samples is given in the legend to Figure 4. Sera were aliquoted and stored at −25 °C.

ELISA

A recombinant form of the 27-kDa *C. parvum* (Iowa strain) protein, termed cp23, was produced from cultured BL21 *Escherichia coli* containing the vector according to the method previously described [9]. Acceptable purity of the protein product (absence of other visible bands) was determined by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomasie Blue staining. Specificity was confirmed by Western blot assay using the C6B6 monoclonal antibody [30, 31]. A standard ELISA protocol was used for the determination of IgG antibody in sera. Briefly, chequerboard titration of a pooled, high-titre human serum was used to determine optimal antigen, serum and conjugate dilutions. cp23 antigen was coated onto Immunlon 2 HB microtitre plates (ThermoLabsystems, Franklin, MA, USA) diluted in carbonate buffer (pH 9·6) overnight at 4 °C. Plates were blocked with 5% skimmed milk powder (SMP) in PBS (pH 7·4) for 1 h at 37 °C. After washing three times with PBS + 0·05% Tween-20 (PBS-Tw), serum samples in duplicate were added at 1/100 in PBS-Tw + 5% SMP. After 2 h at 37 °C and a further three washes, rabbit anti-human IgG horseradish peroxidase (Dako, Ely, Cambs, UK) at 1/2000 in PBS-Tw + SMP was added and incubated for a further 2 h. Plates were developed using *O*-phenylenediamine in citrate buffer (pH 5) and read at 492 nm. A serially diluted high-antibody titre standard of human sera [10] was included in each plate. The standard serum was given an arbitrary unitage of 1000 U and regression analysis used to assign antibody concentration to test sera. Test sera with antibody concentrations >1000 U were re-screened at higher dilutions. Two approaches were taken to designate sera as seropositive or seronegative. The first by observation of a frequency distribution of total antibody concentrations as used previously [32, 33]. The second was to employ the cut-off used in previous Western blot-based studies where a threshold of 10% of the positive control was deemed to be a reliable indicator of antibody presence [10].

Western blot

The method used for immunoblot analysis of sera was based upon that previously described [8] with some modifications. *Cryptosporidium* sporozoite proteins were separated on 15% SDS–PAGE and electrophoretically transferred to polyvinylidene membrane which was cut into 2-mm strips for use. Sera were incubated with membrane strips at 1/50 dilution in PBS + 0·3% Tween and 5% SMP (PBS-Tw-SMP) for 3 h with agitation at room temperature and then overnight at 4 °C. Following three 10-min washes with PBS-Tw, rabbit anti-human IgG horseradish peroxidase conjugate (Dako) at 1/500 in PBS-Tw-SMP was added for 2 h at room temperature. Freshly prepared diaminobenzidine, 6 μg/ml, in PBS + 1 μl/ml 30% H_2O_2 was used as substrate to visualize bands followed by washing in distilled water. The intensity of bands was measured using a digital image analysed with UVISoft UniBand V99.01 (UniTech, Cambridge, UK) to estimate band intensity. Intensities of the 27-kDa bands obtained were expressed as a percentage of the positive control sera, as used in the ELISA [10], included in each run.

RESULTS

Results from seven sera screened by immunoblot using native antigen are shown in Figure 1. There was a strong correlation between the immunoblot and ELISA methods used ($r = 0{\cdot}88$, $P < 0{\cdot}005$) but it appears that antibody concentrations determined by ELISA were more likely to be positive than by immunoblot in the threshold region for seropositivity and, overall, were a higher proportion of the positive control (Fig. 2). Titration of the positive control (Fig. 1) demonstrated that antibody could be detected

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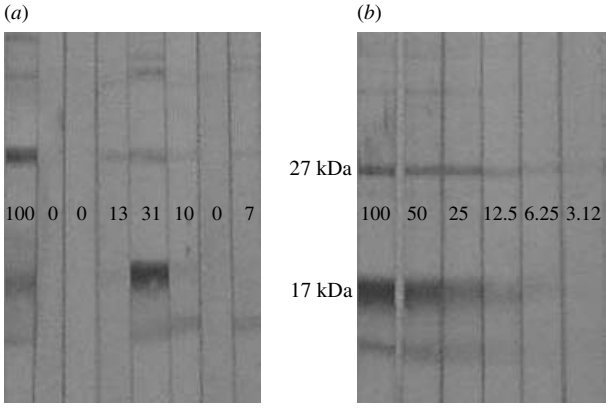

Fig. 1. Western blot of (*a*) serum samples from Sao Paulo and (*b*) a titration of a high titre positive control, using proteins extracted from purified oocysts. The location of the 27-kDa and 17-kDa antigens are indicated.

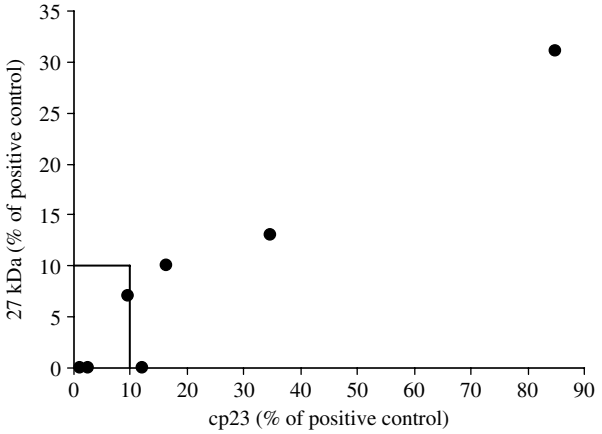

Fig. 2. Comparison of antibody concentrations, using the same sera from Figure 1, obtained from Western blot analysis using sporozoite antigen (intensity of the 27-kDa band) *vs.* the recombinant form of the 27-kDa antigen, cp23.

at levels ~3% of the positive control, which is below the cut-off level of 10% used previously [10] and also in this study.

Scatter plots of all samples screened in the cp23 ELISA are shown in Figure 3. High antibody titres in cord sera can be seen and at very low levels in a proportion of infants. There was a significant correlation between antibodies found in the 39 maternal-cord pairs ($r=0{\cdot}84$, $P<0{\cdot}001$) but no significant difference was evident between geometric means (antibody transfer ratio = 0·99) [34]. A broad range of concentrations was seen for all ages but there was no clear delineation between populations of seronegatives and seropositives, particularly in infants where primary infection occurred. A frequency distribution (not shown) of antibody concentrations for the total sample set did not reveal any suitable threshold for seropositivity. It was, therefore, decided that a threshold, previously evaluated [10], of 10% of the positive control standard would be applied. Figure 4 shows the proportion of seropositives for all age groups. Maternal antibody rapidly declines leaving ~60–80% of subjects seronegative until a steady rise in seropositivity occurs around 18 months of age. This reaches 60% by 5 years of age and ~80% throughout the adult age classes. Regression analysis on seropositive individuals aged >1 year revealed a steady increase in average antibody titre with age, although this was not significant. However, mean antibody titres in seropositive 1- to 10-year-olds ($n=119$, $x=2{\cdot}47$ log units) was significantly lower than in individuals aged ⩾20 years ($n=50$, $x=2{\cdot}61$, $t=1{\cdot}97$, $p=0{\cdot}026$).

DISCUSSION

Detection of IgG antibody to a recombinant immunodominant sporozoite antigen of *C. parvum* has been used to reflect previous exposure to infection. The age-related acquisition of antibody was similar to that recently described for infants in Guatemala using cp23 [28]. It is also similar to patterns observed for other common childhood infections where primary infection occurs early in life such that the majority of individuals have been infected by 10 years of age. It is, therefore, somewhat surprising that seroprevalence throughout adulthood does not increase much above 80%. However, this might be due to an underestimate of the extent of previous exposure due to the rapid decay of antibody to sonicated oocsyts [18] and sporozoite antigens [26, 35] that has previously been observed. Individuals infected some time in the past may now have antibody levels close to, or below, the threshold for seropositivity. The broad range of antibody concentrations seen throughout all age groups in this study, which was not found for other infectious agents using these sera [32], is consistent with a rapid loss of antibody. This may be confounded by the possibility that primary infection produces only moderate antibody responses which are boosted upon subsequent exposure such that average population baseline levels increase with age. Some evidence for this was seen in this study and has been observed in other surveys [13], it has also been reported for other childhood infections such as respiratory syncytial virus [32, 36].

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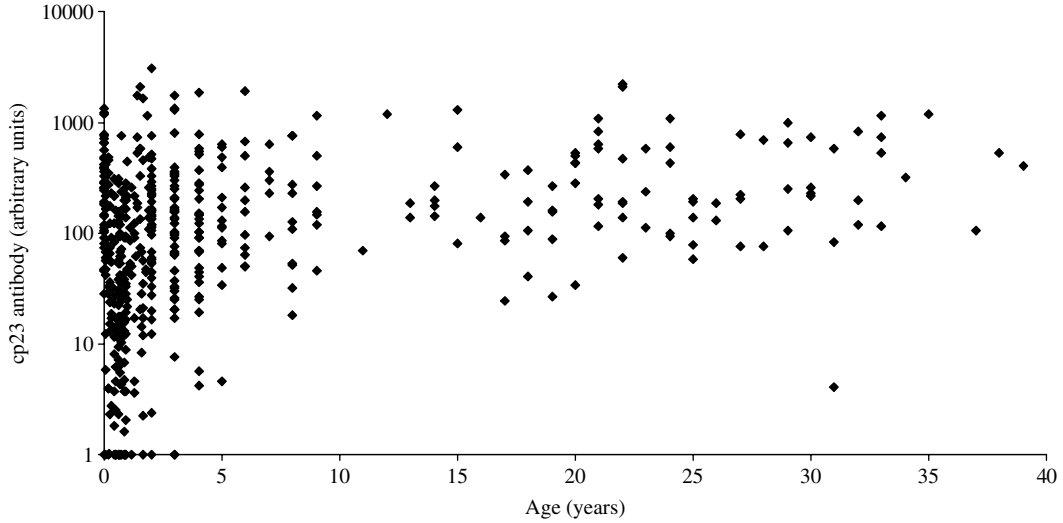

Fig. 3. Scatter plot of antibody concentrations in all subjects, using recombinant cp23 antigen in an ELISA. Antibody concentrations are in arbitrary units with 1000 U being equivalent to the undiluted high-titre positive control; 100 U denotes the cut-off for seropositivity.

Transplacental antibodies correlated with maternal antibodies but there was no evidence of active transport across the placenta. Factors that have previously been suggested to effect placental transfer such as gestational age and low birth weight, HIV and malaria [37, 38] are not applicable here as ratios >1 were seen in these sera for other viral pathogens [32, 33]. The lack of observed active antibody transport has only been previously reported for *Streptococcus pneumoniae* and *Haemophilus influenzae* [38]. Further research will determine whether this observation is ubiquitous and perhaps associated with rapid decay in adult antibody levels.

The intensity of transmission of *Cryptosporidium* around Sao Paulo is not known. It might be predicted that infection levels are high within the community as environmental studies have found high levels of oocysts in ground water [39] and treated water [40] supplies. All samples examined from raw sewage and creek water in Sao Paulo were contaminated with oocysts [41] which is of particular concern given that around 30 million people in Brazil do not have access to treated drinking water [42].

It was not an objective of this study to compare the sensitivity and specificity of the recombinant antigen with the immunoblot formats but to use the blot to confirm the identity of the cp23 antigen. Comparisons between immunoassays have been discussed elsewhere [8, 9, 43] and have led to some debate [44, 45]. However, this study suggests that cp23 protein in ELISA may be more sensitive than oocyst-derived protein in the immunoblot when detecting low levels of antibody

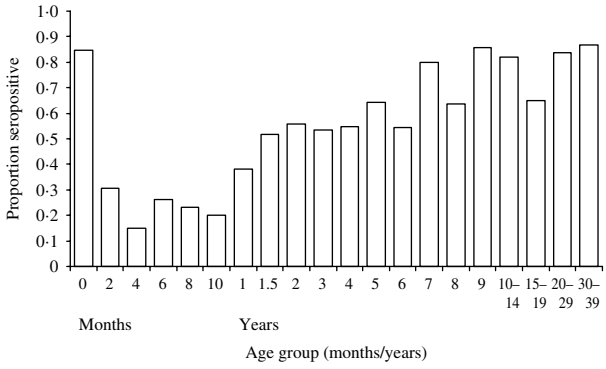

Fig. 4. The proportion of seropositive subjects using a threshold at 10% of the positive control. Sample sizes for each age group were as follows (months/no.): 0/39, 2/26, 4/20, 6/23, 8/43, 10/30; (years/no.): 1/21, 1·5/31, 2/68, 3/43, 4/31, 5/14, 6/11, 7/5, 8/11, 9/7, 10–15/11, 16–19/20, 20–29/43, 30–39/15.

and this requires further evaluation. It is clear that antibody is detectable below the threshold used for seropositivity in this and other studies but the correlation between such low levels of antibody and possible previous exposure is not understood. Therefore, although the use of a known standard serum and a common threshold for seropositive samples is suitable for comparative epidemiological studies, large-scale cross-sectional and longitudinal serological surveys with more in-depth statistical analysis and mathematical modelling are required.

The interpretation of serological assays is compounded by the complex epidemiology of transmission and infection as well as the dynamics of the immune response to the parasite. The confounding

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factors of repeated, non-seasonal and asymptomatic infection, coupled with a rapid loss in IgG suggest that using seroepidemiological studies to investigate the transmission dynamics of *C. parvum* within populations need further evaluation. However, this study shows that such surveys can be informative and could be even more so if coupled with surveillance and the recent developments in molecular epidemiological analysis of isolates [46].

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