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A two-year follow-up survey of antibody to *Cryptosporidium* in Jackson County, Oregon following an outbreak of waterborne disease

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SUMMARY

To estimate the duration of *Cryptosporidium*-specific antibody, a Western blot assay measured antibody in paired sera from 124 residents of Jackson County, Oregon collected 0·5 and 2·5 years after the end of an outbreak in Talent, Jackson County. The outcome measure was the intensity of antibody responses, (which may approximate to a titre), to 27-kDa and 15/17-kDa antigens. Intensity of response to the 27-kDa antigen(s) declined to 54% of the 1992 value while responses to a 15/17-kDa antigen(s) remained close to the initial values. Increasing age of the donor predicted higher intensity of antibody to the 15/17-kDa antigen(s) in both the initial ($P = 0{\cdot}004$) and follow-up ($P = 0{\cdot}038$) surveys. No relationship was observed between age and antibody intensity for the 27-kDa antigen(s) during either survey ($P > 0{\cdot}10$). Both the initial and follow-up surveys showed significant elevations in antibody intensity for Talent residents, possibly indicating a high endemic rate of infection/re-infection or high levels of chronic infection.

INTRODUCTION

Cryptosporidium is a protozoan that is widely distributed in the environment and is increasingly recognized as a cause of epidemic gastroenteritis in humans [1]. It can be transmitted through contact with infected animals, person-to-person, or through ingestion of contaminated food or water [2–5]. Numerous waterborne outbreaks have been reported in the USA, Canada, and the UK [6, 7]. Surveys of untreated surface water sources and treated drinking water have shown that the parasite is widely distributed, perhaps exposing a large proportion of the population through contaminated drinking water [8, 9].

The implications of the widespread distribution of the parasite in drinking water and the absence of outbreaks among consumers of the water are unclear. Repeated *Cryptosporidium* infection may reduce the severity of symptoms, perhaps resulting in predominantly asymptomatic infections in populations with high endemic rates of infection [10]. Since both symptomatic and asymptomatic infections usually result in a serological response [11], endemic levels of infection in a population can be studied by estimating the sero-prevalence of antibody to different *Cryptosporidium* antigens. Interpretation of these studies has been hindered by uncertainty over the duration of antibody response. A paired serosurvey was conducted examining antibody response to the whole oocyst [12], but these results provide little information

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on the duration of antibody response to specific *Cryptosporidium* antigens. The purpose of this study was to characterize the duration of serological responses to two *Cryptosporidium parvum* antigen groups. These responses were studied because a prior study suggested that antibody to these groups increase following infection [13]. Immune responses were ascertained using a Western blot assay for sera drawn 0·5 and 2·5 years after the outbreak. A secondary goal was to identify risk factors by relating serological responses to questionnaire data obtained at the time of the initial sera collection, 0·5 years after the outbreak.

METHODS

Collection of sera

During the period January–June 1992, an outbreak of waterborne cryptosporidiosis was identified and investigated in Jackson County, Oregon [14]. Epidemiological evidence implicated the drinking water supply of Talent and inconclusive epidemiological and microbiological evidence implicated the water supply of Medford, a neighbouring city [15]. Following approval by an ethical review board, sera were collected in November, 1992, 6 months after the outbreak ended, from 380 Jackson County American Red Cross (ACR) blood donors. These individuals completed a consent form and a brief questionnaire about foreign travel, swimming, young children in the household, household children in day care, untreated drinking water consumption, occupational exposure to cattle, and the participant's age, sex and race. Participants were also asked about their residence, work locations, and the source of their municipal drinking water.

In August 1994, following a second ethical review board approval, the ACR recruited these individuals for a follow-up study. A total of 124 of the 380 blood donors agreed to participate by donating sera.

Serological analysis

In all cases, sera were separated and frozen at −20 °C for later analysis. Western blot analyses of the initial sera were performed at the Centers for Disease Control and Prevention, Atlanta, Georgia and the follow-up analyses were performed at The Southwest Center for Managed Care Research, Albuquerque, New Mexico. The initial Western blot analyses used SDS–PGGE (polyacrylamide gradient gel electrophoresis), while the follow-up analyses used SDS–PAGE (polyacrylamide gel electrophoresis) mini-gels. Twenty duplicate samples were tested to validate the two methods. *C. parvum* oocysts for both the initial and follow-up assays were obtained from a calf [16]. The oocysts were purified and antigens were extracted from the purified oocysts using methods previously described [17, 18]. Extracted proteins were treated with sodium dodecyl sulfate (SDS) without reducing agents, as previously described [19].

Western blots with SDS–PGGE

The SDS-treated proteins were separated by SDS–PAGE using 3–25% gradients in a discontinuous buffer system [17]. After electrophoretic transfer onto polyvinylidene fluoride (PVDF) membrane, 2 mm wide strips were cut from the sheets and the transferred proteins were exposed to 1/200 dilution of the patient's serum in PBS/0·3% Tween. Bound *Cryptosporidium* antibodies were reacted with biotinylated mouse anti-human IgG. The bound secondary antibodies were exposed to strepatavidin alkaline phosphatase and then visualized with 5-bromo-4-chromo-3-indolyl phosphatase as a substrate and nitro-blue tetrazolium as chromagen. The PGGE system separated three antigen groups: 27-kDa, 17-kDa and 15-kDa.

Western blots SDS–PAGE mini-gel

The SDS-treated proteins were separated by SDS–PAGE using a 15% mini-gel in a discontinuous buffer system. Unlike the Western blots for the initial analysis, the sheets containing transferred proteins were not cut, but placed in a Bio-Rad Multi-Screen apparatus. This device isolates separated proteins for analysis of different sera. Each isolated area of the sheet was exposed to human sera at a 1/50 dilution in PBS/0·3% Tween. Bound human anti-*Cryptosporidium* antibodies were reacted with biotinylated mouse anti-human IgG diluted 1/500 in 0·3% Tween. The secondary antibodies were detected as described above.

Positive and negative controls

Positive control serum for the PGGE was collected from a patient with confirmed cryptosporidiosis who had a strong serological response. Negative control

serum was collected from a person whose stool was negative for oocysts and had no detectable antibody to these antigens. Positive and negative controls were included on each blot. For the mini-gel analysis, positive control sera from several individuals were mixed so that the intensity of the response was comparable ($\pm 10\%$) to the intensity of PGGE positive control sera. Although the PGGE system in the initial analysis clearly separated the three antigen groups, the Western blots with mini-gels separated the 27-kDa, but did not separate the 17-kDa from the 15-kDa group. Even though the mini-gels did not resolve the 15-kDa group from the 17-kDa group, antibody responses to antigens in comparison to the positive control were almost identical in the two systems. For this study, these antigen groups are referred to as 27-kDa and 15/17-kDa markers.

Data generation

Both the initial and follow-up sera were analysed by the IS-1000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, CA). The IS-1000 was calibrated using a grey scale card in the spot densitometry mode. When calibrated, the blot is imaged and stored as a tagged image file format (TIFF) file. The TIFF file is then digitally analysed. For the follow-up analysis, the 27-kDa and 15/17-kDa markers were manually selected for analysis. The bands were represented as peaks, with the baselines and limits set manually prior to integration by the IS-1000. For the initial analysis, the peak height of the 15-kDa and 17-kDa markers were determined together, with the greater of the peak heights of the two bands selected. In the follow-up analysis, a single peak height for the 15/17-kDa marker was recorded. In both cases, the peak height generally was associated with the 17-kDa marker. The data were then transferred to an Excel™ file for analysis. Band intensities were expressed as the ratio of the unknown serum value to the positive control serum.

Statistical analysis

Statistical analysis was conducted using SPSSPC™ version 5.0. Results are reported as the actual ratios of the unknown to the positive control. For statistical testing, the square root of this ratio was used so that the distribution of the markers approximated that of the normal distribution. Relationships between the band intensities of each antigen marker and the risk factors (age, sex, occupational exposure to cattle, Medford or Talent exposure, and foreign travel) were tested using an analysis of variance. Statistical tests for differences in the fraction of positive individuals by risk factor were tested using a χ^2, Fisher's exact test or a Mantel–Haenszel test for linear association, as appropriate.

RESULTS

The duration of the immune responses to the antigen groups varied. In general, the response to the 15/17-kDa marker was very stable over time and the mean intensity of the ratio on the repeat sample from blood donors was 91% that of the initial value for the 15/17-kDa marker (Table 1). In contrast, the mean antibody level against the 27-kDa marker on the second sample was 54% of the initial value (Table 1). The decline in mean intensity over the 2-year period did not show any relationship with increasing age or other risk factors.

Risk factor information from questionnaires was compared to serological responses. An increased ratio of serological response was observed with increasing age for the 15/17-kDa marker from both the initial and follow-up analyses (Table 1). This increase was statistically significant ($P < 0{\cdot}02$). For the 27-kDa marker, an increasing ratio of serological responses with age was also observed, but was not statistically significant for either the initial or repeat analyses ($P > 0{\cdot}15$). Foreign travel, swimming, cattle exposure, children in day care, children in the household, and untreated water consumption between January and November, 1992 were not predictive or increased serological responses during either the initial or follow-up surveys.

Residence in Talent was associated with an increased intensity for both markers at both time periods (Table 2). The difference was statistically significant for the 15/17-kDa marker in both the initial ($P = 0{\cdot}004$) and the repeat ($P < 0{\cdot}04$) samples and for the 27-kDa marker in the second ($P < 0{\cdot}006$) but not the initial samples ($P = 0{\cdot}06$). The decline in the follow-up antibody response was modest for the 15/17-kDa marker for both Talent (85% of initial values) and non-Talent residents (91% of initial values) and for the 27-kDa marker among Talent residents (89% of initial values). In contrast, the intensity of serological response for the 27-kDa marker declined to 51% of its initial value for non-Talent residents. The rank correlation between the

Table 1. *Persistence of IgG antibody response to* Cryptosporidium *antigens by age of donor*

		Nov. 1992		Sept.–Oct. 1994	
Age	*n*	15/17-kDa	27-kDa	15/17-kDa (% of 1992)	27-kDa (% of 1992)
All donors	124	0·316	0·574	0·287 (91%)	0·309 (54%)
18–39	39	0·189	0·488	0·167 (88%)	0·224 (46%)
40–49	41	0·282	0·568	0·259 (92%)	0·335 (59%)
50–59	25	0·411	0·624	0·359 (87%)	0·330 (80%)
60+	19	0·481	0·701	0·511 (106%)	0·413 (59%)
P-value*		0·015	0·357	0·001	0·186

* Results are displayed as the average ratio of the unknown to the positive control. *P*-values are based on analysis of variance or *t*-tests of the square root of this ratio.

Table 2. *Serological responses by city of residence*

		Nov. 1992		Sept.–Oct. 1994	
City of residence	*n*	15/17-kDa	27-kDa	15/17-kDa (% of 1992)	27-kDa (% of 1992)
Talent	6	0·719	0·931	0·613 (85%)	0·827 (89%)
Other	118	0·295	0·556	0·270 (91%)	0·282 (51%)
P-value*		0·004	0·064	0·038	0·006

* Results are displayed as the average ratio of the unknown to the positive control. *P*-values are based on analysis of variance or *t*-tests of the square root of this ratio.

initial and repeat serological intensities were statistically significant ($P < 0{\cdot}001$) for both the 17-kDa and 27-kDa markers.

DISCUSSION

In this study, we found relatively high initial serological responses to the 27-kDa marker among individuals tested 6 months after the outbreak. In most individuals these responses declined over the following 2 years. The mean response during the follow-up survey was 54% of the mean initial value. Antibody to the 15/17-kDa marker was initially much lower relative to the positive control and did not decline further. One possible reason for this different response is that the 27-kDa marker may have remained high for an extended time after the outbreak, whereas the 15/17-kDa marker may have declined to baseline prior to November, 1992.

Based on routine surveillance by the county health department, evidence of an outbreak of illness did not extend beyond June 1992, but residents of Talent have maintained high antibody levels 2·5 years later. Since only limited improvements in the treatment plant operation were possible without changes in equipment [15], Talent residents may have continued to receive exposure to *Cryptosporidium*, thus maintaining their higher serological response by re-infection. Alternatively, they may have developed chronic, asymptomatic infections.

The implications of high levels of asymptomatic infections should be considered when assessing the risks of drinking oocyst-contaminated water. During the initial Talent outbreak there were indications that residents were less susceptible to illness than were out-of-town guests to a wedding party, and Medford residents provided Talent drinking water were at high risk of illness [20]. If a history of multiple prior infections protects against illness from subsequent infection, as has been suggested [10], then failure to detect illness among consumers of oocyst contaminated drinking water may not imply that the oocysts are incapable of causing illness in susceptible persons. Long-term residents of communities such as Talent, who may receive recurrent exposure to oocysts, may eventually experience minimal risk of illness from re-infection.

From this study, the baseline serological responses to *Cryptosporidium* antigens appear to increase with increasing age. The high correlation between individual serological responses in the initial and follow-up surveys suggest that the responses may remain relatively stable for an individual. Higher serological intensities with increasing age may reflect the indi-

vidual's anamnestic immune response to prior *Cryptosporidium* infections. Because older people tended to have an increased likelihood of remaining seropositive over the 2-year period, they may be at increased risk of carrying ongoing, asymptomatic infections or may be at greater risk of infection than are younger persons.

This study suggests that sero-surveys may be a useful tool to characterize the relative importance of various risk factors for *Cryptosporidium* infections. Additional studies are needed to more fully characterize the serological response and to examine characteristics of the serological responses during outbreaks. Unfortunately, sera have not been routinely collected for *Cryptosporidium* serological analysis during outbreaks. Future outbreaks should include the collection of sera, including, if possible, sera for the period prior to, during and after the outbreak.

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