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ENZYME IMMUNOASSAY OF *CRYPTOSPORIDIUM*-SPECIFIC IMMUNOGLOBULIN G ANTIBODIES TO ASSESS LONGITUDINAL INFECTION TRENDS IN SIX COMMUNITIES IN BRITISH COLUMBIA, CANADA

CORINNE S. ONG,* ANNA S. LI, JEFFREY W. PRIEST, RAY COPES, MOHAMAD KHAN, MURRAY W. FYFE, STEPHEN A. MARION, JACQUELIN M. ROBERTS, PATRICK J. LAMMIE, AND JUDITH L. ISAAC-RENTON

Department of Pathology and Laboratory Medicine and Department of Health Care and Epidemiology, University of British Columbia, Vancouver, British Columbia, Canada; Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; British Columbia Centre for Disease Control, Vancouver, British Columbia, Canada

Abstract. A newly developed enzyme-linked immunosorbent assay (ELISA) that detects immunoglobulin G antibodies to the 27-kDa *Cryptosporidium parvum* sporozoite surface antigen was used to test 4,097 sera collected from pregnant women in 6 communities in British Columbia, Canada, between January 1996, and December 1997. Waterborne outbreaks of cryptosporidiosis occurred in two of the study communities during the period of follow-up, and ELISA seropositivity was high in all six communities during the study period (77% positive to 92% positive). In the community with the largest outbreak, levels of antibody to the 27-kDa antigen increased rapidly and then decayed to background levels within 3–4 months of the peak of the epidemic curve. Trends in serologic reactivity were complex in all communities, and increased antibody levels not related temporally to known waterborne outbreaks were also observed. Serological assays may provide more accurate information regarding community levels of *Cryptosporidium* infection.

INTRODUCTION

Infections with the protozoan *Cryptosporidium parvum* are characterized by a relatively low infectious dose,[1] high numbers of parasite oocysts in host feces,[2] and the absence of an effective drug therapy. Oocysts survive well in the environment[3] and frequently contaminate raw surface-water sources and some treated drinking water supplies.[4] Large waterborne epidemics are well described.[5–10] The number of recognized outbreaks, however, is less than expected given the widespread distribution of oocysts in the environment.[11] Population level immunity may be an important determinant of epidemic waterborne transmission of cryptosporidiosis.

Recent studies suggest that a serological approach may be useful for the study of the epidemiology of cryptosporidiosis.[12–17] Two newly developed enzyme-linked immunosorbent assays (ELISAs), one that uses a partially purified, native *C. parvum* 17-kDa antigen and another that uses a recombinant form of the *C. parvum* 27-kDa antigen, reliably detect immunoglobulin G (IgG) serum antibodies after infection.[18] The 27-kDa antigen ELISA was shown to be slightly more sensitive and specific (92% and 100%, respectively) than the 17-kDa ELISA (77% and 88%, respectively) when compared with the gold standard: large-format Western blot. In addition, 96% of stool-confirmed cryptosporidiosis patients who donated a serum sample between 3 and 13 weeks of symptom onset were positive for antibodies to the 27-kDa antigen by ELISA, and antibodies to the 27-kDa antigen were shown to remain above the threshold of detection for an extended period of time after infection.[18] Because many persons with cryptosporidiosis may not be identified by traditional clinical laboratory means or by public health surveillance, we suggested that serologic assays may provide more accurate information regarding the level of community infection.[19]

In 1996, waterborne outbreaks of cryptosporidiosis were reported in British Columbia (BC).[20] Sera that were collected from pregnant women between 15 and 45 years of age resident in outbreak and non-outbreak communities were tested by ELISA for antibodies to the 27-kDa antigen. Results were used to characterize trends in serologic reactivity during a 24-month period that spanned the identified outbreak periods. The community levels of antibody to *C. parvum* were compared with reported case data obtained through the British Columbia (BC) public health surveillance system.

MATERIALS AND METHODS

Ethical review. The Clinical Screening Committee for Research Involving Human Subjects, University of British Columbia, approved this project (certificate C 96-0404). Samples used in this study were not linked to personal identifiers.

Community identification and serum collection. Six British Columbia communities were targeted for study based on the source of public drinking water: one community (Community 1) uses deep-well sources, whereas the other five (Communities 2, 3, 4, 5 and 6) use surface-water sources (Table 1). None of the community drinking water supplies are filtered prior to distribution, but all are chlorinated. Cryptosporidiosis outbreaks that were linked to drinking water by epidemiologic studies occurred in two of the study communities (Communities 4 and 5) during 1996.

Sera collected for routine monitoring for rubella in pregnant women between 15 and 40 years of age were used to provide a cross section of the adult population in each of the 6 study communities. Banked sera, collected from confirmed residents of the study communities during a 2-year period between January 1996 and December 1997 were selected by the diagnostic laboratory that performed the rubella testing. Serum aliquots were labeled by community and by month and year of collection and were stored frozen at –20°C until needed for testing. We attempted to test 23 samples per month from each community, but obtaining the requisite number of samples from the two smallest communities (Communities 3 and 5) was problematic, especially in 1997. For most communities, equal numbers from sera were selected for

* Address correspondence to Corinne S. Ong, British Columbia Centre for Disease Control, 655 West 12th Avenue, Vancouver, British Columbia, Canada V5Z 4R4. E-mail: cong@interchange.ubc.ca

TABLE 1
Community and watershed details

Community	Population	Outbreak identified (year)	Drinking water source and treatment
Com 1	103,625	No	Deep well, unfiltered, chlorinated
Com 2	546,211	No	Surface, protected watershed, unfiltered, chlorinated
Com 3	13,900	No	Surface, unfiltered, chlorinated
Com 4	49,800	Yes (1996)	Deep lake intake, unfiltered, chlorinated
Com 5	25,277	Yes (1996)	Surface, unfiltered, chlorinated
Com 6	206,583	No	Surface, protected watershed, unfiltered, chlorinated

each week whenever this was possible so that the samples were evenly distributed over the 2-year study period. We tested an average of 120 samples per month from the largest community (community 2; Table 1) during the first year of the study. Samples collected from Communities 1–3 in 1996 were used in an earlier mini-blot analysis of *C. parvum* antibodies.[16]

ELISA assays. The IgG antibody response to the parasite 27-kDa antigen was tested by recombinant protein ELISA as described elsewhere.[18,21] Briefly, 96-well plates (Immulon II, Dynatech Industries, McLean, VA) were coated overnight at 4°C with 50 μL of 0.1 M $NaHCO_3$ buffer (pH 9.6) containing purified protein at 0.14–0.2 μg/ml. After blocking and washing, diluted sera (1:50 in 0.05% Tween-20 with 0.85% NaCl and 10 mM Na_2HPO_4 at pH 7.2) were loaded in duplicate wells and were incubated at room temperature for 2 h. Two buffer blanks and a battery of three Western-blot-positive sera and two Western-blot-negative sera were included on each ELISA plate to control for intra- and inter-assay variation. A twofold serial dilution (1:50 to 1:12,800) of a strong positive control was used to generate a nine-point standard curve on each ELISA plate. Bound antibodies were detected using a biotin-labeled, mouse monoclonal anti-human IgG secondary antibody and alkaline phosphatase-labeled streptavidin as previously described. The ELISA absorbance values of test sera were converted to arbitrary unit values using the standard curve with a four-parameter curve fit. The 1:50 dilution of the standard curve serum was assigned a value of 6,400 arbitrary units. Unknown sera were retested if the coefficient of variation for the duplicate well readings exceeded 15%. Based on previous work with longitudinal serum samples from stool-confirmed cryptosporidiosis patients from British Columbia, an ELISA value > 86 arbitrary units was considered positive for antibodies to the 27-kDa antigen.[18]

Public health surveillance. Cryptosporidiosis is a reportable communicable disease in British Columbia. Under provincial legislation, diagnostic laboratories are required to report cases to public health officials in the jurisdiction of residence of the patient. All diagnostic parasitology laboratories routinely test for cryptosporidiosis using an acid-fast staining technique for examination of fecal specimens by microscopy.[22] Data from cases are entered into a central electronic communicable disease surveillance system. The number of confirmed cases reported by diagnostic laboratories to the regional public health units in the six communities was recorded for the study period.

Statistical analysis. The percent of patients who were positive for antibodies was calculated and compared by community, month of sample, and year using Sudaan version 8.0 to take into consideration the clustering effect of monthly sampling. Multiple comparison *t* tests were used to compare communities. The significance level was set at alpha = 0.003 for 1996 community comparisons (Communities 1–6) and at alpha = 0.005 for 1997 community comparisons (Communities 1, 2, 4, 5, and 6) to maintain an overall experiment-wide alpha level of 0.05 for each year.

The analysis of antibody responses from the six communities was approached two ways: 1) the overall (regardless of month) mean antibody level from Community 1 (deep well) was used as a baseline against which each year/month mean antibody level from Communities 2, 3, 4, 5, and 6 (surface water) was compared; 2) the mean antibody level from January through May 1996 for each community was used as a baseline against which each subsequent month in the same community was compared. All means were computed based on the natural log of the antibody responses of individual patients. Dunnett's multiple comparison test[23] was used to test for differences between monthly antibody levels and their respective baseline holding the experiment-wise error rate to a level not exceeding the alpha level of 0.05. Months that had ≤ 10 samples collected were not analyzed.

RESULTS

Serum samples from 4,097 pregnant residents of the 6 study communities were tested by ELISA for IgG antibodies to the 27-kDa *Cryptosporidium* antigen. Using the previously described ELISA cutoff value of 86 arbitrary units,[18] 3,464 of the tested serum samples (85%) were positive for *C. parvum*–specific antibodies. Community 1, with a deep-well drinking water supply, had the lowest overall prevalence of positive sera in the study period (77%), whereas Community 4, which had a large outbreak of waterborne cryptosporidiosis in 1996, had the highest overall prevalence of positive sera (92%) (Table 2). When the monthly percentages of positive sera were compared by year, Community 4 was significantly different from Communities 1 and 2 in 1996, and Communities 4 and 5 were significantly different from Communities 1 and 6 in 1997.

Figure 1 shows the relationship between the number of cases reported to public health in the 6 study communities and the monthly 27-kDa antigen ELISA median values and geometric mean values for the selected community residents. Despite the detection of low numbers of cryptosporidiosis cases in each of the communities in the late fall and early winter of 1996, no peaks in community antibody levels were observed in monthly data from the residents of the deep-well control community (Community 1) or from residents of two of the surface-water communities (Communities 2 and 6). In contrast, residents of Community 4, where a large waterborne outbreak of cryptosporidiosis occurred between June and October of 1996, had a sharp rise in serologic antibody response in September of 1996 that decayed over the following 2–3 months. Community levels of antibody in September, October, and November were significantly higher than levels

TABLE 2
Mean prevalence and range of antibody responses to the 27-kDa *Cryptosporidium* antigen

Community	1996 No. tested (monthly median)	1996 Prevalence (%) (monthly range)	1997 No. tested (monthly median)	1997 Prevalence (%) (monthly range)	Total no. tested	Total prevalence (%)
1	283 (22	75 (45–89)[b]	269 (23)	79 (65–91)[d,e]	552	77
2	1,445 (120)	84 (79–89)[c]	274 (23)	86 (74–96)	1,719	84
3	219 (17.5)	83 (50–94)	61 (4)	95 (NR)[a]	280	86
4	272 (23)	92 (78–100)[b,c]	276 (23)	91 (74–100)[d,f]	548	92
5	262 (23)	88 (78–100)	195 (16)	91 (75–100)[e,g]	457	89
6	272 (23)	83 (61–96)	269 (23)	79 (65–95)[f,g]	541	81

[a] No range given because of the small number of samples.
[b–g] Mean prevalence values indicated by the same footnote letters (b to g) were found to have significant differences.

found in the deep-well control community (Community 1), and antibody levels in September and October were significantly above the January to May pre-outbreak baseline for this community. Thus, the serological response in Community 4 mirrored the surveillance-based epidemic curve with a time lag of 1–2 months.

Serological trends from the remaining two study communities varied from the previously described pattern. Community 3, which is physically adjacent to Community 4 and draws its drinking water from a different water source, also had an increase in the number of detected cryptosporidiosis cases (peak in August 1996) and a significant increase in community antibody reactivity (July 1996). However, the peak antibody level in Community 3 was low in comparison to that observed in Community 4, and its timing was shifted relative to case detection: the antibody response peak was in July, but the highest number of detected cases did not occur until August. In contrast to the outbreaks in Communities 3 and 4, the outbreak in Community 5 in June of 1996 did not result in a detectable increase in 27-kDa antigen–specific antibody response among the selected residents. A peak in the community antibody level was detected in April of 1997 by comparison to the deep-well control community, but this peak was not associated with more cryptosporidiosis cases. We were unable to determine whether this peak persisted in the following month (May) because of an insufficient number of serum specimens.

In earlier work on the kinetics of the 27-kDa antigen response in stool-confirmed cryptosporidiosis patients, we noted that while more than half of the patients (54%) had antibody levels above 1,000 arbitrary units within 92 days of symptom onset, only 7% had such high levels 1 year later.[18] Using this antibody level as a benchmark for recent infection, we determined the proportion of sera (expressed as a monthly percentage) from the 6 communities that had antibody responses of greater than 1,000 arbitrary units (Figure 2). The 3 communities (Communities 3, 4, and 5) that had the highest rate of *Cryptosporidium* cases detection all had months where more than 25% of the selected residents were high responders (ELISA response > 1,000 arbitrary units). In Community 4, approximately half the community (48% and 57%, respectively) had antibody responses above 1,000 arbitrary unit level in September and October 1996, 1–2 months after the reported number of cases peaked (Figures 1 and 2). In Community 3, increased numbers of high responders were evident in a 3-month period that spanned the peak of the epidemiologic curve. Community 5 had only 1 month (July 1996) where high responders were 26% of the sample, and, as in Community 4, this occurred after the peak of case detection.

DISCUSSION

In an earlier study, Isaac-Renton and others used a mini-blot assay format to show that residents of two communities who received water from a surface-water supply with occasional oocyst contamination were more likely to have a *C. parvum*–specific antibody response than residents of a community who received water from a deep-well supply that was oocyst free.[16] They were also able to detect an increase in seroprevalence that was temporally associated with an increase in the number of reported cases of cryptosporidiosis in one of the surface-water communities. In the current work, we have added a second year of surveillance to two of these communities, and we have included surveillance from three additional surface-water communities, two of which had waterborne outbreaks of cryptosporidiosis during the period of study. We decided to use a newly developed *C. parvum* 27-kDa antigen ELISA for the current study because the ELISA is fast and relatively inexpensive to use, uses a recombinant antigen that can be produced cheaply and in large quantities, has a wide response range, and is more sensitive (relative to the gold standard large-format Western blot assay) than the mini-blot format assay used in the earlier work.[18] In fact, when the 1,944 samples previously assayed by mini-blot were reexamined using the 27-kDa antigen ELISA, a substantial number of additional positive sera were detected (50.5% positive versus 82.6% positive, respectively). Even if the ELISA cutoff value was increased by 50% to 129 arbitrary units, more positives were detected by ELISA in these three communities (73.6% positive) than by mini-blot. We believe that the numerical results provided by the ELISA make it a more useful assay format for the types of epidemiologic studies described here.

The percentage of sera that were positive for antibodies to the *C. parvum* 27-kDa antigen was high in all of the communities studied in this work (≥77%). This observation is consistent with other serological studies[11,15] and suggests that infection with *Cryptosporidium* may be very common and that the antibody response may be long lived. Comparisons of the number of laboratory-confirmed cases with the serologic results from residents of each of the study communities (Figure 1) showed that reports of disease to public health agencies were lower than would be predicted from the serologic data. For instance, only 157 laboratory-confirmed cases (0.3% of the population) were reported to public health during the 1996 outbreak in Community 4, yet more than 50% of the selected resident sera had evidence of recent *Cryptosporidium* infection in the September–October period (Figure 2). This is consistent with a significant underestimation of *Cryp-*

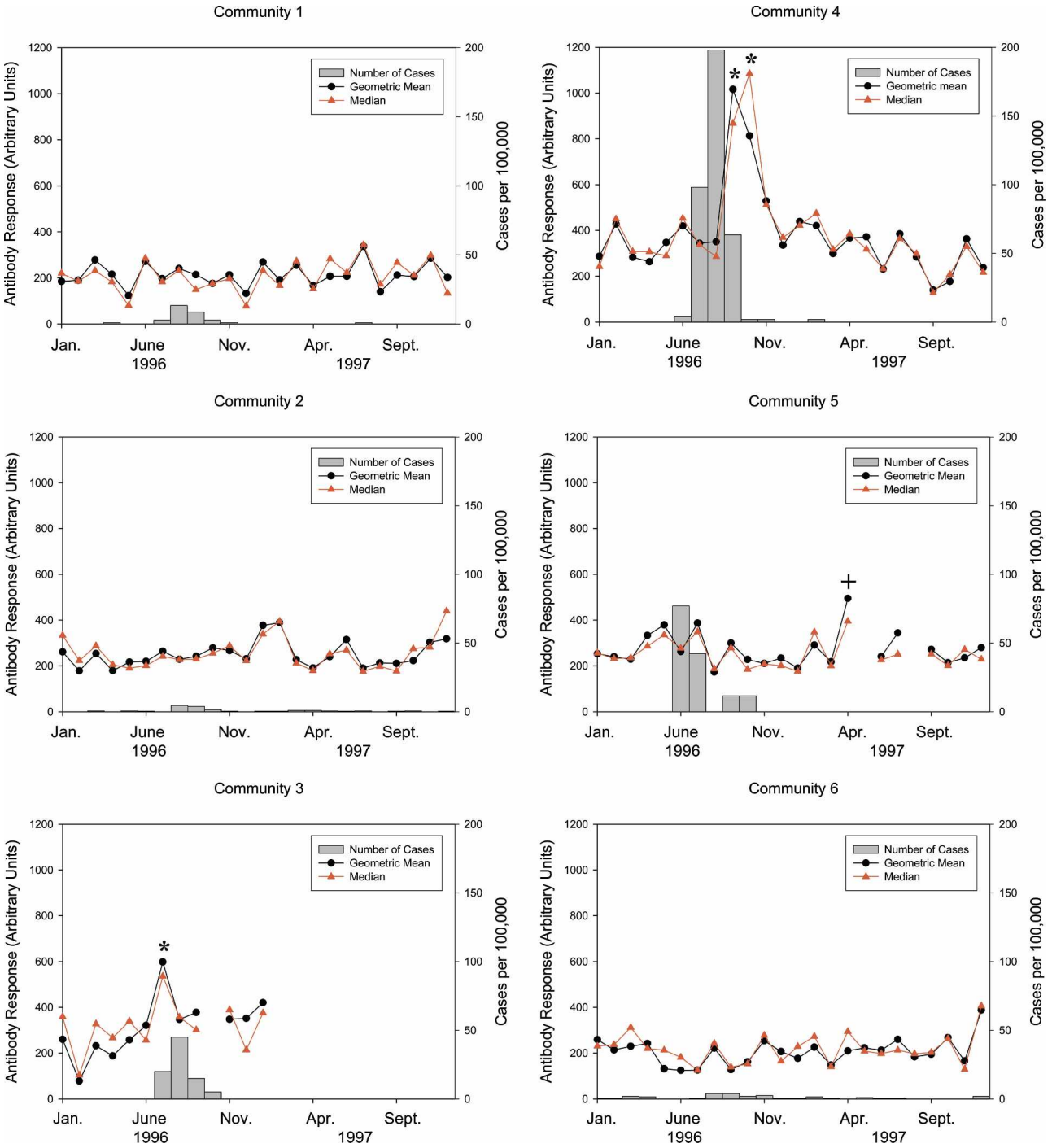

FIGURE 1. Serological responses to the recombinant 27-kDa antigen in 6 British Columbia communities. Responses for months in which less than 10 sera were tested have not been plotted. Bars indicate the number of laboratory-confirmed cases of cryptosporidiosis that were reported to public health. Lines show the antibody response by month plotted as the geometric mean (filled circles) or as the median (filled triangles). Geometric mean responses that are significantly different from the overall value for control Community 1 and from the community-specific baseline (includes same-community responses from January to May 1996) are marked by an asterisk (*). Geometric mean responses that are significantly different from the overall value for control Community 1 only are marked by a plus sign (+). This figure appears in color at www.ajtmh.org.

tosporidium infection with the laboratory diagnostic tools currently in use throughout North America and Europe and with the suggestion that many community infections, possibly clusters of infection, are not being recognized by public health.[24] Current surveillance methods are insensitive, detecting far fewer infections than are actually occurring. Thus the morbidity caused by this gastrointestinal infection may be underestimated significantly.[25]

The persistence of the antibody response to the 27-kDa antigen probably also contributes to the high antibody levels that were detected by ELISA, especially in communities where no recent, large-scale cryptosporidiosis outbreaks are

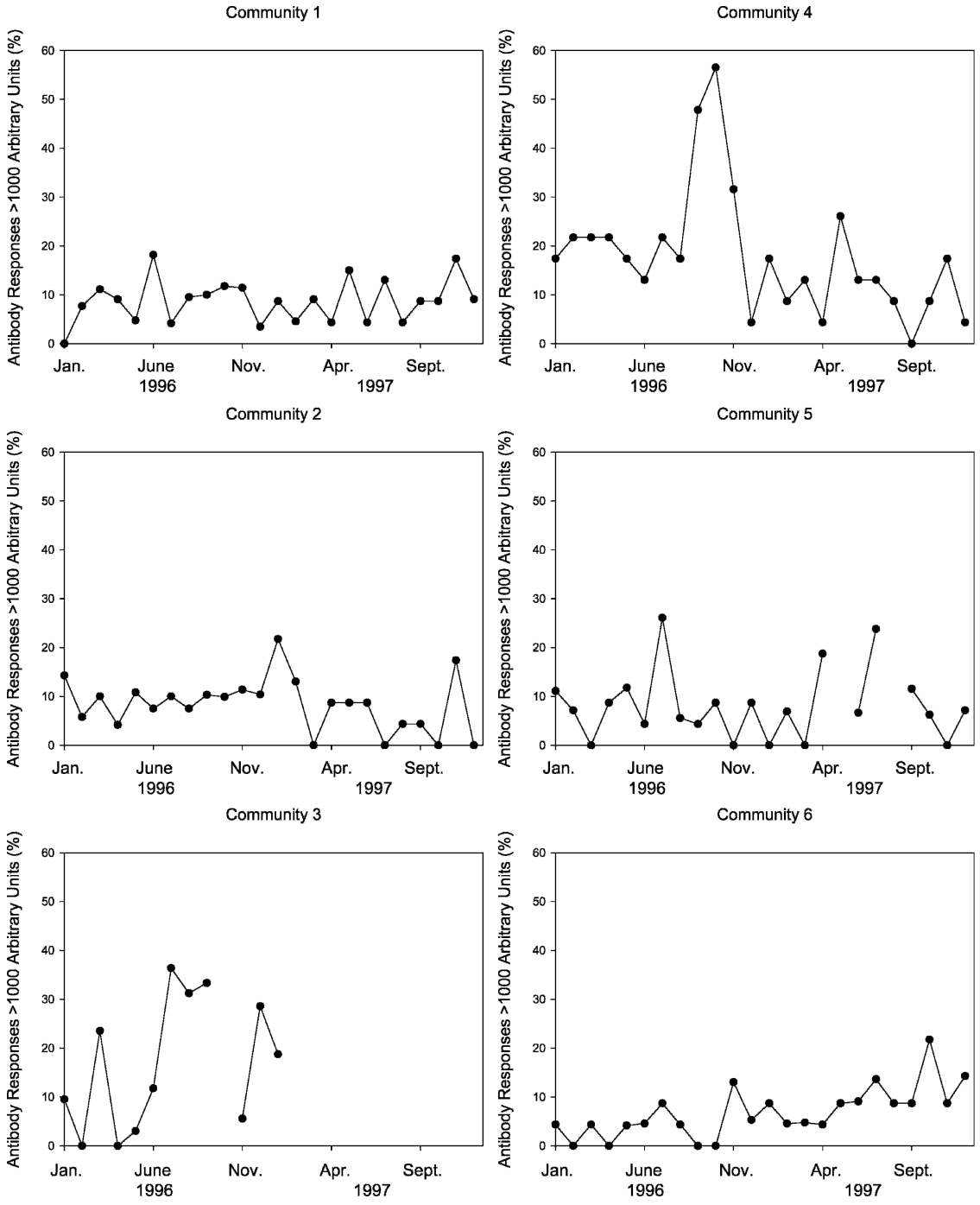

FIGURE 2. Percentage of samples having antibody responses to the recombinant 27-kDa antigen greater than 1,000 arbitrary units. Responses for months in which less than 10 sera were tested have not been plotted.

known to have occurred. In a study of laboratory-confirmed cryptosporidiosis patients,[18] immunoglobulin G antibody levels to the 27-kDa antigen tended to remain above the detection threshold for at least 2 years after infection, whereas levels of antibody to the other immunodominant antigen, the 17-kDa antigen, decreased more rapidly. Our initial inclusion of Community 1, the deep well water community, as the control community was based on the assumption that the risk of waterborne cryptosporidiosis would be lower in this community because of its protected water supply (no oocysts were detected in tap water samples collected in 1996).[16] As suggested by the previous mini-blot study, no peaks of antibody were apparent in the monthly analysis nor were large numbers of high antibody responders or cryptosporidiosis cases found in Community 1 during the 2-year study. Similarly, surface-water Communities 2 and 6 had high overall seroprevalence rates (84% and 81%, respectively) despite the absence of identifiable cryptosporidiosis outbreaks either by case detection or by monthly serologic analysis. Based on these results, we believe that the high seroprevalence rates in these communities are indicators of historic exposures within the different populations. This conclusion is supported by the

recently reported observation of an age-dependent increase (to a maximum between 70% and 80%) in *C. parvum*–specific seroprevalence in the general U.S. population.[26]

Of the three communities in our study that experienced an outbreak of cryptosporidiosis in 1996, Community 4 had the highest attack rate. Using telephone surveys to assess clinical disease, the 22.5% attack rate in Community 4 (Fyfe MW, unpublished data) was similar to the 26.2% rate reported for the 1993 Milwaukee outbreak.[8] Serum specimens drawn monthly from the Community 4 population showed a sharp rise in antibody levels that lagged 1–2 months behind the number of cases reported to public health followed by a gradual decay. In addition, between 48% and 57% of the selected community samples had ELISA responses > 1,000 arbitrary units in September and October 1996. The antibody profile for this community is very similar to that observed for children who were exposed to contaminated water on the south side of Milwaukee in 1993: within 3–4 weeks of the peak of case detection, more than 50% of the Milwaukee children had a response in excess of 1,000 arbitrary units.[19] We believe that the outbreak in Community 4, like the massive outbreak in Milwaukee, affected the majority of the exposed population.

The outbreaks in Communities 3 and 5 were smaller in scale and were more difficult to characterize using the serologic antibody assay. Community 3, which is physically adjacent to Community 4, experienced an outbreak at the same time as Community 4 even though the two communities use different sources of drinking water. A statistically significant elevation in the community serologic response, as well as an elevated number of high responders, were noted in Community 3 in July, 1 month before the peak in the epidemiologic curve and 2 months before the peaks in the corresponding Community 4 profiles. These results imply that some level of increased disease transmission, possibly unrelated to the waterborne outbreak in Community 4, was occurring in Community 3 before an increase in the number of cases was reported. However, these results must be interpreted with some caution, because the small size of the community often restricted the number of samples that could be collected for analysis.

Community 5, which had more cryptosporidiosis cases per capita than Community 3, experienced a waterborne outbreak with an attack rate estimated by telephone survey of only 11.6%.[27] Although a peak in the proportion of high antibody responders was evident in the month after the maximum number of identified cases, the monthly median and geometric mean serologic responses in Community 5 did not show convincing evidence of an increase when compared with the deep-well community or to the early 1996 Community 5 baseline. It is interesting to note that both the Milwaukee and the Community 4 outbreaks were caused by *Cryptosporidium hominis* (previously called *C. parvum* genotype 1) whereas the Community 5 outbreak was caused by *C. parvum* genotype 2 (or bovine genotype).[20,28] The Community 5 outbreak is also the only reported bovine genotype waterborne outbreak in North America to date.[29]

A significant increase in community antibody level that was not temporally related to a known outbreak was observed in Community 5 in April 1997. However, less than 25% of the samples were from high responders, and the observed peak in community reactivity was only significant when compared with the deep-well control community (Community 1) results. At present, although we are uncertain of the significance of the observation, we think that it is most likely due to increased cryptosporidiosis transmission in the community that was not detected by conventional surveillance. Some high responders may have asymptomatic infections, as antibodies to *Cryptosporidium* antigens have been reported to reduce oocyst shedding and severity of clinical symptoms.[30] As well, there is the possibility of unrecognized cases due to asymptomatic carriage occurring in individuals who have acquired antibodies from previous exposures. Protection from more severe clinical symptoms has been observed in individuals challenged with a second infectious dose of *Cryptosporidium* oocysts.[31]

Although it is possible that some unknown bias based on age (inclusion criteria 15–40 years of age) or pregnancy may explain our results, we believe that the weak antibody responses triggered by the outbreaks in Communities 3 and 5 are probably reflective of a low rate of infection that is superimposed on a high background seroprevalence. Risk factors such as exposure to children with diarrhea, contact with cattle, international travel, and freshwater swimming have been reported to have a positive association with cryptosporidiosis.[32] Women of reproductive age may be more likely to have contact with children with diarrhea. However, it is anticipated that their behaviors and exposures are representative of the general population. In a study using sera collected in the U.S. National Health and Examination Survey (NHANES), a weak gender relationship was found between women and a higher prevalence of serological responses to the *Cryptosporidium* 27-kDa antigen.[26]

In the Milwaukee outbreak, children who resided on the north side of the city and who were, therefore, exposed to lower levels of oocyst-contaminated water had a lower peak seroprevalence (47% versus 87%), a lower median antibody response (147 versus 1,036 arbitrary units), and a lower proportion of high responders (17% versus 53%) than children who lived on the south side of the city. However, in the context of children (median age of 2 years) who had little or no pre-existing antibody to the parasite, pre- and post-outbreak differences in antibody levels were easy to demonstrate, even on the north side where exposure levels were relatively low. The high baseline seroprevalence in the adult women of our study, compared with the 20% level in the Milwaukee children, may raise the threshold infection level that is needed to stimulate a detectable increase in the community-wide antibody response.

A consistent theme of seroepidemiologic studies of *Cryptosporidium* infection has been the unexpectedly high antibody seroprevalence, especially in contrast to case surveillance reports or reports of outbreaks. Based on current knowledge, we believe that these antibody responses are specific and reflect infection with viable oocysts. Because the serologic antibody detection method appears to be more sensitive than conventional disease surveillance techniques, data generated from this study and from future studies should provide a more accurate estimate of disease transmission rates at the community level. This should help us identify communities with potential public health problems such as contamination in drinking water supplies, inadequate sanitation, or poor food hygiene. Given the high levels of antibody reactivity seen in settings with protected water sources (such as the

deep well water community, Community 1), further studies of the seroepidemiology of *Cryptosporidium* transmission seem warranted to ascertain the incidence of cryptosporidiosis more accurately.

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Authors' addresses: Corinne S. Ong, Anna S. Li, Ray Copes, Mohamad Khan, Murray W. Fyfe, and Judith L. Isaac-Renton, British Columbia Centre for Disease Control, 655 West 12th Avenue, Vancouver, British Columbia, Canada V5Z 4R4. Stephen A. Marion, Department of Health Care and Epidemiology, University of British Columbia, Mather Building, 5804 Fairview Avenue, Vancouver, British Columbia, Canada V6T 1Z3. Jeffrey W. Priest, Jacquelin M. Roberts, and Patrick J. Lammie, Division of Parasitic Diseases, Centers for Disease Control and Prevention, Mail Stop F-13, Building 23, Room 1025, 4770 Buford Highway N.E., Atlanta, GA 30341-3724.

Reprint requests: Corinne S. Ong, Laboratory Services, British Columbia Centre for Disease Control, 2nd Floor, 655 West 12th Avenue, Vancouver, BC, V5Z 4R4, Canada, Telephone: (604) 660-1524, Fax: (604) 660-6073, E-mail: cong@interchange.ubc.ca.

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