Cryptosporidium parvum–Specific Antibody Responses among Children Residing in Milwaukee during the 1993 Waterborne Outbreak

Alicia C. McDonald,1,2,a William R. MacKenzie,1,a David G. Addiss,1 M. Stephen Gradus,1 George Linke,3 Elizabeth Zembrowski,3 Margaret R. Hurd,1,a Michael J. Arrowood,1 Patrick J. Lammie,1 and Jeffrey W. Priest1

A major gastroenteritis outbreak among >400,000 residents of Milwaukee, Wisconsin, in April 1993 was attributed to Cryptosporidium parvum oocysts in drinking water. Plasma specimens obtained from children (6 months to 12 years old) for routine blood lead level surveillance March–May 1993 were assayed by ELISA for levels of IgG antibody against the immunodominant Triton-17 and 27-kDa C. parvum antigens. Over a 5-week period, the seroprevalence for antibodies to the 2 antigens increased from 15% to 82% and from 17% to 87%, respectively, in samples from children living in southern ZIP code areas (n = 218), whereas smaller increases (20% to 43% and 22% to 46%, respectively) were noted among samples from children living in northern ZIP code areas (n = 335; P < .0001). The results demonstrate that C. parvum infection was much more widespread than previously appreciated and confirm that infection was associated with residence in the area served by the southern water treatment plant.

Cryptosporidium parvum is a parasitic protozoan that causes diarrhea in both immunocompetent and immunocompromised persons [1]. In addition to diarrhea, infection is associated with abdominal cramps, fever, nausea, dehydration, and weight loss. C. parvum infection can lead to premature death in immunocompromised persons, especially AIDS patients, because the illness is not self-limited in these patients, as it is in immunocompetent persons [1]. Infection can be acquired from infected persons or animals and from food- and waterborne routes of transmission [2–9].

In April 1993, in Milwaukee, Wisconsin, a major gastroenteritis outbreak was attributed to the presence of C. parvum oocysts in water supplied by the Howard Avenue Purification Plant, a water treatment plant that primarily served the southern region of the city. As determined on the basis of a random digit–dialing telephone survey of the greater Milwaukee area, ~400,000 Milwaukee residents (26% of the population) were ill and met the cryptosporidiosis case definition (watery diarrhea) [10]. The magnitude of the outbreak in Milwaukee established C. parvum as a significant threat to the US water supply and emphasized the need for better drinking water treatment practices and for the establishment of surveillance programs for waterborne parasites [11].

The conclusion that C. parvum was the cause of the outbreak in Milwaukee was reached on the basis of (1) a strong epidemiologic link between drinking water and diarrheal illness, (2) demonstration of C. parvum in the stools of >700 ill persons, (3) the absence of demonstrable infections with other pathogens, and (4) detection of C. parvum in ice made from water collected during the outbreak period [10]. However, the relatively nonspecific nature of the clinical case definition and the finding of oocysts in only 29% of stool specimens submitted for C. parvum testing has raised questions in the minds of some investigators about the magnitude of the estimate of the number of infected persons. The existence of banked plasma specimens, which were obtained from Milwaukee children in 1993 for routine blood lead level determination, provided us with an opportunity to conduct a retrospective analysis of the Milwaukee outbreak, using recently developed serologic assays for Cryptosporidium infections.

Methods

Study population. A major gastroenteritis outbreak among >400,000 residents of Milwaukee in April 1993 was attributed to C. parvum oocysts in drinking water. During the same time period (25 March through 2 June 1993), blood samples were obtained from children in Milwaukee for routine surveillance of blood lead...
levels. Residual blood lead specimens (5–20-μL volumes) were retrieved for serologic analysis at the time of the outbreak investigation. The plasma specimens were centrifuged to remove particulate material and were stored at −80°C until the time of assay. Personal identifiers were not associated with the specimens; however, age, sex, date of collection, and ZIP codes of residences were available.

A total of 1484 plasma samples was available from children ≤12 years old who lived in the greater Milwaukee area. The samples were grouped by ZIP code of residence and by week of collection. To increase the available numbers of samples in the first and last sets, we added samples collected on 25 and 26 March to the week 1 set (29 March to 2 April), and samples from 31 May to 2 June were added to the week 9 set (24–28 May). Samples from weeks 1 (25 March to 2 April), 3 (12–16 April), 5 (26–30 April), 7 (10–14 May), and 9 (24 May to 2 June) from 4 contiguous southern ZIP code areas (n = 218) and from 6 contiguous northern ZIP code areas (n = 335) were chosen for ELISA analysis. The number of samples collected in each time period is indicated in figure 1A. Samples from the southern and northern ZIP code areas were assayed separately in the approximate order of the date of collection.

An additional 120 residual blood lead samples obtained from children (median age, 3 years; range, 6 months to 5 years) between 8 March and 8 April 1999 were available for antibody testing. As with the 1993 samples, the 1999 samples were not associated with personal identifiers, but age, sex, date of collection and, and ZIP codes of residences were available. The samples were subdivided into residents of the southern (n = 60), middle (n = 30), and northern (n = 30) ZIP code areas before ELISA analysis, but they were not divided by date of collection.

**Laboratory analysis.** ELISA antibody responses to the 17- and 27-kDa antigens were tested, as described elsewhere [12], using a partially purified preparation of a native antigen preparation enriched for a 17-kDa antigen (Triton-17 antigen) and a recombinant form of 27-kDa antigen. In brief, each antigen was diluted in 0.1 M sodium bicarbonate buffer (pH 9.6), was added to 96-well, flat-bottom microtiter immunoassay plates (Immulon 2; Dynex Technologies), and was incubated overnight at 4°C. Triton-17 and 27-kDa antigens were used at 14 and 17 ng per well, respectively. After blocking for 1 h at 4°C with 100 μL of 0.3% Tween-20 in buffer containing 0.85% NaCl and 10 mM Na2HPO4 (PBS; pH 7.2), plasma specimens (diluted 1:50), 3 positive and 4 negative controls (diluted 1:50), and an 8-point standard curve (2-fold serial dilutions from 1:50 to 1:6400 of a strong positive specimen) were loaded onto the plate in duplicate wells (50 μL/well). Tween-20 (0.05%) in PBS was used for all washes and dilutions.

The samples were incubated in the wells for 2 h at room temperature and then were incubated for 1 h at room temperature with biotin-conjugated monoclonal anti-human IgG (50 μL/well of a 1:1000 dilution; clone HP6017; Zymed Laboratories) and with alkaline phosphatase–labeled streptavidin (50 μL/well of a 1:500 dilution; Life Technologies) with washing steps in between. Finally, p-nitrophenyl phosphate substrate (50 μL/well; Sigma) in 3 mM MgCl2 and 10% diethanolamine (pH 10) was added. The plates were read when the optical density of the 1:5 dilution of the standard curve control serum reached an absorbance of 1.5 at 405 nm, and this point on the standard curve was assigned a value of 6400 arbitrary units (AU). AU values for unknown samples were derived from the 8-point standard curve, using a 4-parameter fit, and were expressed per microliter of serum. Mean values were calculated for each set of available specimens in each subset are indicated in parentheses on the respective bars in A. Significant differences (P < .05) in the prevalence of positive antibody responses to the Triton-17 and 27-kDa C. parvum antigens between children from northern and southern Milwaukee were observed from the week of 26 April through the week of 24 May (including 31 May to 2 June) 1993.
to the immunoblot [12, 13], and the results were reproducible from
day to day and from one laboratory to another [13].

Statistical analysis. The $\chi^2$ test was used to assess the signifi-
cance of differences in antibody response to Triton-17 and 27-kDa
antigens between children who resided in ZIP code areas primarily
served by southern and northern Milwaukee water treatment plants
during the 9 weeks of sample collection. Antibody responses were
compared by use of the Kruskal-Wallis test. Statistical significance
was defined as $P < .05$. Statistical analysis was done with Epi Info
(version 6.04a; CDC) and SAS for Windows (version 6.12; SAS
Institute).

Results

Plasma specimens from 553 children were assayed for anti-
body against the Triton-17 and 27-kDa C. parvum antigens. The children’s ages ranged from 6 months to 12 years (median,
3 years); 46.6% were \( \approx \) 2 years old, 46.8% were 3–5 years old,
and 6.6% were 6–12 years old. Samples were examined from
children residing in ZIP code areas primarily served by the southern water treatment plant ($n = 218$, representing 6 ZIP
codes) and northern water treatment plant ($n = 335$, repre-
senting 4 ZIP codes). Of the specimens from southern Mil-
waukee, 62.8% came from children who resided in one partic-
ular ZIP code (53204) area. Likewise, 34.8% and 30.7% of
specimens from northern Milwaukee were from children resid-
ing in areas covered by ZIP codes 53206 and 53212, respectively.

The earliest plasma samples that were available to us (begin-
ning 25 March 1993) corresponded to the beginning of the period
during which increases were observed in the number of cases of
laboratory-confirmed cryptosporidiosis (figure 2) and clinically
defined cases of watery diarrhea (as determined by random
digit-dialing telephone surveys) [10]. Figure 2 shows the reported
date of illness onset for all laboratory-confirmed cases of cryp-
tosporidiosis (both adults and children; $n = 270$) in the greater
Milwaukee area between 16 March and 15 May 1993.

In this study, samples collected during weeks 1 (29 March
to 2 April, including 25 and 26 March), 3, 5, 7, and 9 (24–29
May, including 31 May to 2 June) of the outbreak were ana-
lyzed by ELISA. For the period between 25 March and 2 April,
15% and 17% of children served by the southern water treat-
ment plant ($n = 46$) and 20% and 22% of children served by
the northern water treatment plant ($n = 98$) had positive IgG
antibody responses against the Triton-17 and 27-kDa C. parvum
antigens, respectively (figure 1). No significant difference in the
prevalence of IgG antibody responses between children residing
in areas served by the 2 water treatment plants was observed
during this period for either the Triton-17 antigen ($P = .61$) or
the 27-kDa antigen ($P = .63$). Similarly, the median Triton-17
and 27-kDa antigen ELISA responses for the first time period were below the respective cutoff levels in both the north and south (figure 3).

To determine whether the age of the child was related to the antibody response, the ELISA responses in the first time period (25 March to 2 April) from the north and south were combined and were grouped by donor age, in years (n = 144; median age, 3 years; range, 7 months to 6 years). No significant age-related differences were detected in the prevalence of positive responses (P = .59 and P = .92) or in the median antibody responses (Kruskal-Wallis test; P = .123 and P = .755) for either the Triton-17 or 27-kDa antigen, respectively. The only 6-year-old child in the set had responses of 52 AU (Triton-17 antigen) and 127 AU (27-kDa antigen).

The prevalence of IgG antibody responses to the Triton-17 and 27-kDa antigens increased rapidly after the first week. By week 5 of serum collection (beginning 26 April), 82% and 87% of children served by the southern water treatment plant and 43% and 46% of children served by the northern water treatment plant had IgG antibody responses to the Triton-17 and 27-kDa C. parvum antigens, respectively (figure 1). The prevalence of antibody responsiveness in both the south and the north remained at higher-than-baseline levels through week 9 of serum collection. The prevalence of positive antibody responses in children served by the southern water treatment plant was significantly greater than that of children residing in the north (P < .05) during weeks 5–9 of serum collection.

For both the Triton-17 and 27-kDa C. parvum antigens, the median antibody response for children served by the southern water treatment plant peaked during week 5 of serum collection (week beginning 26 April; figure 3); however, the median antibody responses of children residing in the north peaked during week 7 of serum collection (week beginning 10 May). As with antibody prevalence, median antibody responses of children residing in the southern part of the city were greater than those of children residing in the north for plasma samples collected from week 5 (beginning 26 April) through week 9 (beginning 24 May) for both antigens. To rule out the influence of antibody prevalence on these comparisons, we restricted the analysis to children with positive antibody responses. Among antibody-positive children, those in southern Milwaukee had significantly higher median responses (P < .05) than those in northern Milwaukee to both the Triton-17 and 27-kDa antigens during weeks 5–9 of sample collection (table 1).

To determine the current prevalence of antibody-positive children in Milwaukee, we used Triton-17 and 27-kDa antigen ELISAs to test residual blood lead specimens (n = 120) that were collected in 1999. Samples from children <6 years old were chosen for this work, so that any children who may have been infected with C. parvum during the 1993 outbreak would be excluded. Among the children, 7% and 6% were positive for antibodies to the Triton-17 and 27-kDa antigens, respectively (data not shown). No significant differences in prevalence were observed between residents of the northern, middle, and southern ZIP code areas for either the Triton-17 or 27-kDa responses (P = 1.0 and P = .43, respectively).

Discussion

We assessed acquisition of C. parvum infection among children who resided in Milwaukee during the 1993 waterborne cryptosporidiosis outbreak, using newly developed serologic as-
silkens to C. parvum infection [14]. Thus, serologic responses to the 17-kDa and 27-kDa antigens develop after both symptomatic and asymptomatic infection [15]. Previous studies using human volunteers have shown that serologic responses to the 17-kDa and 27-kDa antigens develop after both symptomatic and asymptomatic infection (figure 1). These increases in IgG antibody prevalence and median antibody level (figure 3) paralleled the epidemic curve of illness onset shown in figure 2, with a lag time of ∼3 weeks (∼4 weeks after the likely time of exposure). This result is consistent with the amount of time required to mount an IgG antibody response [14]. The fact that increased antibody responses were observed to 2 distinct C. parvum antigens over this time period decreases the likelihood that changes in antibody reactivity were a result of cross-reactivity to other agents (e.g., Microsporidia species, Giardia species, etc.).

Evidence from animal studies suggests that antibody responses to C. parvum antigens require inoculation with viable oocysts [15]. Previous studies using human volunteers have shown that serologic responses to the 17-kDa and 27-kDa antigens develop after both symptomatic and asymptomatic C. parvum infection [14]. Thus, serologic responses to C. parvum in children probably reflect infection and not merely exposure to nonviable oocysts. On the basis of this conclusion, children had greater levels of infection with C. parvum than those predicted from the random digit–dialing survey of diarrheal illness [10]. However, the attack rates determined from the telephone survey may not be directly comparable to the rates of seropositivity found in this study, because, although the telephone survey included residents from all the ZIP code areas in the region, our study concentrated on children from only a few ZIP code areas in the northern and southern Milwaukee regions. Thus, our results may not be fully representative of their respective regions. Despite this potential limitation, the rates of serologic positivity in both regions were substantially greater than the attack rates from the telephone survey, even when the attack rates were weighted for the ZIP codes used in our study (W. R. MacKenzie, unpublished data).

From a comparison of baseline seroprevalence values from the first week of sample collection with peak seroprevalence, we estimate that children served by the southern and northern water treatment plants had, at a minimum, C. parvum infection rates of 70% and 37%, respectively, during this outbreak. If the outbreak in Milwaukee began in early March, as is suggested by the occurrence of sporadic cases of diarrheal illness earlier in the month, the seroprevalence for the period beginning 25 March was not a true baseline, and these numbers probably underestimate the rate of infections due to the outbreak. Unfortunately, no samples predating the 1993 outbreak are available, and it may be impossible to accurately determine the pre-outbreak baseline seroprevalence in Milwaukee. To demonstrate that the assays perform well in the absence of an outbreak, a baseline seroprevalence was determined for children, using plasma samples more recently collected from Milwaukee (1999). However, we would suggest that a direct comparison between these results and those of the 25 March to 2 April 1993 time period is not valid, because Milwaukee instituted significant changes to improve the overall quality of the water supply in the aftermath of the 1993 outbreak.

No serum samples from adult populations were available for measurement of C. parvum–specific responses for the time period represented by the blood lead specimens from children. Nonetheless, given the higher attack rate for diarrheal illness among adults in the greater Milwaukee area (28% for persons ≥20 years old), compared with that among children (19% for children <10 years old) [10], it seems likely that the antibody

### Table 1. Median antibody response to the Triton-17 and 27-kDa Cryptosporidium parvum antigens among antibody-positive children in 1993 in Milwaukee.

<table>
<thead>
<tr>
<th>Dates of sample collection</th>
<th>No. of children with positive Triton-17 responses</th>
<th>Median antibody response to Triton-17 antigen, AU</th>
<th>No. of children with positive 27-kDa responses</th>
<th>Median antibody response to 27-kDa antigen, AU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>North</td>
<td>South</td>
<td>North</td>
<td>South</td>
</tr>
<tr>
<td>25 March to 2 April</td>
<td>20</td>
<td>7</td>
<td>102</td>
<td>329</td>
</tr>
<tr>
<td>12–16 April</td>
<td>9</td>
<td>14</td>
<td>139</td>
<td>548</td>
</tr>
<tr>
<td>26–30 April</td>
<td>23</td>
<td>31</td>
<td>196</td>
<td>460</td>
</tr>
<tr>
<td>10–14 May</td>
<td>46</td>
<td>44</td>
<td>221</td>
<td>420</td>
</tr>
<tr>
<td>24 May to 2 June</td>
<td>22</td>
<td>44</td>
<td>210</td>
<td>432</td>
</tr>
</tbody>
</table>

* a Positive response for Triton-17 ELISA defined as >57 arbitrary units (AU).
* b Median among children with a positive ELISA response.
* c Positive response for 27-kDa antigen ELISA defined as >160 AU.
* d Level in southern Milwaukee children is significantly greater than that in northern Milwaukee children (P < .05). The analysis was restricted to children with positive antibody responses to the respective antigen.
prevalence among adults would be comparable in magnitude to that among the children. In fact, Frost et al. [16] recently demonstrated that, in the weeks after the 1996 outbreak of cryptosporidiosis in Collingwood, Ontario, Canada, 69% and 88% of the adult residents who were tested by Western blot assay were positive for antibodies to the 17- and 27-kDa C. parvum antigens, respectively [16]. Both the fraction of positive residents and the mean antibody responses to these 2 markers were higher in residents of Collingwood than in residents of Toronto, where no outbreak occurred. These serologic responses were detected despite the fact that only 16% of the non-nursing home adult population had a laboratory-confirmed infection. Thus, as noted by Mac Kenzie et al. [10], the total number of infected persons in the Milwaukee population probably was higher than the initial estimate of 400,000 people.

Differences between published rates of infection from epidemiologic studies (based on measures of illness such as watery diarrhea) and antibody prevalence probably reflect the range of clinical manifestations of infection (from watery diarrhea to asymptomatic infection) in the population. These approaches provide complementary public health information. Case definitions based on illness are better for quantifying the degree of morbidity associated with C. parvum. In contrast, serologic studies provide better measures of infection.

Unfortunately, we do not know whether children in our study experienced symptoms of cryptosporidiosis. Although we have no reason to believe that these children experienced symptoms at a higher rate than the rate determined by telephone surveys, we cannot make strong inferences regarding the rate of asymptomatic infection. Nonetheless, the rate of diarrheal illness was lower in children than in adults. Perhaps this disparity is related to the smaller number of organisms ingested by children, because development of symptoms was related to the oocyst dose in studies of Cryptosporidium-infected adult volunteers [17]. Alternatively, the absence of symptoms in children may reflect some degree of prior exposure to infection, as suggested by Morris et al. [18]. Moss et al. [14] suggested that the presence of preexisting antibody responses may be associated with protection from illness. In the absence of specimens collected before 25 March 1993, our data do not address this possibility directly. We postulate, however, that prior outbreaks would be reflected by an increase in C. parvum seroprevalence with age at baseline (period beginning 25 March). Although our sample numbers were small, we saw no age-related differences in antibody prevalence or level in samples collected during the first week, as we would have expected if prior exposures had occurred.

There were several potential limitations to our study. Because our study was based on plasma specimens collected for blood lead level surveillance in March through May 1993, only a single specimen was available from each child. Therefore, we were unable to document seroconversion to Cryptosporidium per se. However, we have no reason to believe that there were any systematic differences in the way that specimens were collected or analyzed over the course of the study that could explain the temporal variation in antibody prevalence and level. In addition, we cannot control for the possibility that children residing in northern Milwaukee were exposed to infection in southern Milwaukee or vice versa. Likewise, because pipes in northern and southern Milwaukee are interconnected, children residing in northern Milwaukee may have had some level of exposure to water coming from the southern water treatment plant while they were at home. However, both the movements of children and of water between northern and southern Milwaukee would tend to diminish the magnitude of the differences in seroprevalence and antibody level that we did observe. Finally, as noted above, there were no serum specimens from adults available to us, so we were unable to document the level of infection in adults over the same time period and, thus, were unable to estimate the overall infection level in the community.

Despite these limitations, our results provide very strong evidence that C. parvum was the cause of the 1993 Milwaukee outbreak. These results emphasize the utility of serologic assays for C. parvum for epidemiologic studies and surveillance. Further investigations are needed to have a better understanding of the relationship between seroprevalence at the population level and community susceptibility to outbreaks of symptomatic disease.

References


