ENZYMELINKED IMMUNOELECTROTRANSFER BLOT ANALYSIS OF A CRYPTOSPORIDIOSIS OUTBREAK ON A UNITED STATES COAST GUARD CUTTER

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Abstract. Symptoms consistent with an outbreak of cryptosporidiosis (diarrhea, vomiting, nausea, and abdominal cramps) occurred on a U.S. Coast Guard cutter within 0–18 days after the cutter filled its tanks with Milwaukee, Wisconsin city water in March 1993. At three-weeks postdocking (PD), the suspected water was removed, and serum samples and stool specimens were collected from 47 of the 58 crew members, as well as questionnaire data on their water consumption and symptoms aboard the cutter. At 10-weeks PD and/or at 28-weeks PD, additional serum specimens were collected. Intensitometric data from enzyme-linked immunoelectrotransfer blot (EITB) were obtained on IgA responses to a 17-kD antigen group, IgM responses to a 27-kD antigen group, and IgG responses to 27-, 17-, and 15-kD antigen groups extracted from oocysts. In addition, IgG responses to crude oocyst antigens were obtained by ELISA. Based on reported symptoms, EITB results, and stool examination, the crew members were classified as confirmed (10), probable (10), suspected (22), and noncases (16). Of the 10 confirmed cases (all symptomatic) and the 10 probable cases (eight symptomatic) whose stools were positive and negative, respectively, for Cryptosporidium oocysts by microscopy, all showed changes in EITB intensities to the antigen groups and were considered EITB positive. The remaining 38 crew members, 22 suspected cases (all symptomatic), and 16 noncases (all asymptomatic), if tested, had negative stool examinations and were considered EITB negative. Of the 10 confirmed cases, only four showed a significant change in IgG responses (P < 0.05) between three-weeks PD and follow-up serum specimens by ELISA. Crew members considered confirmed cases consumed significantly more water (P ≤ 0.005) aboard the cutter than noncases. Crew members considered EITB positive consumed more water (P ≤ 0.04) than crew members considered EITB negative while there was no significant difference in water consumption (P ≥ 0.19) between crew members considered ELISA positive and ELISA negative. Using the EITB, the observation of changes in intensity of IgA responses to the 17-kD antigen group, IgM responses to the 27-kD antigen group, and IgG responses to the 27-, 17-, and 15-kD antigen groups from C. parvum oocysts between acute and convalescent serum specimens appears useful for immunodiagnosis of Cryptosporidium infection and for prospective epidemiologic studies designed to monitor infection risk.

Cryptosporidiosis is a disease caused by Cryptosporidium parvum, an intestinal protozoan parasite that infects both humans and animals throughout the world.1–16 For those infected, some have symptoms characterized by watery diarrhea, nausea, vomiting, headache, or abdominal cramps, while others have no symptoms. In immunocompetent humans, cryptosporidiosis is usually self-limited. In contrast, immunocompromised patients such as those with acquired immunodeficiency syndrome may have life-threatening cryptosporidiosis. To date, no treatment other than hydration and hyperalimentation is available.

Cryptosporidium has been identified as the second most common infectious agent in outbreaks of diarrhea in cattle.7,8 In humans, it has caused many water-borne outbreaks of cryptosporidiosis,9–14 including the massive outbreak in 1993 in Milwaukee, Wisconsin, where it was estimated that more than 400,000 residents had watery diarrhea.11

The most commonly used method to confirm Cryptosporidium infection is stool examination by microscopy, which may require multiple stool specimens due to the intermittent and relatively short duration of oocyst excretion.15 This method is not only labor-intensive, but it also requires trained personnel. In addition, the sensitivity of microscopic detection of oocysts in stools using a modified formalin-ethyl acetate concentration and immunofluorescent techniques has been shown to be less than 100% when samples were seeded with less than 50,000 and 10,000 oocysts per gram of formed and watery stool, respectively.16

Other methods such as the ELISA17–21 and the enzyme-linked immunoelectrotransfer blot (EITB)22–27 have assessed humoral immune responses to crude antigens extracted from oocysts. In a previous qualitative EITB study, crew members who were involved in a cryptosporidiosis outbreak aboard a U.S. Coast Guard cutter developed IgG responses that were predominantly IgG128 to the 27- 17-, and 15-kD antigen groups from C. parvum oocysts. In addition, IgA and IgM responses were shown to be directed against the 17- and 27-kD groups, respectively.29 Over time, these responses diminished. Here, in an extension of that study, we used EITB and intensitometry to quantify the kinetics of IgA, IgM, and IgG responses to these antigen groups and an ELISA to determine IgG responses to oocyst antigens. The relationship of these responses to clinical and infection outcome and to consumption of drinking water was examined.

MATERIALS AND METHODS

Study population. The study population consisted of 58 crew members aboard a U.S. Coast Guard cutter that docked in Milwaukee on March 21, 1993 and filled its tanks with city water during that city’s cryptosporidiosis outbreak. On April 12, three-weeks postdocking (PD), epidemiologists were asked to investigate an outbreak of gastrointestinal illness among the crew members. Questionnaire data were collected from the crew members on symptoms, the estimated date of onset of symptoms, and fluid consumption both while on leave in Milwaukee and while aboard the cutter. Severity of illness was arbitrarily given a score of 1 for each reported...
symptom of nausea, abdominal cramps, and vomiting, and a score of 2 for reported diarrhea. Thus, a cumulative score for severity of illness could range from 0 (no illness) to 5 (maximum illness).

At three-weeks PD, the suspected water aboard the cutter was removed, and its tanks were disinfected. Approximately 40 liters of the water was analyzed for the presence of Cryptosporidium oocysts.

**Serum and stool specimens.** At three-weeks PD, serum specimens were collected from 54 crew members who were available. Additional serum specimens were collected from 45 crew members at 10-weeks PD, and from 39 crew members at 28-weeks PD. Two or more serum specimens were collected from 50 crew members.

Within five days after collection of the three-weeks PD serum specimens, one or more stool specimens were submitted by each of 47 crew members. The stool specimens were examined by cold acid-fast Kinyoun stain as previously described and by direct immunofluorescent assay (Merifluor Cryptosporidium kit; Meridian Diagnostics, Cincinnati, OH) as instructed by the manufacturer.29 Crew members with oocyst-positive stools were classified as confirmed cases of cryptosporidiosis.

**Oocyst purification and antigen preparation.** Oocysts (Iowa strain)27 were collected from experimentally infected Holstein calves and were purified by discontinuous sucrose gradients as described elsewhere.30

Proteins were extracted from purified oocysts by sonication, followed by three freeze/thaw cycles, and then centrifuged to remove particulate matter as previously described.31 The clarified supernatant was used in the ELISA and EITB.

**Protein determination.** The protein content of the antigen preparation was determined by previously described methods using dye reagent obtained from Bio-Rad Laboratories (Rockville Centre, NY) and human albumin and globulin (Sigma Chemical Co., St. Louis, MO) as standards.32

**Enzyme-linked immunosorbent assay.** Microtiter plates were sensitized overnight at 4°C with 100 μl of antigen preparation per well at a concentration of 2 μg/ml in 0.05 M Tris-HCl, 0.3 M NaCl, 2 mM disodium EDTA, pH 8.0. All washes and subsequent incubation steps were performed in 0.3% Tween 20 in phosphate-buffered saline. Human serum was exposed to bound antigens at a 1:200 dilution. After reacting bound primary antibodies with biotinylated goat anti-human IgG, bound secondary antibodies were exposed to streptavidin–alkaline phosphatase. After addition of p-nitrophenyl phosphate to the bound enzyme, optical densities (ODs) were obtained at 405 nm. All serum specimens were tested in triplicate.

**Electrophoresis.** The antigen preparation was treated with sodium dodecyl sulfate (SDS) under nonreducing conditions and separated by polyacrylamide gradient gel electrophoresis (PGGE) using 3–25% gradient gels in a discontinuous buffer system.33 Gels (0.75-mm thick) were loaded with 545 ng of protein per millimeter lane width.

**Enzyme-linked immunoelectrotransfer blot and intensitometry.** The methods used in the EITB have been previously described.34 Briefly, separated proteins on SDS-PGGE gels were electrotransferred to polyvinylidene fluoride sheets (Millipore Corp., Bedford, MA). After cutting the sheets into 2 or 3 mm–wide strips, the transferred proteins were exposed to human serum at a 1:200 dilution for IgG and a 1:50 dilution for IgA and IgM. To confirm the identification of sporozoite-derived proteins monoclonal antibody (MAb) C6B6, specific for the 27-kD antigen group,27,28 and MAb C6C1, specific for the 17- and 15-kD antigen groups,26,35 were added to the strips. Bound MAbs were detected with goat anti-mouse immunoglobulin conjugated with horseradish peroxidase and visualized with hydrogen peroxide as the substrate and diaminobenzidine as the chromagen. Bound human antibodies were detected with biotinylated goat anti-human IgG (Organon Teknika Corp., West Chester, PA), mouse anti-human IgA (clone GA112; Zymed Laboratories, Inc., South San Francisco, CA) or mouse anti-human IgM (clone HP6083; Zymed Laboratories). Streptavidin alkaline phosphatase was used to probe for bound biotinylated secondary antibodies that were visualized with 5-bromo-4-chloro-3-indolyl phosphate as the substrate and nitro blue tetrazolium as the chromagen. Development was stopped by rinsing the strips with water.

Human serum specimens, used as positive and negative controls on each EITB, were collected from a confirmed cryptosporidiosis patient whose stools were oocyst positive and from a person whose stools were oocyst negative by microscopy. These individuals were not members of the U.S. Coast Guard cutter crew but lived in Milwaukee during the cryptosporidiosis outbreak of 1993.

Protein profiles and protein standards on EITB strips were developed using Aurodye solution (Janssen Life Sciences, Piscataway, NJ) according to the manufacturer’s instructions.

Intensitometry was used to quantitate EITB intensities of IgG responses to the 27-, 17-, and 15-kD antigen groups, IgM responses to the 27-kD group, and IgA responses to the 17-kD group. After development, a tagged image format file (TIFF) was created on each EITB using a Scanjet IIc scanner and DeskScan II software (Hewlett Packard, San Diego, CA). On each EITB, scanner intensity values of the bands, which were gray level intensity measurements from the TIFF ranging from 0 (white) to 256 (black), were obtained using SigmaScan software (Jandel Scientific, San Rafael, CA). The EITB intensity was expressed as the ratio of the highest scanner intensity value within an antigen group of the crew member’s serum specimen to the highest scanner intensity value within the corresponding antigen group and isotype of the positive control serum. Net EITB intensity was defined as the difference between the maximum and minimum EITB intensity observed using three-10-, or 28-week PD serum specimens that were available. The EITB intensities of confirmed cases changed in follow-up serum specimens and were considered EITB positive and were used to define EITB changes among other groups of crew members: IgA or IgM net EITB intensity to the 17- and 27-kD groups of 0.054 and 0.117, respectively, and IgG net EITB intensity to at least two of the three antigen groups of 0.159, 0.107, and 0.128 for 27-, 17-, and 15-kD groups, respectively.

**Statistical analysis.** Questionnaire data collected from the crew members and intensitometry data obtained from EITB were analyzed using the Kruskal-Wallis test. The ELISA data comparing three-week and 10-week PD serum specimens, or if the latter was not available, between three-week and 28-week PD serum specimens were analyzed using the Student’s t-test.
RESULTS

Study population. Of 58 crew members, 55 reported no symptoms consistent with cryptosporidiosis before boarding the cutter (day 0 PD), which had docked in Milwaukee and filled its tanks with city water. Of the 55 previously asymptomatic crew members, 37 (67%) reported the onset of symptoms consistent with cryptosporidiosis 0–18 days PD (mean = 5.8 days PD). Of the 58 crew members, scores for severity of illness ranged from 0 to 5 (mean = 2.1). Examinations of three-week PD stool specimens confirmed Cryptosporidium infection in 10 (17%) of 58 who were considered confirmed cases of cryptosporidiosis. Cryptosporidium oocysts were not identified in the approximately 40 liters of water that was removed from the cutter’s tanks.

Antigen identification and EITB positivity. In a previous study, qualitative changes in antibody responses over time to small molecular weight antigens were observed in these crew members.28 In this study, quantitative changes by intensitometry were evaluated for immunoglobulin responses to these antigen groups shown in Figure 1: IgA to the 17-kD group (strip J); IgM to the 27-kD group (strip K);
and IgG to the 27-, 17-, and 15-kD antigen groups (strip L). Intensitometric data were not collected on immunoglobulin responses to the higher molecular weight antigens because these responses were neither as consistent nor as intense as responses to the lower molecular weight antigens. The MAbs specific for proteins on the surface of sporozoites recognized the antigen groups: MAb C6B6 specific for the 27-kD antigen group (Figure 1, strip H) and MAb C6C1 reactive with the 17- and 15-kD antigen groups and to a 45-kD protein (Figure 1, strip I). 27, 28

**Clinical case classifications.** Summarized in Table 1 are the clinical case classifications of the crew members aboard the cutter along with age, symptoms, onset of symptoms, severity of illness, and EITB and ELISA results. As described above, 10 (17%) crew members whose stool specimens were oocyst positive were considered confirmed cases. Serum samples from all 10 were EITB positive. Another set of 10 (17%) crew members whose serum specimens were EITB positive, but whose stool specimens, if tested, were oocyst negative, were considered to be probable cases of cryptosporidiosis. Of the remaining 38 crew members, 22 (38%) who reported symptoms consistent with cryptosporidiosis, but whose stool and serum specimens were oocyst and EITB negative, respectively, if tested, were considered to be suspected cases of cryptosporidiosis, and 16 (28%) who reported no symptoms and whose stool and serum specimens were oocyst and EITB negative, respectively, if tested, were considered to be noncases of cryptosporidiosis. The confirmed cases showed a mean score for severity of illness that was significantly higher ($P = 0.0005$) than that of suspected cases while the probable cases had a lower mean score for severity of illness ($P = 0.02$) than confirmed cases (Table 1). There was no significant relationship between the clinical case classifications and age or the days PD of onset of symptoms.

**Water consumption.** Listed in Table 2 is the mean number of glasses of water consumed daily aboard the cutter by each of the clinical case classifications: water only and total water, which includes beverages prepared with unboiled water. Also listed in Table 2 is the mean number of glasses of water consumed in Milwaukee before boarding the cutter. The crew members classified as confirmed cases consumed significantly more water and more total water per day aboard the cutter ($P = 0.0008$ and $P = 0.003$, respectively) than noncases. Although the probable cases consumed more water daily (not significant; $P = 0.45$) than noncases, they consumed significantly less water ($P = 0.02$) than confirmed cases. Crew members who were EITB positive, 10 confirmed cases and 10 probable cases, drank significantly more water per day ($P = 0.01$) than crew members who were EITB negative (16 suspected cases and 14 noncases). In contrast, crew members who were ELISA positive showed no significant difference ($P \geq 0.19$) in water and total water consumption per day when compared with ELISA-negative crew members.

**Enzyme-linked immunoelectrotransfer blot.** The confirmed cases showed consistent changes in antibody reactivity to the targets antigens and were considered EITB positive (Table 1). Shown in Figure 2 are EITB profiles (strip panels IgA, IgM, and IgG) and intensities (graphs IgA, IgM, and IgG) on one confirmed case, crew member CGC4. The mean net EITB intensities are shown in Table 3. The confirmed cases showed decreasing IgA and IgM responses to the 17- and 27-kD groups, respectively, from three-weeks to 10-weeks PD, and IgM responses to the 27-kD group continued to decrease through 28-weeks PD for all except one whose

### Table 1

<table>
<thead>
<tr>
<th>Cases*</th>
<th>Confirmed (n = 10)</th>
<th>Probable (n = 10)</th>
<th>Suspected (n = 22)</th>
<th>Non (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diarrhea (%)</td>
<td>10 (100%)</td>
<td>7 (70%)</td>
<td>16 (73%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Symptoms other than diarrhea (%)</td>
<td>10 (100%)</td>
<td>8 (80%)</td>
<td>17 (77%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>No symptoms (%)</td>
<td>0 (0%)</td>
<td>2 (20%)</td>
<td>0 (0%)</td>
<td>16 (100%)</td>
</tr>
<tr>
<td>Severity of illness score§ (mean)</td>
<td>4.4</td>
<td>2.7</td>
<td>2.6</td>
<td>0.0</td>
</tr>
<tr>
<td>Onset, days postdocking (mean)</td>
<td>7.8</td>
<td>5.9</td>
<td>4.4</td>
<td>N/A*</td>
</tr>
<tr>
<td>EITB positive (%)</td>
<td>10 (100%)</td>
<td>10 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>ELISA positive (%)</td>
<td>0 (0%)</td>
<td>7 (70%)</td>
<td>4 (18%)</td>
<td>5 (31%)</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>23</td>
<td>25</td>
<td>30</td>
<td>23</td>
</tr>
</tbody>
</table>

* Not applicable.
* Multiple serum specimens were not available for six crew members.
† Multiple serum specimens not available for two crew members.
‡ A score of 2 was given for reported symptom of diarrhea and a score of 1 was given for each reported symptom of nausea, abdominal cramps, or vomiting.
§ Includes beverages prepared using unboiled water.

### Table 2

<table>
<thead>
<tr>
<th>Cases*</th>
<th>Confirmed (n = 10)</th>
<th>Probable (n = 10)</th>
<th>Suspected (n = 22)</th>
<th>Non (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glasses of water in Milwaukee (mean)</td>
<td>5.9</td>
<td>4.4</td>
<td>4.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Glasses of water daily on cutter (mean)</td>
<td>8.4†</td>
<td>3.2</td>
<td>5.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Total glasses of water daily on cutter (mean)</td>
<td>16.3‡</td>
<td>10.4</td>
<td>9.7</td>
<td>5.9</td>
</tr>
</tbody>
</table>

* For definitions of cases, see Table 1.
† Statistically significant, $P \leq 0.003$ when compared with noncases.
‡ Includes beverages prepared using unboiled water.
IgM responses increased from 10-weeks to 28-weeks PD for unknown reasons. The IgA responses to the 17-kDa group disappeared the fastest of the three isotypes and were either weak or not detectable at 10-weeks PD. Confirmed cases also showed decreasing IgG responses to at least two of the three antigen groups from three-weeks to 28-weeks PD with three exceptions whose IgG responses to at least two of the three antigen groups were most intense at 10-weeks PD and then decreased. These three also showed no IgA activity with the 17-kDa group. The confirmed cases showed higher net EITB intensities to the antigen groups ($P \leq 0.01$) than noncases (Table 3).

As defined above, probable cases showed no evidence of oocysts in their stools, but were EITB positive. Shown in Figure 3 are EITB profiles (strip panels IgA, IgM, and IgG) and intensities (graphs IgA, IgM, and IgG) on one probable case, crew member CGC8. The EITB profiles of the probable cases, including the two who reported no symptoms, were similar to those of the confirmed cases, and their net EITB intensities were significantly higher ($P \leq 0.003$) than noncases (Table 3). In addition, at three-weeks PD, the probable cases showed higher EITB intensities for IgG responses to the 27-, 17-, 15-kDa groups ($P = 0.03$, $P = 0.11$, and $P = 0.03$, respectively) and for IgA and IgM responses to the 17- and 27-kDa groups ($P = 0.04$ and $P = 0.10$, respectively) than confirmed cases (Table 4).

Of the remaining 38 crew members, 22 were classified as suspected cases and 16 as noncases. Despite consuming more water than noncases, the suspected cases showed no statistically significant differences ($P > 0.05$) in net EITB intensities (Table 3) or in EITB intensities at three-weeks PD (Table 4) when compared with noncases. As shown in Figure 4, in crew member CGC2, a noncase, antibody responses to the antigen groups remained relatively static throughout the

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**Table 3**

Mean net enzyme-linked immunoelectrotransfer blot intensity from three-weeks to 29-weeks postdocking on the clinical case classifications

<table>
<thead>
<tr>
<th>Isotype/antigen group</th>
<th>Cases*</th>
<th>Confirmed (n = 10)</th>
<th>Probable (n = 10)</th>
<th>Suspected (n = 22)†</th>
<th>Non (n = 16)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG/27-kD</td>
<td></td>
<td>0.321§</td>
<td>0.195¶</td>
<td>0.043</td>
<td>0.048</td>
</tr>
<tr>
<td>IgG/17-kD</td>
<td></td>
<td>0.400§</td>
<td>0.341¶</td>
<td>0.080</td>
<td>0.065</td>
</tr>
<tr>
<td>IgG/15-kD</td>
<td></td>
<td>0.365§</td>
<td>0.295¶</td>
<td>0.066</td>
<td>0.038</td>
</tr>
<tr>
<td>IgA/17-kD</td>
<td></td>
<td>0.226§</td>
<td>0.453¶</td>
<td>0.003</td>
<td>0.018</td>
</tr>
<tr>
<td>IgM/27-kD</td>
<td></td>
<td>0.503§</td>
<td>0.392¶</td>
<td>0.066</td>
<td>0.084</td>
</tr>
</tbody>
</table>

* For definitions of cases, see Table 1.
† Multiple serum specimens were not available for six crew members.
‡ Multiple serum specimens were not available for two crew members.
§ Statistically significant, $P \leq 0.01$ when compared with noncases.
¶ Statistically significant, $P \leq 0.003$ when compared with noncases.
three- to 28-weeks PD period for 16 suspected cases and 14 noncases who were tested by EITB. Although these 30 crew members showed no relative change in immunoglobulin responses to the three antigen groups, 22 showed light-to-moderate IgG activity to at least one of the three antigen groups that remained relatively static throughout the three- to 28-weeks PD period, and of these 22, 12 showed light-to-moderate IgM activity to the 27-kDa group that remained static throughout the same period. Only eight crew members, whose water and total water consumption per day aboard the cutter was similar to the 22 with light-to-moderate IgG activity \( (P \geq 0.17) \), showed no reactivity to these antigens by EITB.

**Enzyme-linked immunosorbent assay.** The results of the ELISA were not in agreement with those of the EITB. Of the 10 confirmed cases who were considered EITB positive, only four showed a significant change \( (P < 0.05) \) in ELISA OD between three-weeks and follow-up serum specimens, as shown in Table 1. Furthermore, of the 10 probable cases who were also considered EITB positive, only seven showed a significant change \( (P < 0.05) \) in ELISA OD between three-weeks PD and follow-up serum specimens, as shown in Table 1. On the other hand, of the 16 suspected cases and the 14 noncases who were EITB negative, four of the former and five of the latter showed a significant change \( (P < 0.05) \) in ELISA OD between three-week PD and follow-up serum specimens (Table 1).

**DISCUSSION**

Water obtained in Milwaukee, Wisconsin during the 1993 cryptosporidiosis outbreak appeared to be the etiologic source of a cryptosporidiosis outbreak aboard a U.S. Coast Guard cutter. Although *Cryptosporidium* was not identified in the approximately 40 liters of water taken from the cut-

### TABLE 4
Mean enzyme-linked immunoelectrotransfer blot intensities at three-weeks postdocking by clinical case classifications

<table>
<thead>
<tr>
<th>Isotype/antigen group</th>
<th>Confirmed ( (n = 10) )</th>
<th>Probable ( (n = 10) )</th>
<th>Suspected ( (n = 22) )</th>
<th>Non ( (n = 16) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG/27-kDa</td>
<td>0.612</td>
<td>0.838( ^6 )</td>
<td>0.387</td>
<td>0.407</td>
</tr>
<tr>
<td>IgG/17-kDa</td>
<td>0.631</td>
<td>0.956</td>
<td>0.320</td>
<td>0.406</td>
</tr>
<tr>
<td>IgG/15-kDa</td>
<td>0.521</td>
<td>0.864( ^6 )</td>
<td>0.249</td>
<td>0.340</td>
</tr>
<tr>
<td>IgA/17-kDa</td>
<td>0.235</td>
<td>0.509( ^6 )</td>
<td>0.029</td>
<td>0.069</td>
</tr>
<tr>
<td>IgM/27-kDa</td>
<td>0.670</td>
<td>0.969</td>
<td>0.465</td>
<td>0.511</td>
</tr>
</tbody>
</table>

* For definitions of cases, see Table 1.
† Multiple serum specimens were not available for six crew members.
‡ Multiple serum specimens not available for two crew members.
§ Statistically significant, \( P \leq 0.04 \) when compared with confirmed cases.
FIGURE 4. Enzyme-linked immunoelectrotransfer blot (EITB) and intensitometric profiles of CGC2, a crew member classified as a noncase of cryptosporidiosis. The EITB strip panels include IgA responses to the 17-kDa antigen group, IgM responses to the 27-kDa group, and IgG responses to the 27-, 17-, and 15-kDa groups at three-, 10-, and 28-weeks postdocking (PD). Brackets denote antigen groups. Graphs show EITB intensities at three-, 10-, and 28-weeks PD. hexagon = IgM responses to the 27-kDa group; ● = IgA responses to the 17-kDa group; ○, □, and △ = IgG responses to the 27-, 17-, and 15-kDa groups, respectively.

Crew members’ exposure to water likely containing *Cryptosporidium* oocysts provided an opportunity to investigate serologic responses to infection in a relatively homogenous population with a well-defined period of exposure. In confirmed and probable cases, humoral immune responses to small molecular weight antigens from *C. parvum* oocysts changed dramatically with time. Small molecular weight antigens from oocysts and sporozoites have been shown by EITB to elicit humoral immune responses in infected animals and humans. Here, relatively static IgG and IgM responses to high molecular weight antigens were observed in some crew members with parasitologically confirmed cryptosporidiosis (e.g., Figure 2, strip panels IgG and IgM). Based on these observations, intensitometric data was collected on IgA responses to the 17-kDa antigen group, IgM responses to the 27-kDa antigen group, and IgG responses to the 27-, 17-, and 15-kDa antigen groups. These antigen groups are known to contain surface proteins from sporozoites as shown by indirect immunofluorescent assay using MAbs C6B6 and C6C1. The 17-kDa antigen group studied here may be the same as an 18- or 20-kDa antigen extracted from stools of infected calves and humans, suggesting that this antigen is produced in large quantities during colonization of the intestines and may, therefore, be likely to elicit antibodies. In addition, it is known that surface proteins on both sporozoites and merozoites share common epitopes, and since sporozoites and merozoites are invasive forms, it seems likely that these surface proteins would elicit immune responses.

Antibodies to the antigen groups may have existed in some crew members before their consumption of water aboard the cutter. The light-to-moderate IgG and IgM responses observed in 22 of 30 crew members who were EITB-negative may have been a result of previous *Cryptosporidium* exposure. This suggests that antibodies to *Cryptosporidium* may have been present in a portion of the crew members before consumption of water aboard the cutter. For
example, one could speculate that the probable cases had antibodies to Cryptosporidium before exposure on the cutter because at three-weeks PD their mean EITB intensities were higher than in the confirmed cases (Table 4), indicating a possible secondary antibody response. In addition, severity of illness scores were significantly lower for probable cases than for confirmed cases (Table 1). It is known that oral administration of antibodies to Cryptosporidium decreases the intensity of Cryptosporidium infection in mice\(^{40-42}\) and ameliorates clinical symptoms in humans.\(^{43,44}\) However, the Cryptosporidium antibody status of the crew members before exposure was not known because serum specimens were not available before the outbreak.

The reason for observed discrepancies between the ELISA and EITB is not known. The ELISA collects data from all signals emitted directly or indirectly by an immunoglobulin subclass responding to complex native antigens binding hydrophobically to a solid phase. Therefore, when using complex antigens such as crude oocyst extracts, the ELISA may generate signals from many bound antigens that mask signals from a subset of antigens that provide useful diagnostic information. The EITB, on the other hand, allows visualization of the responding immunoglobulin subclass to specific SDS-treated antigens, but the SDS treatment may irreversibly destroy important conformational epitopes. Lack of concordance between the ELISA using crude antigen and the EITB using densitometry on a 23-kD antigen was also shown in another study.\(^23\) In this study, ELISA results showed no relationship to water consumption aboard the cutter while water consumption was significantly related to EITB results. Until more definitive data evaluating the use of the ELISA and the EITB are generated, both may be required in a complementary fashion to evaluate humoral immune responses elicited by Cryptosporidium.

The sensitivity of the EITB can not be estimated from the present study. Of 47 crew members who submitted stool specimens, only 10 were positive for Cryptosporidium by microscopy. The low prevalence of confirmed infection relative to the number of crew members with symptoms reflects both the insensitivity of diagnostic techniques and the fact that three weeks had passed since the initial exposure to the water aboard the cutter. It is likely that a number of crew members had cleared their infections before collection of stool specimens at three weeks PD. Thus, the number of parasitologically confirmed infections probably underestimates the actual number infected. Also, EITB sensitivity cannot be determined based on clinical case classification in this study because we were not able to exclude other agents as possible causes of clinical manifestations. In addition, human studies have shown that not all infected persons experience typical symptoms of clinical cryptosporidiosis.\(^15\) Finally, we cannot be certain which crew members, if any, were free of infection nor can we be certain of the level of antibody that was related to the current outbreak or to the potential contribution of cross-reactive antibodies elicited by other agents.

A number of factors may underlie the heterogeneity of antibody responses we observed in this study. The number of oocysts to which individual crew members were exposed is unknown and certainly varied from individual to individual, based upon patterns of water consumption. These differences may have contributed to some of the variation in antibody responses. In addition, we do not know the pre-exposure immune status of the crew members or how pre-existing anti-Cryptosporidium antibodies may influence susceptibility to infection or antibody production. Answers to these questions will require a study involving controlled parameters.\(^15\) Nonetheless, it is clear that symptomatic infection was associated with consistent changes in antibody responses. Based on this and on the differential clearance of IgA and IgG, the EITB may be useful for epidemiologic studies designed to monitor exposure to Cryptosporidium at the population level.

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REFERENCES


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