A Comparison of an Immunoassay with Acid-Fast Staining to Detect Cryptosporidium

Brian J. Harrington, PhD, MPH,1 Hailu Kassa, PhD, MPH, MSOH2
1Department of Public Health, Medical College of Ohio, Toledo, OH, 2Department of Public and Allied Health, Bowling Green State University, Bowling Green, OH

Cryptosporidium, a protozoan parasite first identified in mice in 1907, has become an important cause of human enteric disease, cryptosporidiosis, which may range from sub-clinical or self-limiting mild disease to a cholera-like diarrhea characterized by dehydration, fever, and abdominal pain.1 While Cryptosporidium parvum is the most prevalent cause of cryptosporidiosis in humans,2 other species of Cryptosporidium have also been found in feces of immunocompromised patients.3,4 There are 2 genotypes of C. parvum that cause cryptosporidiosis in humans, a genotype specific to humans and a zoonotic (bovine) genotype.2

A dose as low as 30 oocysts of C. parvum may be sufficient to cause infection in healthy persons with no serologic evidence of past infection with C. parvum.5 Infection may occur with C. parvum either by ingesting contaminated food or water; by direct contact with infected persons, animals; or with environmental surfaces contaminated with feces of infected animals or humans.6,7 Since the oocysts C. parvum remain viable and infective at below freez-
ing temperatures, the potential risk of infection may not diminish due to seasonal changes in temperatures.

While cryptosporidiosis is usually a self-limiting diarrheal disease in immunocompetent persons, it is a serious, even life-threatening, disease in some immunocompromised patients, especially in those with late-stage AIDS. In some AIDS patients, the oocysts may spread to the lungs and cause pulmonary cryptosporidiosis. At the present time, there are no safe and effective treatments for cryptosporidiosis.

Cryptosporidium parvum is emerging as an important cause of human gastroenteritis in the United States. It causes an estimated 300,000 cases of cryptosporidiosis each year, of which 10% are attributed to food-borne transmission and 90% are due to consumption of contaminated water and person-to-person transmission. Failure of the water filtration system in Milwaukee, WI, and subsequent contamination with C. parvum caused the first large outbreak of waterborne cryptosporidiosis in the United States in 1993, and 2 years after the outbreak the mortality rate with cryptosporidiosis as the underlying or contributing cause increased significantly among the immunocompromised population.

In addition to the Milwaukee outbreak caused by drinking contaminated water, there have been other waterborne outbreaks of cryptosporidiosis in the United States, and these were associated with recreational use of water, including chlorinated swimming pools. As oocysts of Cryptosporidium are not easily killed by chlorination, there is a real potential for contamination of other drinking water systems in many areas in the United States.

At the present time, C. parvum is not a significant cause of food-borne illness in the United States. It accounts only for about 0.2% of the total food-borne illnesses occurring each year. Food-borne outbreaks of cryptosporidiosis may be caused by consumption of food contaminated at the source or food contaminated by infected food handlers in restaurants or the home. Infected food workers were responsible for 2 recent outbreaks of cryptosporidiosis in the United States. Oocysts of C. parvum infectious for humans have been found in oysters from the Chesapeake Bay area, indicating that raw oysters can be a potential source of Cryptosporidium infection in the United States.

Cryptosporidium can be transmitted from many wild animals to humans via the fecal-oral route. Canada goose (Branta canadensis) is one of the animals that might be a potential source of Cryptosporidium infection for humans in the United States. Viable oocysts of C. parvum were found in fecal droppings of geese in 7 of 9 sites in the Chesapeake Bay area and in 13 of 16 (81.3%) sites in and around Toledo, OH. The feces in the Toledo study were collected from places of intense human activities, such as golf courses, parks, work, and residential areas.

Since fecal droppings of Canada geese are dense in many areas used by humans, the authors suggested that goose feces could be sources of Cryptosporidium infection in the Toledo area. However, in the Toledo/Lucas County area, there was only 1 reported case of cryptosporidiosis in 1998, none in 1999, and 5 in 2000 (Ohio Department of Health, Infectious Disease Surveillance Section, February 2001). As a result, we decided to investigate the presence of Cryptosporidium in stool specimens of patients with gastroenteritis seeking medical attention. The stool specimens had not been previously tested for the presence of Cryptosporidium.

Materials and Methods

Between October 1999 and February 2001, 1,450 specimens of human feces, of which 46 were duplicates, were obtained from 2 large clinical laboratories in Toledo, OH. Specimens were either submitted in formalin-fixed or air-dried, fixed, and stained with modified acid-fast carbol fuschin stain reagent following the procedure described for Cryptosporidium oocysts (Remel, T1 #40104). The carbol fuschin acid-fast technique was used here rather than the fluorescent auramine O stain, which is probably slightly more sensitive for cryptosporidial oocysts, as not all laboratories have fluorescence microscopy capabilities. Carbol fuschin will also stain oocysts of Cyclospora, and the presence of WBCs can be easily detected with the Kinyoun stain. Stained smears were examined with the 40x dry objective and oocysts confirmed using the 100x oil immersion objective. If no oocysts were seen on the initial smear of each type, 2 more smears were made and examined. The results in T1 indicate how many slides of each type were examined before a positive was found. “Negative” in the smear results in T1 indicate that no oocysts were found in all smears of that type.

Results

Twelve stool specimens from different patients (0.85%) were positive for Cryptosporidium by the microtiter plate monoclonal enzyme immunoassay (EIA) (ProSpect Cryptosporidium Microplate Assay) following the manufacturer’s procedure (Alexon-Trend M). Results were evaluated visually by comparing the color development in the test wells to the color chart supplied with the kit. These methods, for Cryptosporidium and other pathogens, have been widely accepted for clinical use due to the high degree of specificity and sensitivity associated with monoclonal antibodies and methodology.

Specimens positive for Cryptosporidium antigen by the EIA method were examined microscopically after acid-fast staining. Three types of smears were made from each of the positive specimens.

1) “Suspension” smears were made directly from the formalized specimens after being vortexed thoroughly.

2) “Sediment” smears were made from the sediment after several mL of the formalin suspension had been centrifuged for 5 minutes at 600 g.

3) “Concentrate” smears were made from the sediment after the standard formalin-ethyl-acetate concentration method was used.

Approximately 100 µL of the prepared material were used for each smear. The smears were air-dried, fixed, and stained with modified acid-fast carbol fuschin stain reagent following the procedure described for Cryptosporidium oocysts (Remel, T1 #40104). The carbol fuschin acid-fast technique was used here rather than the fluorescent auramine O stain, which is probably slightly more sensitive for cryptosporidial oocysts, as not all laboratories have fluorescence microscopy capabilities. Carbol fuschin will also stain oocysts of Cyclospora, and the presence of WBCs can be easily detected with the Kinyoun stain. Stained smears were examined with the 40x dry objective and oocysts confirmed using the 100x oil immersion objective. If no oocysts were seen on the initial smear of each type, 2 more smears were made and examined. The results in T1 indicate how many slides of each type were examined before a positive was found. “Negative” in the smear results in T1 indicate that no oocysts were found in all smears of that type.
Stool Specimens Positive for Cryptosporidium by Enzyme Immunoassay (EIA) and Acid-Fast Staining Methods

<table>
<thead>
<tr>
<th>Specimen #</th>
<th>EIA Result</th>
<th>Suspension</th>
<th>Sediment</th>
<th>Concentrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>118</td>
<td>4+</td>
<td>First</td>
<td>First</td>
<td>First</td>
</tr>
<tr>
<td>145</td>
<td>1+</td>
<td>Negative</td>
<td>First</td>
<td>Second</td>
</tr>
<tr>
<td>195</td>
<td>1+</td>
<td>First</td>
<td>Second</td>
<td>Negative</td>
</tr>
<tr>
<td>251</td>
<td>3+</td>
<td>Negative</td>
<td>First</td>
<td>First</td>
</tr>
<tr>
<td>364</td>
<td>1+</td>
<td>Negative</td>
<td>Third</td>
<td>Third</td>
</tr>
<tr>
<td>549</td>
<td>3+</td>
<td>Second</td>
<td>First</td>
<td>Second</td>
</tr>
<tr>
<td>599</td>
<td>1+</td>
<td>Negative</td>
<td>Second</td>
<td>Negative</td>
</tr>
<tr>
<td>476</td>
<td>4+</td>
<td>First</td>
<td>First</td>
<td>Second</td>
</tr>
<tr>
<td>533</td>
<td>1+</td>
<td>Negative</td>
<td>Third</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Note: *Positive means for each preparation, oocysts were observed in either first, second, or third smear; negative means for each preparation, oocysts were not observed in all 3 smears.

Discussion

Nine of the 1,404 (0.64%) stool specimens were positive for *Cryptosporidium* both by the EIA and acid-fast microscopic methods. The reported mean 4-year (1995 to 1998) rate of Cryptosporidiosis in Ohio was ≤0.9 per 100,000 population. Since not all infected persons are symptomatic and since not all symptomatic persons seek medical care, the actual number of persons infected with *Cryptosporidium* remains unknown, and could have been significantly higher than the number reported here. A recent study from Ontario, Canada (the southwest part of this province is geographically close to Toledo), found 1,615 specimens positive for *Cryptosporidium* in more than 260,000 stool specimens examined over 39-month period, a rate approximately 0.6%. A survey conducted in 1999 found a prevalence rate overall for *Cryptosporidium* <1%, with rates of 0.9% in children and <0.5% in adults. We did not know the age or immune status of any of the patients in our study. The true extent of under-diagnosed and under-reported cases of cryptosporidiosis in the United States is not known. Lack of routine examination of stool specimens is a likely reason for under-diagnosis of cryptosporidiosis in the Toledo area.

Although cryptosporidiosis is a reportable disease in Ohio, we found that most clinical laboratories in the Toledo area do not routinely include any stool assay for *Cryptosporidium* unless requested specifically by the ordering physicians. The 1999 survey found that only 27% of respondents included examination of *Cryptosporidium* in their routine parasitology workup. According to another study, laboratories that routinely tested stool specimens for *Cryptosporidium* had higher positive rates than those that did not. In addition, higher positive rates were associated with the use of monoclonal antibody methods and with the use of 2 or more staining procedures.

Enzyme immunoassay (EIA) methods and other immunoassays involving monoclonal antibodies are known for their relatively high specificity and sensitivity. The EIA microtiter plate method can be used to test multiple stool specimens simultaneously, is not labor-intensive, and does not require the high technical skills needed to identify parasites using microscopic examinations of acid-fast stained stool specimens. Although the numbers are small, this study shows the insensitivity of acid-fast staining smears, especially those made from formalin suspension of feces submitted to laboratories. Whether smears made directly from the
undiluted feces would have given higher detection rates is unknown, as all the specimens examined had been previously diluted. The standard formalin-ethyl acetate concentration method was found to be less sensitive than the simple centrifuging formalin suspension. Even with concentration and straight centrifuging methods, examination of only 1 stained smear may not be adequate to detect Cryptosporidium oocysts. Whether or not examining more than 1 stool specimen per person could improve oocyst detection rate is also unknown.

Cryptosporidium is commonly found in surface waters in the United States and in the feces of Canada geese and in other wild and domestic animals, wild and domestic, with which humans have frequent and close contact. Although there is no therapy for cryptosporidiosis, from a public health perspective, detection of cases is important as it could lead to preventing the spread of infection to others. Two large outbreaks of cryptosporidiosis, 1 involving 700 persons in OH, and another involving 225 persons in NE occurred in 2000 and were traced to infected persons using chlorinated swimming pools. These outbreaks might have been avoided if the index cases been identified early.20

A recent review noted the importance of person-to-person spread in households, day-care centers, and hospitals.34 Clinical laboratories should be encouraged to screen for Cryptosporidium routinely. Physicians should have an increased awareness of its presence in the environment, should know whether or not laboratories examining specimens from their patients routinely test for Cryptosporidium, and should specifically request such testing when ordering laboratory studies on diarrheal patients.35

**Conclusion**

Cryptosporidiosis is both under-suspected and under-diagnosed. We recommend that clinical laboratories increase their testing for Cryptosporidium, and encourage physicians to include it in the workup of diarrheal patients. Given the insensitivity of acid-fast stain microscopy, the technical time involved, and the skills required for such testing, use of an immunoassay technique when examining stool specimens for Cryptosporidium would be the preferred procedure.

**Acknowledgment**

We wish to thank Alexon-Trend for providing microtiter immunoassay kits used in this study. The cooperation of Pathology Laboratories of NW Ohio, Promedica Laboratories, and their microbiology technical staffs are also acknowledged.