Evaluation of an Enzyme-Linked Immunosorbent Assay for Detection of Cryptosporidium spp. in Stool Specimens

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Received 17 November 1992/Accepted 2 March 1993

We evaluated a commercially produced enzyme-linked immunosorbent assay (ELISA; LMD Laboratories, Inc.) for the detection of Cryptosporidium spp. in 296 stool specimens submitted to the Mayo Clinic parasitology laboratory for routine examination. The specimens examined were fresh (4 specimens), were stored frozen at -65°C (49 specimens), or were preserved in 10% formalin (243 specimens). Results were compared with those obtained by indirect immunofluorescent antibody detection (MerillFluor Cryptosporidium/Giardia; Meridian Diagnostics, Inc.). One hundred of the specimens were positive by indirect immunofluorescent antibody and ELISA, while 187 were negative by both methods; 91 of these negative stool samples contained 121 parasites of 17 different species. Eight ELISA false negatives and one false positive were observed. The ELISA sensitivity was 93%, specificity was 99%, and the positive predictive value was 99%. Storage of specimens preserved in 10% formalin or frozen fresh at -65°C for up to 18 months did not appear to affect the results. There was no cross-reactivity with Giardia lamblia (54 negative specimens) or with the 16 other parasites present in the ELISA-negative stool samples. The ELISA is a fast, easy-to-read, and accurate method for the detection of Cryptosporidium spp. in stool specimens.

Organisms of the genus Cryptosporidium are small (2 to 6 μm), coccidial parasites which have only recently been recognized as important enteric pathogens of humans. This parasite infects epithelial cells lining the gastrointestinal and respiratory tracts and most commonly produces an acute, though self-limiting, diarrhea in immunocompetent individuals (4, 25). Infection in malnourished or immunocompromised patients is much more serious, often resulting in prolonged, intractable, and severe diarrhea (4, 19). In patients with AIDS, the infection may be particularly debilitating and may spread beyond its usual sites in the small and large bowel to involve the stomach, biliary tract, and respiratory tract (3, 6, 9, 22). There is no proven effective treatment for these patients, although some improvement has been seen with ingestion of hyperimmune bovine colostrum (16). Cryptosporidium spp. was first recognized as a cause of diarrheal disease in poultry and cattle, and one species, Cryptosporidium parvum, is now recognized as an important agent in life-threatening neonatal diarrhea in calves and lambs (4, 14). This organism is an obligate, intracellular parasite residing in parasitophorous vacuoles confined to the microvillous region of the host epithelial cells (5, 17). Infection in humans and other mammals is thought to be caused by ingestion of the thick-walled, environmentally resistant oocysts of C. parvum, after fecal contamination by humans or animals. These oocysts are resistant to most common disinfectants and are not readily killed by routine chlorination of water supplies (10, 12). Zoonotic transmission is probably responsible for cryptosporidiosis occurring in humans having direct contact with animals or for waterborne outbreaks due to contaminated surface water supplies (4, 10, 14). Person-to-person transmission is also probably common and responsible for the frequent occurrence of the infection in children attending day-care centers and for the high prevalence, in general, in pre-school age children (1, 11).

Cryptosporidiosis is also recognized as a cause of traveler’s diarrhea. Cryptosporidiosis is a worldwide infection with prevalence rates being higher in developing (5 to 10%) than in developed (1 to 3%) countries (4, 18, 20). It is more common in young children (ages 1 to 4 years) than in adults, with the exception of patients with AIDS, and occurs more frequently in the warmer months of the year. Diagnostic efforts should probably be focused on those groups with the highest prevalence rather than on the general population of those with diarrheal disease. Laboratory diagnosis of cryptosporidiosis is dependent on detection of the characteristic oocysts in stool specimens. Microscopic examinations of stool samples after concentration and staining with nonspecific acid-fast stains and/or fluorescent antibody stains are the most common techniques used. These methods are complex and can be very time-consuming. Nonspecific stains can be difficult to interpret (false positivity has been a problem on proficiency tests) and may not detect low numbers of oocysts. Availability of a rapid yet simple test to detect the presence of Cryptosporidium spp. in stools would avoid these diagnostic problems and obviate the need for skilled microscopists, special processing methods, and expensive equipment. In this study, we evaluated a commercially produced (LMD Laboratories, Carlsbad, Calif.) enzyme-linked immunosorbent assay (ELISA) that detects antigens of Cryptosporidium spp. present in unprocessed stool samples. The ELISA results were compared with those of an indirect fluorescent antibody (IFA) method (MERIFL UOR Cryptosporidium/Giardia; Meridian Diagnostics Inc., Cincinnati, Ohio) which was considered the "gold standard."

MATERIALS AND METHODS

Stool specimens. Two hundred and ninety-six stool specimens submitted to the Mayo Clinic parasitology laboratory were selected for testing for the presence of Cryptosporidium spp. by ELISA and IFA. The specimens selected were
loose stools from children 5 years old or younger and stools which had a specific request for Cryptosporidium testing. The samples were placed in formalin preservative at the time of collection (n = 243), received in the laboratory in the fresh state and processed immediately (n = 4), or frozen at −65°C for testing at a later date (n = 49). Some stool samples that were frozen or formalin preserved (at room temperature) were stored for periods of up to 18 months.

**ELISA procedure.** Specimens received in formalin were already diluted approximately 1:5 and used for the ELISA without further processing. Fresh and fresh-frozen stool samples were diluted 1:5 in the wash-dilution buffer provided in the kit prior to testing. Per the manufacturer’s instructions, specimens were not concentrated but were vortexed (10 s) and allowed to sediment until clearing of the top of the supernatant (1 to 3 min) before use. Any number of wells can be broken off from the strips and used as needed. To each microtiter well coated with Cryptosporidium polyclonal antibody, 100 μl of diluted wash-dilution buffer was added; subsequently, 50 μl of stool supernatant was added. Controls (positive and negative) were included with each run. After a 45-min incubation at 37°C, the wells were washed and anti-Cryptosporidium monoclonal antibody was added. The wells were then incubated again for 20 min and washed, and anti-second antibody conjugated to biotin was added. After another 10-min incubation and washing, streptavidin-horseradish peroxidase was added; next came another 10-min incubation, washing to remove unbound enzyme, and addition of tetramethylbenzidine (TMB) substrate to develop the reaction. A subsequent 10-min incubation at room temperature allowed for color development, and the reaction was stopped with the addition of sulfuric acid, which changes the blue to yellow; results were read visually. Total time for performing the ELISA was approximately 135 min, with 30 minutes of "hands on" time.

**IFA procedure.** For IFA testing, stool samples were concentrated, per the suggestion of the manufacturer and as part of the complete ovum and parasite examination, by the formalin-ethyl acetate procedure (Evergreen Scientific, Los Angeles, Calif.). This was done prior to freezing the fresh stool samples and within 1 day of receiving the formalin-preserved stool specimens. Ten microliters of stool concentrate and controls was added to the treated slide wells included in the kit. The slides were allowed to dry at room temperature, and fluorescein isothiocyanate-labeled anti-Cryptosporidium monoclonal antibody and counterstain (Eriochrome Black solution) were added to each well. After incubation for 30 min at room temperature, each well was scanned with a fluorescence microscope, with 200× magnification. Total time for performing the IFA was 45 min, with 15 min being hands on time. Tabulation of ELISA results was done without reference to whether Cryptosporidium oocysts were present by IFA.

**RESULTS**

Cryptosporidium spp. was detected in 100 of 296 stool specimens by both ELISA and IFA; 187 stool samples were negative for Cryptosporidium spp. when tested by both methods (Table 1). Of the 187 ELISA-negative specimens, 91 stool samples contained 121 parasites of 17 different species (Giardia lamblia [n = 54], Entamoeba histolytica [n = 16], Endolimax nana [n = 10], Entamoeba coli [n = 7], Entamoeba hartmanni [n = 6], Isospora belli [n = 5], Strongyloides stercoralis [n = 5], Hookworm [n = 4], Isolamnaeobasitischii [n = 3], Blastocystis hominis [n = 2],

**TABLE 1. Comparison of ELISA and IFA for detection of Cryptosporidium spp.**

<table>
<thead>
<tr>
<th>ELISA result</th>
<th>No. of stool specimens with following IFA result:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>100</td>
</tr>
<tr>
<td>Negative</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>108</td>
</tr>
</tbody>
</table>

clonorchis sinensis [n = 2], Dientamoeba fragilis [n = 2], Chilomastix mesnili [n = 1], Diphyllobothrium latum [n = 1], Hymenolepis nana [n = 1], Schistosoma mansoni [n = 1], Trichuris trichiura [n = 1]). The parasite detected most often in the ELISA-negative stool samples was G. lamblia. It was present in 54 samples and did not appear to cause any cross-reactivity.

Eight samples were considered to be false negatives by the ELISA method, i.e., they were IFA positive. All of these were from formalin-preserved specimens. No parasites were present on conventional microscopic examination, yet they were positive for Cryptosporidium spp. by IFA. Small numbers of organisms present in these samples may have been detectable only after concentration which was performed prior to the IFA procedure but not prior to the ELISA.

Only one specimen was falsely positive by the ELISA when compared with the IFA. This stool sample was frozen at −65°C and contained three parasites other than Cryptosporidium spp. These parasites were present in the list of negative stool samples by ELISA (see above), so cross-reactivity was probably not a factor. With the above data, the ELISA sensitivity was calculated to be 93%, the specificity was 99%, and the positive predictive value was also 99%.

ELISA results were similar to those obtained by IFA for specimens which were tested immediately upon receipt, after storage in 10% formalin, or frozen at −65°C for periods of up to 18 months. Therefore, preservation and storage did not appear to significantly alter the results; 97% of these specimens were either true positives or true negatives. Only 3% (nine specimens) gave results which were false positives or false negatives. Eight of the nine (89%) false results were from formalin-preserved specimens. However, since the great majority (82%) of all specimens tested were formalin preserved, one would expect that most of the false results would come from this group.

**DISCUSSION**

Results of this study indicate that the ELISA is comparable to the IFA technique for the detection of Cryptosporidium spp. in stool specimens. The sensitivity, specificity, and positive predictive value of the ELISA (in relation to the IFA) were 93, 99, and 99%, respectively. The slightly low sensitivity rate might be related to the fact that the specimens were not processed before performing the ELISA but were concentrated prior to the IFA procedure. The lack of cross-reactivity with 121 other parasites belonging to 17 different species in 91 ELISA-negative specimens illustrates the specificity of this test. Formalin preservation (or freezing) and storage of specimens did not seem to adversely influence the results.

The IFA method is a valid one for comparison, since it has
been shown to be extremely specific and approximately 20 times more sensitive than acid-fast techniques (7, 8, 24). The nonspecificity of these latter techniques is illustrated by the frequent presence of acid-fast staining artifacts in stool samples. The ELISA, which performs comparably to the IFA method, might be more advantageous for some clinical laboratories, since concentration of the specimen is not performed. Moreover, the expense of fluorescence microscopy equipment and need for experienced microscopists are obviated. It is generally recommended that testing for Cryptosporidium spp. be done on selected patients who may be unlikely candidates for having other parasitic infections. The ELISA may be economically performed on unprocessed specimens from such patients, without the need for more extensive ovum and parasite examinations. In our own laboratory, it is estimated that the cost of the ELISA would be one-third that of a full ovum and parasite study. Our cost for performing the IFA procedure per se is similar to that of the ELISA, but if the specimen is concentrated prior to the IFA, the cost will increase. The total test time for the ELISA (135 min) is considerably longer than that for the IFA (45 min), but again, additional time will be incurred if specimens are concentrated. The choice between these two tests will depend upon the individual laboratory’s criteria for specimen selection and other work practices.

Although this is the first reported evaluation of this commercially produced ELISA, there have been several other reports of experience with other versions of an ELISA for detecting Cryptosporidium spp. in stool specimens. Ungar reported a double-polyclonal antibody ELISA which was 82% as sensitive as detection by two microscopic methods (acid-fast and IFA) (23). This test could not be used on formalin-preserved specimens, and repeated freezing and thawing of specimens decreased the sensitivity. The failure to detect low numbers of oocysts in 11 Peruvian patients was thought due to inaccessible antigens or antigens not recognized by the antisera, possibly because of the presence of different strains or species of C. parvum than those used to produce the antisera. In general, this test could detect approximately 10⁶ oocysts per ml. Anusz et al. developed an ELISA using a monoclonal antibody for capture and studied its efficacy at detecting Cryptosporidium spp. in bovine feces (2). Their ELISA was more sensitive than acid-fast staining microscopy in detecting oocysts (3 × 10⁶/ml of feces versus 10⁶/ml) but was less sensitive than IFA (1 × 10⁶/ml). Others have also determined the detection threshold of the IFA to be around 5 × 10⁴ oocysts per g of stool (24). We did not attempt to determine the detection threshold of the LMD ELISA, but, on the basis of its favorable comparison with the IFA in this study, we would expect its threshold to be similar to that of the IFA. Another ELISA, which is commercially produced in Great Britain, was able to detect 10³ oocysts per ml of seeded tap water (21), a threshold similar to that shown for IFA.

Cryptosporidium species can be readily differentiated antigenically with monoclonal and polyclonal antibodies. However, antigenic differences between isolates of C. parvum, the species which infects humans, are not marked (15). More than 75 electrophoretically distinct C. parvum oocyst or sporozoite antigens are recognized by hyperimmune rabbit sera, but fewer than 20 are usually recognized by immune serum from humans (4). The specific antigen(s) detected in the LMD ELISA has not been determined, and the exact extent of cross-reactivity with different isolates of C. parvum is unknown. However, studies employing oocyst wall-reactive antibodies have shown strong binding to antigens in the 40- to 41-kDa range (2, 15). Others have shown a major antigen with a molecular mass of 47 kDa (13). These are likely to be the oocyst antigens detected by IFA and ELISA methods. These somewhat variable findings are possibly due to slight differences between different isolates of C. parvum, some heterogeneity in antibody responses, and laboratory differences in antigen preparation and performance of Western blots (immunoblots). The presence of these common antigens provides some assurance that immunoassays are efficient in detecting antigens of C. parvum from a variety of different patients and other sources such as water.

REFERENCES

ELISA FOR DETECTION OF CRYPTOSPORIDIUM SPP.


