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# Enzyme-Linked Immunoassay for Detection of *Cryptosporidium* Antigens in Fecal Specimens

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***Cryptosporidium* sp. is a ubiquitous 4- to 6-μm protozoan parasite infecting the intestinal tract of humans. It causes mild to fulminant diarrhea in patients, especially immunocompromised persons, and it may be hard to detect by microscopic fecal examination. An indirect, double-antibody enzyme-linked immunosorbent assay (ELISA) was developed using specifically produced goat and rabbit antisera to detect *Cryptosporidium* antigens in human feces. Of 62 frozen stools from patients with cryptosporidiosis, as detected by at least two microscopic diagnostic techniques, 51 were positive by ELISA; all ELISA-negative specimens came from patients with fewer than five oocysts per 0.01 ml of concentrated fecal sample examined after modified acid-fast or fluorescent monoclonal antibody staining. A total of 182 specimens from persons without *Cryptosporidium* infection were negative by ELISA in 176 instances; 3 ELISA-positive specimens came from patients with cryptosporidiosis diagnosed earlier. The sensitivity of the assay was 82.3%, and specificity was 96.7%. The predictive value of a positive ELISA was 89.5%, and the predictive value of a negative ELISA was 94.2%. The ELISA was not affected by the presence of eight other intestinal parasites but was sometimes affected by repeated freezing and thawing of fecal specimens. All fecal specimens were heated to 100°C for 2 min to reduce proteolytic enzyme activity, although the necessity of this step needs further evaluation. This first-generation ELISA is a simple, rapid, easily standardized test for *Cryptosporidium* antigens in stool samples which will be useful for diagnosis and for large-scale epidemiologic studies.**

*Cryptosporidium* sp. is a 4- to 6-μm coccidian parasite of veterinary importance, recognized worldwide as a human pathogen which causes self-limited diarrhea in immunocompetent patients and potentially intractable diarrhea in immunodeficient hosts (5, 6, 22). Prevalence is greater than 30% in some less-developed countries, although it rarely exceeds 2% in more-developed countries, except in specialized populations, such as acquired immunodeficiency syndrome patients or household members of infected patients, or in outbreaks, either waterborne or in hospitals and day-care centers (5, 6, 22; B. L. P. Ungar, *in* J. P. Dubey, C. A. Speer, and R. Fayer, ed., *Cryptosporidiosis in Man and Animals*, in press).

Until the acquired immunodeficiency syndrome pandemic gave human cryptosporidiosis a new importance, diagnosis relied on the identification of organisms in intestinal biopsy sections, frequently by electron microscopy. In the last decade, more than six concentration methods and more than a dozen staining techniques to detect oocysts in fecal specimens have been developed without consensus as to diagnostic dependability (5, 6). In the United States, as many as three different techniques may be used in a single laboratory; concentration by flotation in Sheather's sugar solution, modified acid-fast and fluorescent stains, and monoclonal antibody-based assays are most often used (7–9, 15, 23, 25). Nevertheless, *Cryptosporidium* oocysts may be hard to detect except in laboratories with the repeated and continuous exposure to positive specimens that allows diagnostic proficiency. An indirect double-antibody enzyme-linked immunosorbent assay (ELISA) was developed to detect *Cryptosporidium* antigens in fecal specimens.

## MATERIALS AND METHODS

**Preparation of immunogen.** *Cryptosporidium* oocysts were purified from calf feces by a modification of an earlier described technique (29). Fecal fat was removed by 10% ethyl acetate in deionized water (7 ml of feces to 25 ml of 10% ethyl acetate). Oocysts were separated from fecal material by flotation in sodium chloride-saturated deionized water (25 ml) and by sedimentation in deionized water (50 ml) after 10 min of centrifugation (4°C) at 1,000 × *g*. Other contaminating material was removed by treatment with 1% sodium hypochlorite in deionized water (20 ml) at room temperature for 1 min. After centrifugation as before, the resulting pellet of organisms was washed three times as follows: it was suspended by shaking in deionized water (50 ml) and recentrifuged. The final pellet was suspended in 1.0 ml of 0.15 M phosphate-buffered saline (PBS) (pH 7.4) and frozen at −20°C immediately after the number of oocysts had been counted with a hemacytometer. Before use, oocyst preparations were pooled, kept on ice, and sonicated at maximum intensity (Sonic Dismembrator model 300 with 1/8-in. [ca. 4.6-cm] microtip, 300-W generator; Fisher Scientific, Springfield, N.J.) for 10-s intervals with 20-s rest periods. Generally, within 2 min, more than 75% of the oocysts had been disrupted, as determined by repeat hemacytometer count.

**Production of antisera.** For each of three New Zealand White rabbits, a total of $10^8$ sonicated *Cryptosporidium* oocysts in Freund complete adjuvant (vol/vol) was inoculated intradermally at six sites in the back. For one Saanan goat, a total of $10^9$ oocysts was inoculated intramuscularly at four deep muscle sites, one per limb. The first booster of $10^7$ to $10^8$ sonicated *Cryptosporidium* oocysts in Freund incomplete adjuvant (vol/vol) was administered at 4 to 6 weeks, with three subsequent boosters at approximately 6-week intervals for all animals.

Sera were tested monthly at a 1:100 dilution by ELISA for anti-*Cryptosporidium* immunoglobulin G until the optical density (OD) reading had at least doubled the preimmuniza-

tion OD (approximately 3 months) and remained stable for at least 3 months (29). Postimmunization sera were then obtained and stored at −20°C until use. Preimmunization antibody-negative sera were also obtained and stored at −20°C.

**Collection and preparation of specimens.** Fecal specimens ($n$ = 244) were collected. Of these, 231 were from 113 persons, aged 6 months to 60 years, presumed or known to be seronegative for human immunodeficiency virus from the United States and from an area in Peru in which cryptosporidiosis is endemic. An additional 13 fecal samples were from human immunodeficiency virus-seropositive patients with diarrhea. Specimens for ELISA were refrigerated or placed on ice immediately after collection and frozen at −70°C within 8 h.

Fresh specimens were examined in the laboratory of original submission by direct microscopy after concentration and/or after modified acid-fast or fluorescent monoclonal antibody staining (8, 15, 23). Cryptosporidiosis was considered to be a definite diagnosis if *Cryptosporidium* organisms were found in two different laboratories or by two different staining processes (modified acid-fast or fluorescent monoclonal antibody). There were 62 specimens (12 from human immunodeficiency virus-seropositive patients) with cryptosporidiosis; for the remaining 182 specimens, confirmed identification (two techniques or two laboratories) of *Cryptosporidium* sp. could not be made. Fecal specimens were also examined in the laboratory of original submission for other parasites by standard techniques. Eight other intestinal parasites were identified: *Giardia lamblia* (67 specimens), *Endolimax nana* (38 specimens), *Entamoeba coli* (34 specimens), *Chilomastix mesnili* (27 specimens), *Ascaris lumbricoides* (8 specimens), *Hymenolepis nana* (7 specimens), *Trichuris trichiura* (4 specimens), and *Isospora belli* (3 specimens).

Fecal specimens for ELISA were thawed immediately prior to use and heated to 100°C in a water bath for 2 min to inactivate bacterial proteolytic enzymes. A fecal suspension was made by vortexing a cotton swab well coated with stool (approximately 0.3 mg of feces per swab) in 0.5 ml of 0.15 M PBS (pH 7.4) for 1 min. The original fecal specimens were immediately refrozen at −70°C. Each specimen was examined between two and four times on different microtiter plates and in duplicate on each microtiter plate.

Oocysts were purified as described above, but not sonicated, for subsequent use as a test antigen. These were serially diluted (twofold) from 500,000 to 2 organisms per 0.1 ml in 0.15 M PBS (pH 7.4).

**ELISA procedure.** The double-antibody ELISA procedure is a modification of assays for the detection of *G. lamblia* or *Entamoeba histolytica* antigens in fecal specimens (30, 31). Optimal concentrations of specific rabbit and goat antisera were determined by checkerboard titration with various numbers of purified *Cryptosporidium* oocysts diluted in 0.15 M PBS (pH 7.4) as a test antigen. Each ELISA washing procedure was performed five times in rapid succession with the addition and immediate vacuum suction of 0.2 ml of 0.15 M PBS (pH 7.4) containing 0.05% Tween 20 (PBS-T).

Alternate double rows of wells on round-bottom polystyrene microtiter plates (Immulon II; Dynatech Laboratories, Alexandria, Va.) were coated with a 1:50,000 dilution of anti-*Cryptosporidium* serum or preimmune rabbit serum in 0.1 ml of 0.1 M carbonate buffer (pH 9.6). Plates were stored at 4°C for at least 12 h but not longer than 5 days before use. After washing, a 1:4 dilution of fecal specimen in PBS-T with 0.5% gelatin (BBL, Becton Dickinson and Co., Cockeysville, Md.) (PBS-T-G) or a dilution of purified oocysts was added to four wells, two coated with immune and two with nonimmune rabbit sera. At least five negative- and one positive-control fecal specimens were also applied to each plate testing fecal specimens. After overnight incubation at 4°C, the plates were washed, and 0.1 ml of a 1:400 dilution of immune goat antisera in PBS-T-G was added to all wells and allowed to incubate for 1 h at 37°C. The plates were then washed, and 0.1 ml of a 1:800 dilution of peroxidase-labeled antibody (made in a rabbit) to goat immunoglobulin G (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) in PBS-T-G was added. After another hour of incubation at 37°C and another wash, 0.1 ml of 2,2-azino-di(3-ethylbenzthiazoline sulfonate) peroxidase substrate (Kirkegaard & Perry Laboratories) was added and allowed to react at room temperature for 60 min. At that time, the amount of color resulting from hydrolysis of the substrate by enzyme bound to the well was measured at a wavelength of 414 nm with a Titertek Multiskan Plus (Flow Laboratories, Inc., McLean Va.) microplate colorimeter.

To calculate results, the mean OD reading of wells coated with preimmune rabbit sera was subtracted from the mean OD of wells coated with immune rabbit sera to yield the specific OD for a given specimen. The mean and standard deviation of the OD for the negative control fecal specimens were calculated as described above. A clinical specimen was considered positive if the specific OD value was 0.08 U greater than the mean plus 2 standard deviations of the specific OD of at least five simultaneously examined negative control specimens (30, 31). A dilution of *Cryptosporidium* oocysts was considered positive if it yielded a value greater than that of the buffer alone.

## RESULTS

**Ability of ELISA to detect known numbers of *Crytoposporidium* oocysts.** Serial twofold dilutions containing from 500,000 to 2 *Cryptosporidium* organisms per 0.1 ml were examined in duplicate. The ELISA was easily able to detect between approximately 2,000 and 4,000 purified *Cryptosporidium* oocysts in six comparable ELISAs run on different days. It was able to detect between approximately 1,000 and 2,000 purified *Cryptosporidium* oocysts in two additional ELISAs run on other days.

**Testing of clinical specimens.** As shown in Fig. 1, 51 of 62 specimens from patients with confirmed cryptosporidiosis were positive by ELISA. All of the 11 remaining specimens of this group came from patients with fewer than five oocysts per 0.01 ml of concentrated fecal specimen, and 10 of these were from Peruvians. Of 182 samples from patients without confirmed cryptosporidiosis, 176 were negative by ELISA. Three of the six ELISA-positive, microscopy-negative specimens came from persons with cryptosporidiosis 30 to 60 days earlier, and two came from persons with *Cryptosporidium* oocysts identified by one, but unconfirmed by a second, stool examination. There was no medical history or additional specimen available from the final patient in this group.

Compared with results of a microscopic examination (Table 1), the overall sensitivity of the assay was 82.3%, with a false-negative rate of 17.7%. Specificity was 96.7%, with a false-positive rate of 3.3%. The predictive value of a positive ELISA was 89.5%, and the predictive value of a negative ELISA was 94.2%.

**Limits of ability of ELISA to detect *Cryptosporidium* antigen.** Some ELISA-positive specimens lost and some retained detectable *Cryptosporidium* antigen after a minimum

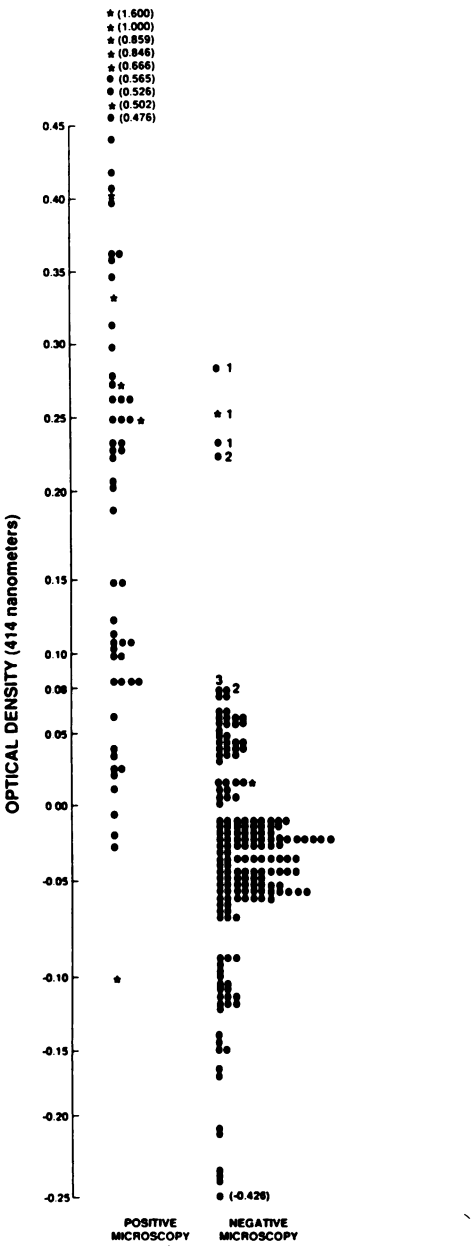


FIG. 1. Results of ELISA performed on fecal specimens collected from patients known to be positive or negative for *Cryptosporidium* sp. by two direct stool examinations. Positive results were those greater than 0.08 absorbance units. To allow for a valid comparison among assays performed at different times, the *y* axis was standardized so that the zero value corresponded to 2 standard deviations above the mean of five simultaneously examined negative-control stool specimens. Symbols: ★, acquired immunodeficiency syndrome patients; ●, all other patients; 1, persons with cryptosporidiosis 30 to 60 days earlier; 2, persons with *Cryptosporidium* infection unconfirmed by a second stool microscopic examination; 3, a person with no available additional information.

of three freeze-thaw cycles and separate ELISAs over a 6-month period; for four positive specimens, positive ELISA readings turned negative by 37 days (earliest) to 158 days (latest) after initial testing. For an additional six specimens, positive ELISA readings remained positive for at least 21 days (last time tested) to 164 days (last time tested) for different specimens. Results for 16 ELISA-negative specimens were not affected by at least three freeze-thaw cycles and ELISAs for up to 56 days. The presence of additional parasites did not affect the positivity of ELISA results for specimens with microscopically diagnosed *Cryptosporidium* sp. or negativity for specimens without *Cryptosporidium* sp. Preservation in 10% Formalin or in 2.5% potassium dichromate destroyed the ability of the ELISA to detect *Cryptosporidium* antigens.

TABLE 1. Comparison of ELISA and microscopic stool examination for detection of *Cryptosporidium*

| ELISA result | No. of microscopy results | | |
|---|---|---|---|
| | Positive | Negative | Total |
| Positive | 51 | 6 | 57 |
| Negative | 11 | 176 | 187 |
| Total | 62 | 182 | 244 |

Examination of positive and negative specimens in various locations on the same microtiter plate showed no difference in overall positivity or negativity of the specimens. OD readings for six positive specimens examined on at least three instances during a 3- to 23-week period showed OD values which ranged from 0.110 to 0.476 absorbance units, and for no specimen did this value vary more than 0.2 U. For 16 negative specimens examined at least three times over an 8-week period, OD readings were greater than 0.002 on only 4 of 48 occasions, with the highest reading at 0.067.

## DISCUSSION

During the past decade, as *Cryptosporidium* sp. has become acknowledged as an important human pathogen, numerous stool concentration and staining techniques that allow direct visualization of *Cryptosporidium* oocysts in fecal specimens have been developed (2, 4, 6, 8, 9, 12, 18, 21–23, 25). These include the most commonly used modified acid-fast stain and monoclonal antibody-based fluorescence detection assays (1, 3, 7, 8, 21). All microscopic diagnoses rely on direct visualization and morphologic recognition of small-size oocysts which may be scant in number, intermittently shed, or inconsistently stained (5; Ungar, in press). The use of fluorescence-based diagnostic tests is limited by expense and frequent lack of a fluorescence microscope, and other staining techniques, which may require examination of three or more fecal specimens to detect *Cryptosporidium* sp. (5), remain standard. Frequently, more than one technique may be necessary to diagnose *Cryptosporidium* sp., which is time-consuming and costly. Furthermore, recent identification of an acid-fast blue-green alga (cyanobacterium) which is only slightly larger than *Cryptosporidium* cells as a putative agent of diarrhea may limit the utility of acid-fast staining in the future (D. R. Shlim, M. T. Cohen, M. Eaton, R. Raja, E. G. Long, and B. L. P. Ungar, submitted for publication).

ELISAs have previously been developed for detection of *E. histolytica* and *G. lamblia* antigens in fecal specimens (10, 11, 13, 19, 20, 24, 26, 30–32). For *E. histolytica*, an ELISA using a single conjugated specific antibody was developed in 1978 (19), followed in 1982 by a double-antibody ELISA system (11), further modified in 1985 to a double-antibody ELISA using a monoclonal antibody (31). For *G. lamblia*, a double-antibody assay which was first described in 1984 (30) has been used with experimentally infected humans (17) and

in a field study performed in a region in Peru in which *G. lamblia* is endemic (M. F. C. Vidal, R. H. Gilman, B. L. P. Ungar, M. R. Verastegui, A. C. Benel, G. Marquis, M. Penny, C. Lanata, and E. Miranda, submitted for publication). At least six other similar tests have been developed (10, 13, 20, 24, 26, 32), and recent modifications include the use of antisera to specific ubiquitous giardia antigens (20, 24).

The ELISA for *Cryptosporidium* antigen detection in fecal specimens described here offers a diagnostic alternative to direct microscopy and represents the first generation of an ELISA for this organism. The sensitivity of the assay was 82.3% and specificity was 96.7%, with two separate positive microscopic examinations as the "gold standard" for positive diagnosis. At least three of the ELISA-positive, microscopy-negative specimens came from persons with previously documented cryptosporidiosis, suggesting either that microscopically undiagnosed *Cryptosporidium* sp. was still present or that the ELISA detected disintegrating organisms or their products; two other specimens of this group came from persons with only one unconfirmed positive microscopic examination, which may imply real infection or an ability to detect free antigen from some extracellular life cycle stage. In these cases, the ELISA may have detected cryptosporidiosis more efficiently than other means of diagnosis and may be important in identifying persons not actively excreting oocysts at the time of specimen collection, which is relevant to the transmission of infection (5; Ungar, in press).

The failure of the ELISA to detect infections in specimens from 11 individuals (10 Peruvians) with fewer than five oocysts per 0.01 ml of concentrated fecal sample may suggest that these specimens contained antigen that was inaccessible to or not recognized by the detecting polyclonal antibodies. These polyclonal antibodies were raised to sonicated *Cryptosporidium* oocysts and therefore to both sporozoites and oocyst wall components. In an infected individual, different antigens may be present and detectable at different life cycle stages of the parasite. Another possibility, particularly in the Peruvian patients, is that *Cryptosporidium* isolates are antigenically distinct from the isolates from the United States and used to produce antisera. Another possibility is that Peruvian patients did not have *Cryptosporidium parvum* infection at all but rather ingested, perhaps through a contaminated water supply, another, possibly antigenically distinct *Cryptosporidium* isolate, such as *C. baileyi*. Some specimens from Peru may have thawed during transport and lost recognizable antigen. Alternately, the inability of the ELISA to detect *Cryptosporidium* antigens from these 11 patients or generally from fewer than 1,000 to 2,000 purified oocysts may simply mean that these samples contained an amount of free antigen below the sensitivity of the assay.

Compared with at least two microscopic techniques to diagnose infection, in terms of personnel time and economy, this ELISA is quite practical. Samples are easy to prepare for the ELISA, although repeated freezing and thawing may adversely affect ELISA reproducibility over time for some positive, but not negative, specimens. A limiting feature may be the inability to use this ELISA when specimens are preserved in Formalin, although this problem has been circumvented in later generations of some other ELISAs (20, 26).

Several studies have now identified *Cryptosporidium* antigens, particularly those in the 20- to 27-kDa molecular mass range, which may be immunogenic in most infected humans (16, 28) and which may be unique to *C. parvum*, the isolate which infects humans (14, 27). Future ELISAs to detect *Cryptosporidium* antigens may be also improved by using polyclonal or monoclonal antibodies raised to such an antigen or by using a pool of monoclonal antibodies with different specifications.

In summary, the ELISA described here is a simple, diagnostic test for *Cryptosporidium* antigen that can be used as a single test in place of two or more microscopic techniques conventionally used. The assay may eliminate some of the skill needed in performing complicated staining procedures and recognizing the morphology of the small *Cryptosporidium* oocyst, which is important as therapeutic modalities become available to treat cryptosporidiosis. This ELISA will be particularly useful in laboratories not accustomed to diagnosing cryptosporidiosis often, in epidemiologic studies in need of diagnostic standardization, and in situations when batch specimen processing may be crucial.

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