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***Cryptosporidium* Antigen Detection in Aids Patients with Diarrhea**

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Abstract: The microscopic detection of cryptosporidiosis is difficult, time-consuming and relatively insensitive, especially in patients with light infections. New techniques based on specific antibodies to *Cryptosporidium* antigens have become available. One method, an indirect immunofluorescence assay, has increased sensitivity over conventional microscopy. A more recent technique, an antigen detection ELISA, has not been adequately evaluated in a clinical setting. The purpose of this study was to compare the antigen detection ELISA to the quantitative IFA (QIFA) using stool samples from HIV-infected patients with diarrhea. The antigen detection test had a high degree of repeatability with a coefficient of variation of 0.08. This method compared very favorably with the QIFA and yielded a sensitivity of 96.4% and a specificity of 100%. The two tests gave comparable results when high numbers of oocysts were present. In contrast, the antigen detection assay was often positive for QIFA-negative samples from patients intermittently shedding oocysts. Hence, in the lighter infections, the antigen detection ELISA appeared to be more sensitive. The ELISA absorbance value was proportional to the number of oocysts present in the test as defined by QIFA. Antigen detection ELISA appears to be an effective alternative to immunofluorescence for detecting fecal oocyst excretion and may be particularly useful when large numbers of stools must be tested.

Key words: *Cryptosporidium*, aids patients, diarrhea

Introduction

Cryptosporidium parvum is an intracellular protozoan parasite, which infects the intestinal epithelium of animals and man (Tzipori, 1983; Kennedy *et al.*, 1977). The parasite life cycle is complex and involves both sexual and asexual stages (Current, 1989). Transmission of the parasite occurs when *C. parvum* oocysts from an infected animal (Current *et al.*, 1983) or person (Janoff *et al.*, 1990; Fafard and Lalonde, 1990) are ingested by a naive host. In some cases, community outbreaks of the infection have been linked to municipal drinking water (D'Antonio *et al.*, 1985; Hayes *et al.*, 1989) as in the U.S. outbreak in Milwaukee. Oocysts, which are resistant to disinfection and chlorination (Angus *et al.*, 1882; Campbell *et al.*, 1982), are incompletely removed by water treatment and filtration (Madore *et al.*, 1987). Also, treated water can become contaminated with untreated water during periodic heavy rainfall and flooding. Another mode of transmission, person-to-person contact, is suspected of causing cryptosporidiosis in day care centers, where it is especially prevalent, as well as in other institutions housing children (Janoff *et al.*, 1990; Fafard and Lalonde, 1990). *Cryptosporidium* is also a recognized cause of traveler's diarrhea (Soave and Ma, 1985).

In immunocompetent individuals, the parasite can cause a self-limited, asymptomatic infection or symptoms which range from a mild to severe. A 7-day incubation period is typically followed by an illness of 2-14 days. The most commonly reported symptoms include diarrhea, nausea and vomiting, abdominal pain and low-grade fever (Jokipii and Jokipii, 1986; Elsser *et al.*, 1986; Fayer and Ungar, 1986). However, infectious oocysts may be excreted for an additional 1-2 weeks after symptoms resolve (Jokipii and Jokipii, 1986). In contrast, immunosuppressed patients with CD4 counts <200/mm³ may have a more severe and chronic disease, which in some cases may be life-threatening (Wolfson *et al.*, 1985; Current *et al.*, 1983).

In many laboratories the diagnosis of *Cryptosporidium* infection currently relies on the microscopic examination of stool samples. Various stool concentration methods and staining techniques, such as the DMSO-modified acid fast stain, are typically employed (Bronsdon, 1984; Anderson, 1981). However, with acid fast stains *Cryptosporidium* can be

confused with other acid fast organisms or with comparably sized yeasts. Evidence also suggests that staining methods are relatively insensitive and that detection requires a large number of organisms to be present, especially in formed stools (Weber *et al.*, 1991).

An indirect immunofluorescence assay (IFA, Meridian Diagnostics, Cincinnati, OH) based on a oocyst, wall-specific monoclonal antibody (Sterling and Arrowood, 1986) has provided enhanced sensitivity and specificity over other techniques (Garcia *et al.*, 1987; Rusnak *et al.*, 1989). Moreover, a direct immunoassay, which requires less incubation time, has replaced the earlier indirect method. We have adapted this method to a quantitative immunofluorescence assay (QIFA) technique for detecting *Cryptosporidium* oocysts. Our data indicate that in HIV-infected patients oocyst excretion is relatively constant from stool to stool and day to day in the same person. However, intensity of infection varies widely from patient to patient ranging from a mean daily oocyst concentration of 2×10^3 /ml to 2.3×10^6 /ml (Goodgame *et al.*, 1993). Patients with very light infections, i.e. excreting fewer than 10^4 oocysts/ml, may pose a diagnostic problem unless the more sensitive techniques are employed.

Improvement in sensitivity may come with the development of new antigen capture techniques, which could be designed to detect soluble and particulate fractions, as well as intact oocysts detected by previous methods. An ELISA-based assay would also provide a less time-consuming and more objective method of *Cryptosporidium* detection. One recent study employed a monoclonal antibody to a 40 kDa cyst wall antigen to detect *Cryptosporidium* in bovine feces (Anusz *et al.*, 1990). The sensitivity of the antigen capture assay exceeded that of conventional stains but was less sensitive than IFA.

The purpose of this study was to compare a commercially-available antigen detection EUSA assay to the quantitative IFA using specimens from HIV-infected individuals. Secondly, in samples positive by both tests, EUSA absorbance values were compared to the number of oocysts present in the micro titer well. And lastly, stools from individuals intermittently shedding oocysts were employed to evaluate the sensitivity of the EUSA assay for low level infections.

Materials and Methods

Four hundred eighty two stool samples were collected from 34 HIV-infected individuals who presented at a local clinic with diarrhea. Stools were weighed and an aliquot was preserved in 10% buffered formalin at a 1:4 dilution. Preserved specimens were stored at 4°C before QIFA testing. Samples were then stored frozen at -90°C until further use.

Samples were divided into 3 groups: 1) 307 stool sample *Cryptosporidium*-positive by QIFA, 2) 15 stool samples from a group of patients with microsporidia infection confirmed by electron microscopy and negative for *Cryptosporidium* by modified acid fast staining, QIFA and colon and small bowel biopsy and 3) 160 stool samples negative by QIFA but from symptomatic patients, some of whom were previously positive (by QIFA) for *Cryptosporidium*.

A commercial kit was used for quantitative immunofluorescence assay as previously described (MeriFluor *Cryptosporidium* Kit, Meridian Diagnostic, Inc., Cincinnati, OH) (Goodgame *et al.*, 1993). In this assay a monoclonal antibody to a *Cryptosporidium* oocyst wall antigen was used to visualize the oocyst via a fluorescein isothiocyanate-labeled conjugated second antibody. Apple green fluorescence and characteristic morphology indicated the presence of an intact oocyst. A 5 µL aliquot of preserved stool was examined; each stool was assayed in triplicate. Oocyst concentration was calculated by counting oocysts in a defined area and correcting for the proportion of the slide examined. Frozen samples were thawed and thoroughly mixed before testing. The Color Vue-Cryptosporidium Kit (Seradyn, Inc., Indianapolis, IN) was used to detect *Cryptosporidium* antigen in stool samples. All reagents used in the following procedure were provided in the kit. This assay employed a double-antibody "sandwich" ELISA performed in a microtiter format. One hundred µL of negative or positive control samples or 50 µL of preserved fecal samples were added to microtiter wells in a plate coated with rabbit anti-*Cryptosporidium* antiserum. The plate was incubated at 37°C for 45 minutes and subsequently washed three times with a phosphate buffered wash solution (supplied with the kit) using a Titertek Microplate Washer S8/12 (Flow Laboratories Inc., McLean, VA). A murine monoclonal antibody to oocyst wall antigen (100 µL) was then added to each well and incubated at 37°C for 20 minutes. Plates were washed free of unbound antibody and 100 µL of biotin-conjugated anti-mouse IgG was added to each well and incubated 10 minutes at 37°C. Following a thorough wash, 100 µL of streptavidin-conjugated horseradish peroxidase was added to each well and incubated 10 minutes at 37°C. Wells were again washed prior to the addition of 50 µL of H₂O₂-activated tetramethylbenzidine solution. The plate was incubated at room temperature for 10 minutes. The reaction was terminated by the addition of 50 µL of phosphoric acid solution and the plates were read at 450 nm by a Microplate Autoreader (Model, Elio Tek Instrument, Inc., Winooski, VT). Positive results were defined as any absorbance value that exceeded 2 SD above the mean negative control.

Results

The antigen detection ELISA assay was standardized using positive and negative control samples. To assess assay variability, 6 replicates of positive and negative control samples were tested in a single plate (Table 1). For plate-to-plate variation, duplicate positive and negative controls (contained in test kit) were included in assays done on 15 different days over a period of 6 months. Well-to-well coefficient of variation was remarkably low, 2.9% and 8.0%, for the positive and negative samples, respectively. A greater degree of variation, 0.354 and 0.264, was seen in the positive

Table 1: Variability in the *Cryptosporidium* antigen detection assay. The mean absorbance values (A450), the standard deviation (SD), coefficient of variation (CV), and the number of trials are given for positive and negative control reagents

	Well-to-well		Plates-to-Plates*	
	Positive Control	Negative Control	Positive Control	Negative Control
Mean A450	1.208	0.087	1.163	0.131
SD	0.035	0.007	0.411	0.035
CV	0.029	0.08	0.354	0.264
N-	6	6	15	15

*Plates were run on different days over a period of 3 months

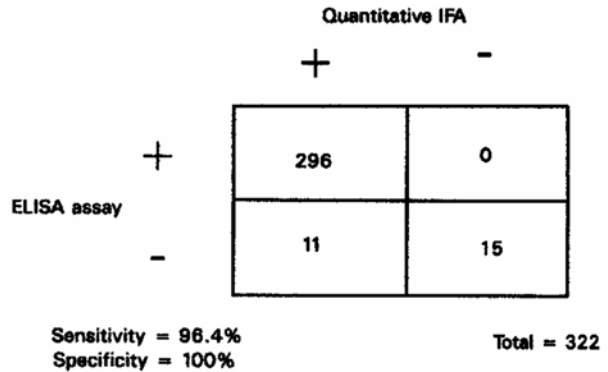


Fig. 1: Comparison of the antigen detection (ELISA) assay to the quantitative immunofluorescence assay (IFA). Three hundred seven stools confirmed positive and 15 stools confirmed negative for *Cryptosporidium* were assessed by both methods. Sensitivity and specificity of the antigen detection assay are indicated

and negative control samples tested on different days. Absorbance values of the positive or negative controls differed as much as 2-fold between some days. This may have been the result of aging of reagents; however, no consistent pattern was seen over the 3 month period of study. It should be noted that even in 2 plates where absorbance values of the positive controls were lower than usual, there was still a 5-fold difference between positive and negative controls. These data suggest that controls should be included at the time of each assay. From among the 482 specimens, 108 of the stool samples were tested in the antigen detection assay 2-3 times. Repeat assays on 60 (65%) of the samples yielded the same result; whereas, discrepancies, i.e. positive versus negative results, were seen between repeat tests in 48 (35%) of the samples. All of the discrepancies, however, were found in samples with low oocyst counts (< 10⁴/50 µL). These data suggest that in those specimens with low oocyst concentrations, sampling of the fecal specimen did not always retrieve a detectable number of oocysts. This non-uniformity of results in low level infections was also noted with the QIFA. The sensitivity and specificity of the *Cryptosporidium* antigen assay was assessed by testing samples previously confirmed to be positive (14 = 307) or negative (N=15) (Fig. 1). Negative samples were from HIV-infected patients with confirmed microsporidiosis. The antigen assay performed well with a sensitivity of 96.4% and a specificity of 100%. There were 11 QIFA-positive samples that the antigen assay did not detect. Seven of these 11 had oocyst counts with a mean and median level of 1.2 X 10⁴ and 5.3 X 10³ per ml, respectively. The remaining 4 had higher oocyst counts (mean = 5.8 X 10⁵ per ml). Since the 322 samples in this analysis were collected

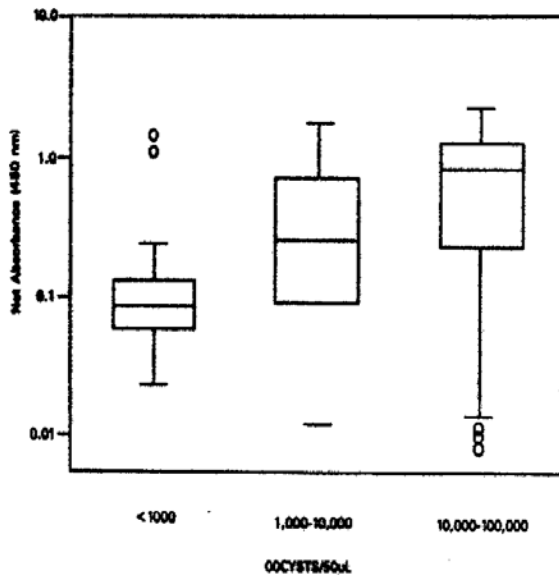


Fig. 2: Comparison of antigen assay absorbance values to the number of oocysts present as defined by quantitative immunofluorescence. Samples were grouped according to infection intensity: <1000 oocysts/50 µL (light infection), 1,000-10,000 oocysts/50 µL (moderate infection) and 10,000-100 oocysts/50 µL (heavy infection). Each box represents the 25-75% quartiles; the median value for each group is shown as the line bisecting the box. The 99% confidence interval is indicated by the vertical lines, rid outliers are indicated by circles

from 30 patients, the data were also evaluated on a per patient basis. Results were similar to the "per specimen" analysis. The relationship of the EUSA abs barb value to the oocyst concentration was examined (Williamson *et al.*, 1989). The number of oocysts in the Miatgitif well was estimated from the QIFA results. For the 294 samples positive by both tests, the number of oocysts per 50 µL was compared to the absorbance value generated in the antigen detection assay. ELISA absorbance values were plotted according to the intensity of infection, which was divided into three groups: $< 2 \times 10^4$ oocysts/ml. (light infection), $2 \times 10^4 - 1 \times 10^5$ oocysts/ml. (moderate infection) and $2 \times 10^5 - 1 \times 10^6$ oocysts/ml. (heavy infection). The number of samples in each group was 43, 158 and 97, respectively. A boxplot (Fig. 2) displays the 25th and 75th quartile (rectangle), the median (line bisecting rectangle and the approximate 99% lie (vertical line) for each group. Outliers are indicated by circles. A positive relationship can be seen in the intensity of infection (number of oocysts present) and the absorbance value worsened in the EUSA assay. A separate group (N = 180) of QIFA-negative samples was tested for the presence of *Cryptosporidium* antigen. One hundred thirty four of these samples were collected from patients that were intermittently positive by QIFA. Twenty six samples were from symptomatic individuals that were QIFA-negative on multiple stool specimens. One hundred five (65.6%) of the temples were positive by the andiron detection assay. These data suggest that the antigen detection assay may be a more sensitive Indicator of infection than the QIFA test.

Discussion

Cryptosporidiosis is now recognised as an important cause of daintiest illness, especially in the immunocompromised, in day

care centers and in traveler's diarrhea. Outbreaks due to drinking water contamination have also been documented. In immunocompromised individuals *Cryptosporidium* infection can be especially serious and even life-threatening. In the past, diagnosis of the disease has been difficult and has required special staining procedures. Documentation of infection in persons with low level infections has been especially problematic. The QIFA detection system is more sensitive than conventional staining procedures, including modified acid fast stains. This approach, however, is time consuming and requires fluorescence microscopy. An EUSA-based assay, such as the antigen detection test reported here, has the advantage of detecting parasite products in addition to whole oocysts and the enzyme-conjugated reporter antibody amplifies the signs several fold. Also, many specimens can be tested on the same plates.

An earlier study (Anusz *et al.*, 1990) found that the ELISA assay was less sensitive than the IFA. In that study bovine feces were seeded with known numbers of oocysts and directly tested in the ELISA. In our experience we have found that if the sample is frozen and thawed before the ELISA assay, the absorbance value is increased 2.3-fold (data not shown). This result is presumably due to the disruption of oocysts and disbursement of antigenic material. Thus, a freeze/thaw step should increase EUSA sensitivity.

To compare the antigen detection assay to the QIFA, well-defined populations were chosen. Patients were first screened for *Cryptosporidium* infection by standard staining techniques at the hospital laboratory (Ben Taub General Hospital, Houston, TX). Patients who tested positive were considered as having a confirmed diagnosis. A small group of patients who went negative for *Cryptosporidium* but symptomatic, were studied extensively and confirmed to have microsporidia infection only. These patients became the negative control group. The antigen detection assay in the above groups compared favorably to the QIFA with a sensitivity of 95.4% and a specificity of 100% (Fig. 1).

The antigen detection assay was evaluated for test repeatability by using the negative and positive control reagents that come with the kit. A minor degree of well-to-well variation (CV = 0.08 or less) was seen within each of the 6 microtiter plates tested (Table 1). A higher variation in absorbance values was seen from day-to-day. That is, negative and positive controls run on one plate may vary as much as 35.4% from values generated in a separate plate on a different day. These data indicate the importance of including positive and negative control wells with each test. Repeat assays on 108 stool samples revealed discrepancies in 35% of these specimens. However, all of the discrepant samples were from lightly infected patients as defined by QIFA and may represent sampling variations. It is likely that most, if not all, of these samples would have been negative by acid fast staining. The QIFA results were based on a mean of 3 separate measurements. In low level infections, the majority (approximately 75%) of stools assessed by QIFA also showed discrepancies among the aliquots tested. In some cases only 1 of the 3 aliquots contained a single oocyst. If any oocysts were seen in any of the WAN measurements, the sample was considered even. Antigen assays were also considered positive if any of the replicates showed positive absorbance values. Thus, both tests have problems detecting oocysts or antigens when working near the lower limit of detection sensitivity. This likely reflects the non-uniformity of oocyst numbers in store aliquots. Hence, duplicate or triplicate assays should be performed on symptomatic patients that are initially negative in either the QIFA or antigen detection assays. It should be noted, however, that the clinical significance of light infections is not clear.

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The association between absorbance value (antigen assay) and the estimated number of oocysts (QIFA) suggests that the absorbance value of a sample may be used to estimate the intensity of the infection, i.e. high, moderate or low level. It was not, however, indicative of the exact number of oocysts present within each range.

It should be noted that 1 patient with *Cryptosporidium* infection had tools uniformly negative in the antigen detection assay despite having a high number of oocysts in the QIFA. The earns monoclonal antibody employed in the antigen detection test was positive when tested by IFA. There may be some unknown factor in the stool that interfered with the antigen assay. Coating antibody could have been adversely affected by pH but this could not be evaluated since the stools were diluted in buffered preservative solution. To our knowledge this patient is not receiving any unusual treatment or dietary supplement that would account for the results. Subsequent to the collection of these stool., the patient developed cytomegalovirus colitis with associated gastrointestinal bleeding. It is possible that excessive blood in the stool may have interfered with the assay.

In summary, the antigen detection assay appeared to be more sensitive and was easier to use than the QIFA procedure. Although both methods have similar incubation times, the antigen detection assay in the microliter format allows many samples to be screened at one time and provides a numerical (objective) assessment of the results.

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