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Immunofluorescence Detection of *Cryptosporidium* Oocysts in Fecal Smears

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An indirect fluorescent antibody (IFA) procedure was developed for the detection of *Cryptosporidium* sp. oocysts in human, nonhuman primate, and bovine fecal smears. The procedure, which takes about 90 min to perform, involves the use of a rabbit antiserum against *Cryptosporidium* oocysts isolated from dairy cattle. Cross-specificity testing of the IFA method revealed no reactivity with yeasts, various amoebae, *Giardia lamblia*, *Chilomastix* sp., or *Blastocystis* sp. and only very weak cross-reactivity with coccidian oocysts of other genera. IFA detection of oocysts in human and nonhuman primate fecal smears was far more sensitive than was dimethyl sulfoxide-carbolfuchsin staining. Moreover, IFA detection was comparable in sensitivity to auramine O staining with samples of high oocyst concentration and somewhat more sensitive than auramine O with samples containing relatively few oocysts. The IFA procedure may be useful in the clinical diagnosis of human and animal cryptosporidiosis and also in the detection of oocysts in environmental samples.

Cryptosporidium sp. is a coccidian parasite of humans and animals and an agent of acute enterocolitis (9, 24, 34). Cases of human infection involving the respiratory tract have also been reported (19, 23). Although formerly believed to be strictly a zoonosis, cryptosporidiosis is now believed to be acquired also through person-to-person transmission (2, 3, 6, 8). The prevalence of detectable *Cryptosporidium* infection in the human population as a whole is very low—probably below 1%—although no survey of the general population has ever been published. However, in patients with gastrointestinal symptoms the prevalence of infection has been reported to range from 1.2 to 9% in developed countries (12, 14, 16, 17, 25, 33; D. P. Casemore, M. Armstrong, and B. Jackson, Letter, Lancet i:734–735, 1984; D. P. Casemore and B. Jackson, Letter, Lancet ii:679, 1983) and from 4 to 13% in developing countries (4, 20, 21, 28, 35; N. Hojlyng, K. Molbak, S. Jepsen, and A. P. Hansson, Letter, Lancet i:734, 1984). A number of reports have shown that the prevalence of infection is generally higher in children than in adults (4, 6, 16, 25, 28, 33; Casemore et al., Letter, 1984; Casemore and Jackson, Letter, 1983) and also higher among children in urban areas than in rural areas (20; Hojlyng et al., Letter, 1984). A number of outbreaks of cryptosporidiosis were reported recently in day-care centers in the United States (7, 32). In patients with acquired immunodeficiency syndrome or other immunodeficient or immunodepressed states, *Cryptosporidium* infection is a serious complication and may result in a life-threatening loss of fluids and electrolytes (8, 18, 22, 29, 36).

Cryptosporidium infection is diagnosed by identifying the oocyst stage of the parasite in host feces or biopsy tissue. Oocysts are most commonly identified in clinical laboratories by acid-fast staining of fecal smears or concentrates with carbolfuchsin (cold Kinyoun stain, dimethyl sulfoxide [DMSO]-carbolfuchsin, or Ziehl-Neelsen stain) (5, 11, 13, 18, 19, 23), by fluorescence staining with auramine-phenol (11, 25, 32, 35; Casemore et al., Letter, 1984; G. Nichols and B. T. Thom, Letter, Lancet i:735, 1984), or by oocyst flotation on Sheather sucrose solution followed by phase microscopy (10, 18). Other effective methods of oocyst detection are also used, including safranin staining (4, 21; D. Baxby and N. Blundell, Letter, Lancet ii:1149, 1983), iodine staining (18), Hemacolor staining (19), methylene blue-eosin staining (26), Giemsa staining (20, 33), negative staining by periodic acid-Schiff reagent (15), and electron microscopy (1). The use of combinations of some of the above methods of oocyst detection is commonly reported. Garcia et al. (11) compared 15 methods of oocyst detection and concluded that a modified (hot) Ziehl-Neelsen staining of 10% Formalin-preserved stool is superior to all other methods tried (safranin was not tested). Bronsdon (5) reported that the DMSO-carbolfuchsin staining method was only slightly less sensitive than auramine staining in detecting oocysts in stool smears; auramine seemed more sensitive for detecting oocysts in smears containing very few oocysts. Ma and Soave (18) recommend a three-step stool examination method for diagnosing cryptosporidiosis, involving an iodine-stained wet mount followed by Kinyoun acid-fast staining and cover slip flotation of oocysts in Sheather sucrose. Ma and Soave also conclude that Sheather sucrose flotation is more effective at concentrating oocysts than is Formalin-ether sedimentation.

Fluorescent antibody (FA) detection is another potentially specific and sensitive way of visualizing *Cryptosporidium* oocysts in fecal smears or concentrates. An FA method tends to have the advantage of high sensitivity, provided that the parasite antigens are stable in stool preparations and that cross-reaction with other organisms does not occur. Sterling and colleagues (30, 31) recently showed that the detection of *Cryptosporidium* oocysts in fecal smears by direct immunofluorescence with a monoclonal antibody is a practical and specific method of diagnosis, although some cross-reactivity was obtained with certain fecal yeasts. An FA detection method has also been reported for the detection of *Giardia lamblia* cysts in fecal smears (27).

By using a rabbit antiserum prepared against *Cryptosporidium* oocysts of bovine origin, we tested the sensitivity and specificity of an indirect FA (IFA) method of detecting *Cryptosporidium* oocysts in fecal smears of human, nonhuman primate, and bovine origin and quantitatively compared

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the sensitivity of this method to that of the auramine and DMSO-carbolfuchsin staining techniques.

MATERIALS AND METHODS

The oocysts used in the preparation of rabbit antiserum were isolated from the manure of Holstein calves (7 to 15 days old) which had previously been confirmed as *Cryptosporidium* infected by DMSO-carbolfuchsin staining (5) of fecal smears. Cattle herds from which manure specimens were obtained were located in the valleys of the Snoqualmie, Skykomish, and Snohomish rivers, approximately 30 miles east by northeast of Seattle, Wash. The oocysts were purified by the following steps: (i) removal of crude particles from manure by filtering an aqueous suspension of manure through several layers of gauze or cheesecloth; (ii) vacuum filtration through brass soil sieves (diameter, 54 mm; pore sizes, 0.5 mm and 125 μm); (iii) passing the filtrate obtained as described above through a 400-mesh nylon net (pore size, 50 μm); (iv) centrifuging the filtrate at 500 × *g* for 5 min; (v) washing the sediment three times with distilled water; (vi) suspending the pellet in distilled water and extracting fats, etc., up to five times by shaking the suspension for 30 s with an approximately 1/10 volume of ethyl acetate, centrifuging after each extraction for 5 min at 500 × *g* (the extracted material was discarded); (vii) floating the oocysts present in the sediment on Sheather sucrose solution (see reference 18 for details); (viii) washing the recovered oocysts three times with distilled water; and (ix) centrifuging the oocysts for 15 min at approximately 650 × *g* over a step-gradient of Percoll (Sigma Chemical Co., St. Louis, Mo.) in water (layers of specific gravity 1.13, 1.09, 1.05, and 1.01) prepared in a 12-ml conical centrifuge tube, followed by removal of the oocyst band from above the 1.09 and 1.13 layers and washing of the oocysts three times with distilled water. After step v, the oocysts were usually suspended and stored in 1% Formalin in phosphate-buffered saline (PBS; pH 7.6) to prevent losses from possible excystation of oocysts and bacterial or fungal overgrowth. The use of rhodamine-albumin (Difco Laboratories, Detroit, Mich.) counterstain in immunofluorescence tests (see below) later revealed that these oocyst preparations were virtually free of yeasts but did contain a small amount of unidentified enteric bacilli. Purification beyond this point was not attempted.

Antioocyst serum was prepared in two adult New Zealand white rabbits by initial intradermal inoculation, at multiple sites, of oocysts mixed 1:1 with Freund complete adjuvant (3×10^6 oocysts per rabbit) followed by intramuscular booster inoculations of 2×10^6 to 5×10^6 oocysts per rabbit in incomplete Freund adjuvant at 3, 6, and 8 weeks after the initial inoculation. The rabbits were bled by cardiac puncture under methoxyflurane anesthesia 5 days after the last booster injection. The serum was stored at −20°C.

Human, nonhuman primate, and bovine fecal specimens for IFA testing were obtained from the following sources. A monkey fecal specimen containing low numbers of *Cryptosporidium* oocysts was obtained from a formula-fed, orphaned infant pig-tailed macaque (*Macaca nemestrina*) through the courtesy of Melinda Bronsdon of the Regional Primate Center, University of Washington, Seattle. This specimen had been stored at 4°C for 8 days before use. A human-source specimen containing *Cryptosporidium* oocysts was obtained through the courtesy of Carolyn Wallis of the Clinical Microbiology Laboratory, Harborview Medical Center, Seattle, Wash., and was stored in 2.5% potassium dichromate for 6 weeks at 4°C before use. This sample was then washed twice with distilled water, pelleted by centrifugation, and suspended in distilled water immediately before use. This specimen was used in the quantitative comparison of oocyst detection methods. Another *Cryptosporidium*-positive human stool specimen was obtained through the courtesy of Sam Eng of the Clinical Microbiology Laboratory, University Hospital, Seattle, Wash., and used within 1 day of collection without dilution in potassium dichromate. Bovine-source *Cryptosporidium*-positive fecal specimens were obtained from Holstein calves as described above and stored for less than 1 week before use. Thin smears of each of the above specimens were prepared on alcohol-cleaned microscope slides (1 by 3 in. [2.54 by 6.62 cm]) by using a cotton-tipped applicator.

Staining of the fecal smears was performed in the following manner. DMSO-carbolfuchsin (acid-fast) staining was done by the method of Bronsdon (5), except that staining with the carbolfuchsin was done for 5 min and staining with the counterstain was done for 2 min. The slides were flamed momentarily before being fixed in the methanol. Prepared solutions of the DMSO-carbolfuchsin and the malachite green counterstain were obtained from Prepared Media Laboratories, Tualatin, Oreg. These reagents had been previously found to give excellent staining results with *Cryptosporidium* oocysts in fecal smears. Staining with auramine O-phenol was done by briefly heat-fixing the smeared slides and then immersing the slides in 0.1% auramine O in 9.5% ethanol–3% phenol for 15 min. After being destained for 2 min in a solution of 0.5% HCl–70% ethanol, the slides were rinsed briefly with tap water and counterstained in 0.5% aqueous potassium permanganate for 3 min. The slides were then briefly rinsed with tap water and air dried. Auramine O powder was obtained from Sigma. The immunofluorescence staining procedure was done as follows. The fecal smears were air dried and then fixed in acetone for 5 min. Acetone-fixed slides were stored until use at −20°C in airtight plastic slide boxes containing desiccant. At the time of use, the smears were flooded with immune rabbit anti-*Cryptosporidium* serum or with preimmune serum diluted 1:40 with PBS (pH 7.6) and incubated in a humid chamber at 37°C for 30 min. Afterward, the slides were rinsed three times, 5 min per rinse, in PBS and air dried. Fluorescein-conjugated goat anti-rabbit immunoglobulin G antiserum (anti-heavy and -light chains; Cooper Biomedical, Inc., Malvern, Pa.) was then applied to the slides at a 1:40 dilution in PBS, and the slides were incubated as before. Rhodamine-albumin counterstain (Difco) was sometimes included at a 1:80 dilution with the conjugated second antiserum. After three final rinses in PBS, the slides were air dried and mounted with a cover glass (25 by 60 mm) and a mounting medium consisting of 90% glycerol in PBS with *p*-phenylenediamine at 0.1 mg/ml. The IFA slides were examined at ×400 magnification with a fluorescence microscope (Carl Zeiss Inc., Thornwood, N.Y.) with epiillumination. Photos were taken at ×190 magnification on Ektachrome 160 tungsten slide film (Eastman Kodak Co., Rochester, N.Y.).

The relative sensitivity of the three methods of oocyst staining was determined by using smears made from both the monkey fecal specimen and the dichromate-preserved human fecal specimen. With each specimen, 15 thin, uniform fecal smears were prepared on alcohol-cleaned microscope slides by using either a second slide (human specimen) or a cotton-tipped applicator (monkey specimen) for smearing. The cotton-tipped applicator was used for the monkey specimen because the stool was formed and thick in consis-

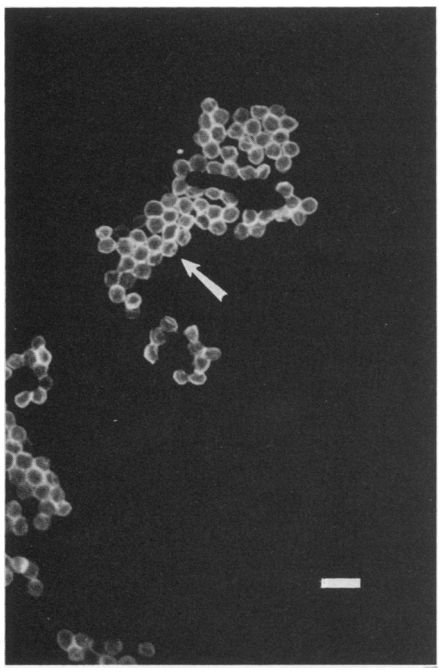

FIG. 1. *Cryptosporidium* oocysts, purified from cattle feces, on the surface of a polycarbonate filter (pore size, 1.0 μm; Nuclepore Corp., Pleasanton, Calif.), as revealed by indirect immunofluorescence. Arrow indicates oocyst. Bar = 10 μm.

tency. Five slides of each specimen were prepared for staining by each method. The number of oocysts on each slide was determined by counting the oocysts visible per field in 27 fields (human specimen) or 150 fields (monkey specimen) examined per slide under ×400 magnification. Bright-field microscopy was used for the carbolfuchsin-stained slides; epifluorescence microscopy was used for the auramine- and IFA-stained slides. The fields counted on each slide were divided evenly among the left, middle, and right thirds of the slide area. Differences between the mean numbers of oocysts counted per slide by each staining method were compared statistically by using a one-tailed Student *t* test.

RESULTS

The antioocyst immune serum bound strongly and specifically to purified *Cryptosporidium* oocysts of bovine origin by indirect immunofluorescence (Fig. 1). Strong reactions were obtained at antiserum dilutions of 1:20, 1:40, and 1:60. At a 1:80 dilution, fluorescence decreased noticeably but was still apparent. Preimmune rabbit serum showed no reaction with oocysts (data not shown). Fluorescence obtained by using the immune serum emanated primarily from the walls of the oocysts. No binding of antiserum to internal components was observed. Binding of antiserum to excysted sporozoites was not tested. Immunofluorescence tests also revealed that the purified bovine-source oocysts used in immunization contained small numbers of bacilli. These bacteria did not bind antiserum but were revealed by the rhodamine counterstain.

The immunofluorescence procedure clearly detected *Cryptosporidium* oocysts in human, monkey, and bovine fecal smears (Fig. 2). The human fecal specimen shown in Fig. 2A was the one not stored in potassium dichromate. *Cryptosporidium* oocysts were virtually the only objects in the fecal smears that were bound by the antiserum. The oocysts observed in all samples were of uniform size (3 to 5 μm in diameter), indicating that the species was probably *Cryptosporidium parvum* in each specimen (34).

Immunofluorescence cross-reactivity testing (with the same dilutions of primary and secondary antisera as noted above) revealed no binding of the antioocyst immune serum to cultured *Candida albicans* yeasts; to human-source *G. lamblia*, *Entamoeba histolytica*, *Entamoeba coli*, or *Endolimax nana* cysts; to *Giardia* sp., *Chilomastix* sp., and *Entamoeba* sp. cysts isolated from a muskrat (*Ondatra zibethica*); to *Blastocystis hominis* cells from a human stool

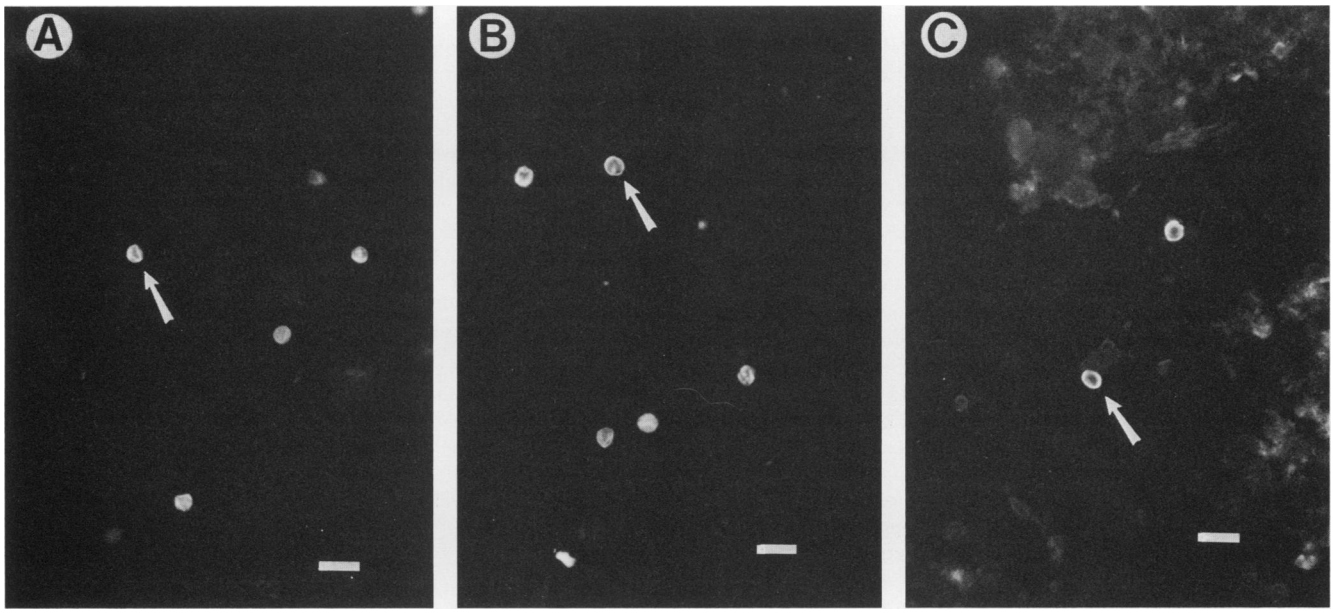

FIG. 2. *Cryptosporidium* oocysts visualized by immunofluorescence in fecal smears of human (A), monkey (B), and cattle (C) origin. Arrows indicate oocysts. Bars = 10 μm.

TABLE 1. Comparison of the sensitivity of IFA, DMSO-carbolfuchsin, and auramine methods in detecting *Cryptosporidium* oocysts

Source and method	Oocyst count		
	$\bar{x}$ Oocysts/slide	SD[a]	P[b]
Human feces			
IFA	210.0	24.9	
Auramine	204.6	35.9	NS[c]
DMSO-carbolfuchsin	15.6	8.3	<0.005
Monkey feces			
IFA	21.6	5.8	
Auramine	8.8	4.6	<0.005
DMSO-carbolfuchsin	4.0	2.8	<0.0005

[a] Standard deviation of the oocyst counts per slide.
[b] From Student's t test one-tailed comparison of mean oocyst counts per slide with mean counts obtained by IFA method; differences considered significant if $P < 0.05$.
[c] NS, Not significant at the 95% confidence level.

specimen; or to yeast cells obtained directly from the small intestine of a laboratory mouse. However, weak-positive immunofluorescence did result with *Eimeria* sp. oocysts derived from cattle (provided by Richard Wescott, Washington State University, Pullman) and with *Caryospora* sp. oocysts derived from a timber rattlesnake (provided by Donald D. Smith, University of Kansas Medical Center, Kansas City).

With both of the fecal specimens used in the sensitivity comparison of the staining methods, the IFA method was much superior to DMSO-carbolfuchsin staining (Table 1). With the human-derived specimen, IFA and auramine gave similar results and were not statistically different in sensitivity. However, with the monkey fecal specimen, in which oocysts were very sparse, the IFA method detected more than twice as many oocysts per slide as the auramine method did. The IFA method detected more than 13 times as many oocysts as did the carbolfuchsin method with the human specimen and more than five times as many with the monkey specimen. Auramine O was also 13 times more sensitive than carbolfuchsin with the human specimen ($P < 0.005$) and was more than twice as sensitive with the monkey specimen ($P < 0.05$). These differences were all statistically significant at or above the 95% confidence level.

DISCUSSION

Indirect immunofluorescence is clearly a sensitive and specific method for detecting *Cryptosporidium* oocysts in fecal smears from human and animal sources. The IFA method was substantially more sensitive in oocyst detection than was the DMSO-carbolfuchsin method of staining. The IFA method yielded results comparable to those of the auramine staining method in smears with high oocyst concentrations and was significantly ($P < 0.005$) more sensitive in smears with low oocyst concentrations.

The IFA method has an obvious advantage in sensitivity over the DMSO-carbolfuchsin staining method. However, it also has several disadvantages. Like the auramine method, it requires the use of a fluorescence microscope, an instrument that is not available in all clinical laboratories. Two other drawbacks of the IFA method are (i) the time (approximately 90 min) needed to perform the test and (ii) the lack of a commercially available antiserum against *Cryptosporidium* oocysts. The use of a direct immunofluorescence method of oocyst staining, employing antiserum conjugated directly to fluorescein, would shorten the procedural time considerably, perhaps to as little as 15 min, by eliminating the requirement for a second incubation. The development of monoclonal antibodies specific for *Cryptosporidium* oocysts, as reported recently by Sterling et al. (30, 31), would eliminate the need for laborious production of polyclonal antiserum and thus should facilitate the clinical application of immunofluorescence detection methods. However, cross-reactivity of monoclonal antibodies with fecal yeasts (as reported by Sterling and colleagues [30, 31]) or potentially with other organisms underscores the importance of extensive testing of the species specificity of antibody reagents and thorough documentation of any consistent patterns of cross-reactivity.

The cysts of most other parasitic protozoa are considerably larger than *Cryptosporidium* oocysts and generally possess characteristic anatomical features that allow rapid identification. Thus, FA methods for the identification of these other, larger protozoan cysts would probably not represent a significant improvement over current identification methods. *Cryptosporidium* oocysts, however, are small enough to be easily confused with yeasts or small *Blastocystis* cells unless special staining methods are used. In our experience, carbolfuchsin failed to stain some of the oocysts in a fecal specimen. Other researchers have reported similar observations (4, 35; Casemore et al., Letter, 1984; Nichols and Thom, Letter, 1984). Iodine or Giemsa staining, negative staining by periodic acid-Schiff reagent, and examination of unstained preparations floated on Sheather sucrose all seem to allow for some error or confusion on the part of inexperienced persons. In contrast, the IFA and auramine methods of oocyst staining are two of the most sensitive, specific, and foolproof methods available for oocyst detection. Because of its greater sensitivity, particularly for smears with low oocyst concentrations, the immunofluorescence method would seem to be the detection method of choice in studies on the prevalence of *Cryptosporidium* infection in human or animal populations. Additional applications of immunofluorescence detection of oocysts lie in the fields of environmental microbiology, environmental engineering, and municipal water and wastewater treatment, in which the technique can be used to detect and quantify *Cryptosporidium* oocysts in municipal water supply sources (J. E. Ongerth and H. H. Stibbs, submitted for publication). In summary, the indirect immunofluorescence method described here represents a useful new technique for *Cryptosporidium* identification.

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