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Comparison of Conventional Staining Methods and Monoclonal Antibody-Based Methods for *Cryptosporidium* Oocyst Detection

M. J. ARROWOOD† AND C. R. STERLING*

Department of Veterinary Science, University of Arizona, Tucson, Arizona 85721

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The sensitivity and specificity of seven microscopy-based *Cryptosporidium* oocyst detection methods were compared after application to unconcentrated fecal smears. The seven methods were as follows: (i) a commercial acid-fast (AF) stain (VOLU-SOL) method, (ii) Truant auramine-rhodamine (AR) stain method, (iii) fluorescein-conjugated C1B3 monoclonal antibody (MAb) direct fluorescence method, (iv) OW3 MAb indirect fluorescence method, (v) biotinylated OW3 indirect fluorescence method, (vi) biotinylated OW3-indirect diaminobenzidine (DAB) method, and (vii) biotinylated OW3-aminoethylcarbazole (AEC) method. A total of 281 randomly collected Formalin-fixed fecal samples (submitted to the Maricopa County Health Department, Phoenix, Ariz.) and 30 known positives (Formalin-fixed and $K_2Cr_2O_7$-preserved stools from our laboratory) were examined in a blind test; 32 of 311 samples (10.3%) were confirmed positive. Of the confirmed positives, 40.6% were identified by the AF method, 93.8% were identified by the AR method, 93.8% were identified by the C1B3 method, 81.3% were identified by the OW3-DAB method, 71.9% were identified by the OW3-AEC method, 100% were identified by the OW3 indirect fluorescence method, and 100% were identified by the biotinylated OW3 indirect fluorescence method. False-positives were encountered by the AF and AR methods (52.0 and 85.7% specificity, respectively), while no false-positives were encountered by the MAb-based methods. Oocysts in infected tissue sections were easily detected by the MAb-based methods.

Cryptosporidium parvum has been recognized as a significant cause of diarrhea in immunocompetent and immunocompromised humans (7). Clinical diagnosis of cryptosporidial infections has been primarily based on the detection of oocysts in stools. A variety of concentration and chemical staining methods have been reported (1, 3–6, 9, 11, 12, 15, 22, 31, 33). Recently, immunofluorescence methods (8, 25) have provided enhanced sensitivity and specificity over those of the conventional staining methods (14, 16, 27, 28), especially when oocyst numbers in stool specimens were low. Prevalence studies should particularly benefit from immunofluorescence assays, since asymptomatically infected individuals may shed oocysts in small numbers.

The present study was initiated to compare fluorescent- and nonfluorescent-monoclonal antibody (MAb)-based methods of oocyst detection with two conventional methods: acid-fast staining and auramine-rhodamine staining. In addition, the MAb-based methods were applied to *C. parvum*-infected intestinal tissue specimens to assay their utility in detecting oocysts in biopsy specimens.

MATERIALS AND METHODS

Specimens. Fecal samples (281 total) collected in 10% Formalin were obtained from the Maricopa County Health Laboratory (Phoenix, Ariz.). These specimens were submitted for examination on a random basis, with many patients exhibiting no diarrheal illness. Also, 30 positive controls (20 human and 10 calf stools) available in this laboratory were included with the above specimens, bringing the total to 311. Fecal specimens were randomly ordered and coded for examination in a blind fashion. Replicate fecal smears of each unconcentrated, vortexed fecal sample were prepared on microscope slides, heat fixed, and assayed by the seven oocyst detection methods described below (1 slide per method). Fecal smears of specimens containing potassium dichromate ($K_2Cr_2O_7$) were rinsed with 0.025 M phosphate-buffered saline (PBS) (pH 7.2) and air dried before proceeding with the assays.

C. parvum-infected tissue was obtained from experimentally infected neonatal BALB/c mice. Mice were infected at 4 days of age with 10^4 purified oocysts (2) and sacrificed 4 days later. The terminal ilea of infected mice were fixed in 10% buffered Formalin, embedded in paraffin, microtome sectioned (5 μm), and dried on albumin-coated microscope slides (23).

Fecal smear examinations. Bright-field and fluorescence observations of fecal smears (1 slide per smear for each detection method) were performed at ×200 and ×400 magnifications. The entire smear was examined to verify the absence of oocysts. When smears contained many oocysts, only a portion of the smear was examined. Oocyst numbers demonstrated per ×400 field were classified as follows: 1+ for <2 oocysts; 2+ for 3 to 5 oocysts; 3+ for 5 to 7 oocysts; and 4+ for >8 oocysts. Epifluorescence microscopy employed an Optiphot microscope (Nikon Inc., Garden City, N.Y.) equipped with a halogen UV light source, a 520-nm-wavelength barrier filter, a 510-nm-wavelength dichroic mirror, and a 450- to 490-nm-wavelength excitation filter.

Acid-fast staining of fecal oocysts. A commercially available acid-fast staining kit (VOLU-SOL; VOLU-SOL Medical Industries Inc., Las Vegas, Nev.) was applied as recommended to fecal smears. Briefly, the primary stain was applied at room temperature to the fecal smear for 2 min, rinsed off with tap H_2O, decolorized for 5 to 10 s, rinsed with tap H_2O, counterstained for 2 min, and rinsed with tap H_2O. After the smears were dried, they were coated with a thin layer of immersion oil and observed by bright-field microscopy.

Auramine-rhodamine staining of fecal oocysts. Acid-fast staining with auramine-rhodamine was based on Truant

* Corresponding author.
† Present address: Department of Animal, Dairy and Veterinary Science, Utah State University, Logan, UT 84322.

auramine-rhodamine stain (21). The stain was prepared by combining 1.5 g of auramine O, 0.75 g of rhodamine B, 75.0 ml of glycerol, 10.0 ml of liquified aqueous phenol (88% [wt/vol]), and 50.0 ml of distilled H_2O. Air-dried, heat-fixed fecal smears were stained for 15 min and rinsed with H_2O. Smears were decolorized for 2 to 3 min with 0.5% HCl (in 70% ethanol) and rinsed with H_2O. The smears were counterstained for 2 to 4 min with 0.5% potassium permanganate (in distilled H_2O), rinsed with H_2O, and air dried. Slides were examined by epifluorescence microscopy.

Direct immunofluorescence assay using MAb C1B3. MAb C1B3 directly conjugated with fluorescein isothiocyanate [FITC]) was employed as previously described (25). Briefly, the C1B3-FITC conjugate was diluted 1:100 with PBS and applied to fecal smears in 50-μl volumes. The slides were incubated at room temperature for 15 min in a humid chamber, rinsed three times with PBS (for over 9 min), mounted with PBS-glycerol (1:1) (pH 8.0), and covered with cover slips. Slides were observed by epifluorescence microscopy.

MAb OW3. Hybridoma OW3 (secreting MAb OW3, an immunoglobulin M [IgM]) was derived in this laboratory as previously described (8). MAb isotyping was performed by using biotinylated isotype-specific reagents (Zymed Laboratories, Burlingame, Calif.) in indirect immunofluorescence (IIF) assays. Oocyst specificity of MAb OW3 was determined in IIF assays against mammalian isolates (*C. parvum*) available in this laboratory, a mammalian isolate (*C. muris*) kindly supplied by Bruce Anderson (University of Idaho, Caldwell), an avian isolate (*C. baileyi*) kindly supplied by William Current (Lilly Laboratories, Greenfield, Ind.), and avian isolates from turkeys and quail kindly supplied by Byron Blagburn (Auburn University, Auburn, Ala.).

IIF assays. Undiluted OW3 culture supernatant or biotinylated OW3 (diluted in PBS) was applied to fecal smears (50 μl) and incubated for 30 min in a humid chamber. Slides were washed three times with PBS, and the excess PBS was wiped off. FITC-labeled goat anti-mouse IgM (American Qualex Inc., La Mirada, Calif.) or streptavidin-FITC (Zymed) diluted 1:100 in PBS was applied to the fecal smears (50 μl) and incubated for 30 min in a humid chamber. Slides were washed three times with PBS, mounted with PBS-glycerol, and covered with cover slips. All slides were observed by epifluorescence microscopy.

Purification of OW3. Ascitic tumors were produced in irradiated, pristane-primed BALB/c mice (10). Ascitic fluids were delipified with silicone dioxide (SiO_2; Cab-O-Sil M-5 scintillation grade; Eastman Kodak Co., Rochester, N.Y.) in Veronal-buffered saline (prepared from complement fixation test diluent tablets; Oxoid U.S.A. Inc., Columbia, Md.) and stored at −70°C (19). MAb OW3 was purified from ascitic fluid by polyethylene glycol precipitation (19). Briefly, a 17.5-ml volume of treated ascitic fluid was mixed with an equal volume of 13% polyethylene glycol (in Veronal-buffered saline) and immersed in an ice bath for 30 min. Precipitated immunoglobulin was recovered by centrifuging at 3,900 × *g* for 20 min, decanting the supernatant, and dissolving the pellet into 8 ml of acetate buffer (100 mM sodium acetate, 100 mM NaCl [pH 5.5]). Protein concentration was determined by the BCA assay (Pierce Chemical Co., Rockford, Ill.). The acetate buffer volume was adjusted to 12 ml (protein concentration of 1.53 mg/ml) and placed in an ice bath.

Biotin hydrazide labeling of OW3. Antibody labeling with biotin hydrazide (Molecular Probes Inc., Eugene, Oreg.) was adapted from a method for labeling oligosaccharide moieties on immunoglobulins (20). Sodium *m*-periodate was added to the purified OW3 solution (on ice) to a final concentration of 10 mM. Oxidation proceeded on ice for 20 min. The mixture (6 ml) was applied to a Sephadex G-25 (Sigma Chemical Co., St. Louis, Mo.) column (15-ml bed volume, 19-cm length) equilibrated with acetate buffer. The oxidized protein peak (minus periodate) was collected into a volume of 7 ml from the column eluent. Solid biotin hydrazide was added to 3.5 ml of the oxidized OW3 preparation, yielding a final concentration of 10 mM. The mixture was reacted in the dark on a rotating tray (500 rpm) at room temperature for 2 h. The reaction mixture was applied to a Sephadex G-25 column (15-ml bed volume, 19-cm length) equilibrated with PBS-azide (PBS supplemented with 0.02% sodium azide). The protein peak was collected in a 4-ml eluent volume which followed the 6.5-ml void volume. The OW3-biotin preparation was split into two portions; one was stored at 4°C, and the second was mixed with an equal volume of glycerol and stored at −20°C. The OW3-biotin titer was determined by an IIF assay.

DAB staining of fecal oocysts using OW3-biotin. Immunoperoxidase staining methods (32) were adapted for diaminobenzidine (DAB) labeling of cryptosporidial oocysts. Stock 3,3′-diaminobenzidine tetrahydrochloride (Sigma) was prepared by dissolving DAB in 50 mM Tris buffer (pH 7.6) to a concentration of 45 mg/ml, dispensing in 100-μl aliquots into plastic vials, and storing at −20°C until used. OW3-biotin was diluted in PBS, applied to fecal smears (50 μl), and incubated for 30 min in a humid chamber. Slides were washed three times with PBS, and the excess PBS was wiped off. Streptavidin-horseradish peroxidase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was diluted in PBS, applied to the smears (50 μl), and incubated for 30 min in a humid chamber. Slides were washed three times with PBS, and the excess PBS was wiped off. Working DAB solution was freshly prepared by thawing a stock DAB vial, diluting it to 10 ml with 50 mM Tris buffer (pH 7.6) or with PBS, and adding 100 μl of 3% H_2O_2. Working DAB was applied to the smears (100 μl), and the slides were incubated in the dark for 15 min. Slides were washed once with PBS, followed by two H_2O washes. Copper sulfate (0.5% $CuSO_4$ in 0.85% NaCl) was applied to the smears for 5 min, and the slides were washed twice with distilled H_2O. Slides were counterstained with 1% aqueous methylene blue for 15 to 30 s and washed briefly under tap H_2O. After the fecal smears were dried, they were mounted with Permount (Fisher Scientific Co., Pittsburg, Pa.) or simply coated with a thin layer of immersion oil and observed by bright-field microscopy.

AEC staining of fecal oocysts, using OW3-biotin. A 3-amino-9-ethylcarbazole (AEC) substrate and hematoxylin counterstaining kit was obtained from Tago Inc. (Burlingame, Calif.) and employed per instructions on air-dried, heat-fixed fecal smears. Fecal smears were treated with OW3-biotin before AEC substrate application essentially as described for the DAB method. Stained slides were dried, mounted with PBS-glycerol, covered with cover slips, and observed by bright-field microscopy.

Oocyst detection method evaluation. Seven fecal oocyst detection methods were compared in their ability to correctly detect oocysts in fecal smears on microscope slides: (i) VOLU-SOL cold acid-fast staining, (ii) auramine-rhodamine staining, (iii) direct immunofluorescence assay employing C1B3-FITC, (iv) DAB labeling employing OW3-biotin and streptavidin-horseradish peroxidase, (v) AEC labeling employing OW3-biotin and streptavidin-horseradish

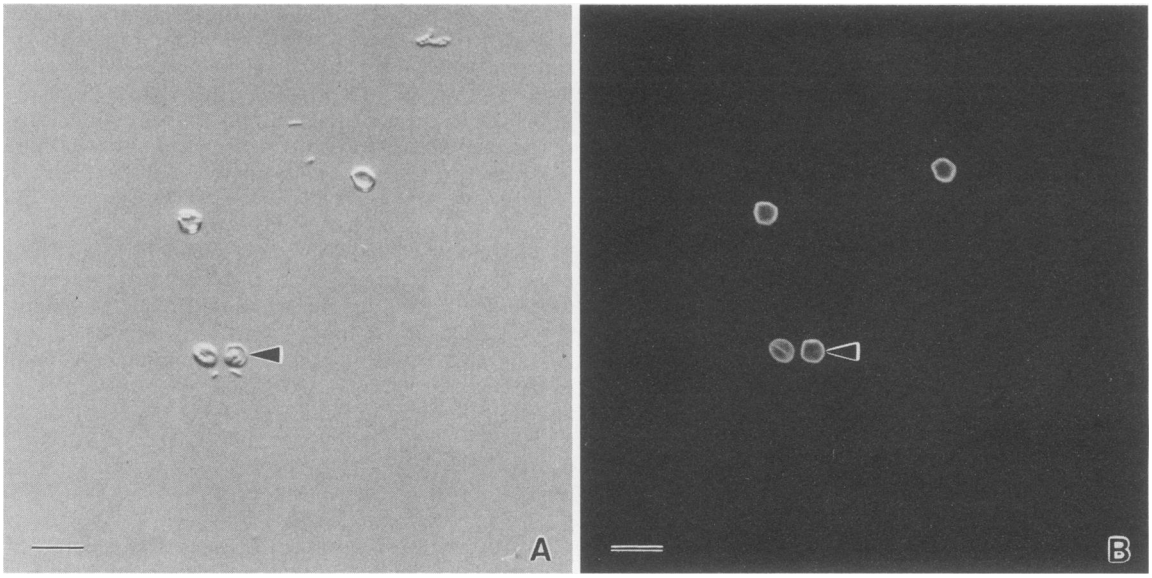

FIG. 1. Interference-contrast (A) and immunofluorescence (B) appearance of *C. parvum* oocysts labeled by MAbs C1B3, OW3, and OW3-biotin. Panel A illustrates the clearly distinguished oocyst wall in relation to the sporozoites within the oocyst (indicated by the arrowhead). The same oocyst (immunofluorescently labeled) is indicated by the arrowhead in panel B. Oocysts were bright, apple green spherical objects 4 to 5 μm in diameter. Bar = 10 μm.

peroxidase, (vi) an IIF assay employing OW3 hybridoma culture supernatant and FITC-labeled goat anti-mouse IgM, and (vii) an IIF assay employing OW3-biotin and streptavidin-FITC. Fecal specimens (311 total) were randomly ordered and coded for examination in a blind fashion. Slides were examined at magnifications of ×200 and ×400. Results were evaluated by correlating positive and negative results among the seven methods and assessing the potential for false-positive identifications. Sensitivity and specificity were assessed for each method (24).

Detection of oocysts in paraffin-embedded tissues. Slides containing paraffin sections of infected tissue were warmed to 65°C for 5 min, immersed in xylene (room temperature), and agitated for 5 min. Slides were transferred to cold (4°C) 95% ethanol for 5 min and rinsed three times with PBS at room temperature. Excess PBS was removed, and immunofluorescence or immunoperoxidase (DAB or AEC) assays were performed.

Oocyst wall antigen analysis. Oocyst walls were purified from oocysts isolated from experimentally infected calves using discontinuous sucrose gradients and isopycnic Percoll (Pharmacia, Piscataway, N.J.) gradients as previously described (2). Purified oocyst walls were counted on a hemacytometer and solubilized by using a variation of the urea-dithiothreitol method for solubilizing *Eimeria tenella* oocyst walls (29). Briefly, 2.5×10^9 oocyst walls were suspended in 1 ml of solubilization solution (25 mM phosphate buffer, 1% sodium dodecyl sulfate, 140 mM 2-mercaptoethanol, 0.015% bromophenol blue, 6.0 M urea, 10% glycerol, 250 mM dithiothreitol), degassed, and placed in a 100°C water bath for 12 h. The reaction mixture was centrifuged at 20,000 × *g* for 5 min, and the supernatant was decanted and stored at 4°C. This antigen preparation was subjected to polyacrylamide gel electrophoresis and Western blot (immunoblot) analysis as previously described (17). Western blot analyses employed MAbs C1B3 and OW3 and hyperimmune sera from the mouse from which MAb OW3 was derived.

RESULTS

Antioocyst C1B3 hybridoma. Hybridoma C1B3 secreted an IgG1 MAb which recognized an oocyst wall surface determinant in immunofluorescence assays (Fig. 1). Western blot analyses of solubilized oocyst wall antigens using MAb C1B3 showed reactivity to a wide band of 70 to 200 kilodaltons (Fig. 2). Amido black-stained nitrocellulose replicas showed no distinct bands or smears, perhaps indicating the absence of protein or the presence of undetectable levels of protein (data not shown). Oocysts of all *Cryptosporidium* isolates tested to date have been labeled by C1B3. Oocyst labeling was apparent in stools preserved with $K_2Cr_2O_7$ or

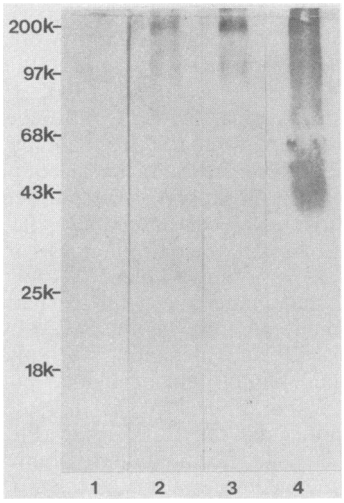

FIG. 2. Western blot analysis of solubilized oocyst wall material with normal mouse serum (lane 1), hyperimmune RBF/Dn mouse serum (from the mouse that hybridoma OW3 was derived from) (lane 2), MAb OW3 (lane 3), and MAb C1B3 (lane 4). k, Kilodaltons.

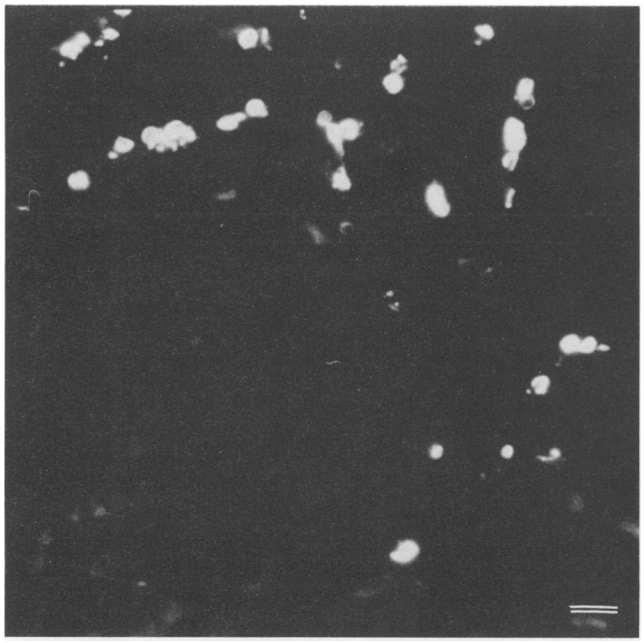

FIG. 3. Immunofluorescent appearance of *C. parvum* oocysts along infected villus surfaces of BALB/c mice, using OW3-biotin in an IIF assay. Bar = 10 μm.

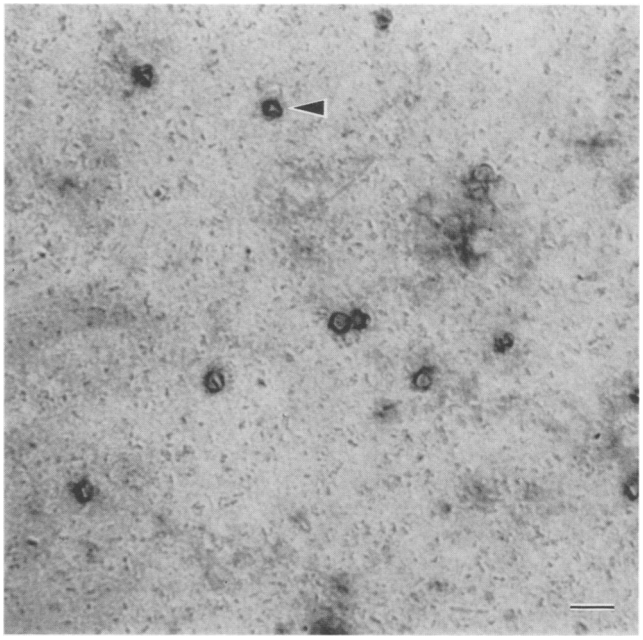

FIG. 4. Immunoperoxidase (DAB)-labeled *C. parvum* oocysts (indicated by the arrowhead) in a fecal smear using OW3-biotin. Bar = 10 μm.

fixed with Formalin. Although C1B3 recognized a determinant on oocyst walls, it also recognized determinants on unidentified yeasts and bacteria encountered in a small number of stool specimens. Stool samples containing cross-reactive yeasts and bacteria had been previously fixed in Formalin, preventing any attempt to isolate, cultivate, or identify these organisms.

Antioocyst OW3 hybridoma. Hybridoma OW3 secreted an IgM MAb which recognized an oocyst wall surface determinant in immunofluorescence assays (appearance identical to Fig. 1). Western blot analyses of solubilized oocyst wall antigens using MAb OW3 (and sera from the immunized mouse that MAb OW3 was derived from) showed reactivity to a band of >200 kilodaltons (Fig. 2). Oocysts of all *C. parvum* isolates tested to date have been labeled by OW3, while *C. baileyi* oocysts were not labeled (16). Oocysts of the turkey and quail isolates were labeled by OW3 but at a lower fluorescence intensity (data not shown). The *C. muris* isolate was also labeled by OW3 but is distinguished by its larger oocyst size (7.4 by 5.6 μm) compared with oocysts of *C. parvum* (5.0 by 4.5 μm) (30). Oocyst labeling was apparent by using $K_2Cr_2O_7$-preserved and Formalin-fixed stools.

Biotin hydrazide labeling of MAb OW3 and application in immunofluorescence assays. Biotinylated OW3 displayed the same labeling characteristics as the unconjugated MAb (appearance identical to Fig. 1). Stock OW3-biotin (Sephadex G-25 column recovered) displayed an IIF titer of ≥4,096 when diluted in PBS. The conjugate was stable for at least 8 months when stored at 4°C. Storage at −20°C in 50% glycerol was unsuccessful. IIF assays were successful with 30-min incubation times for reagent binding. Oocysts appeared as 4- to 5-μm spherical objects fluorescing bright apple green against a dark background, facilitating rapid scanning of fecal smears (especially at low power [×200]). Immunofluorescence assays were successfully performed using histologic sections of infected murine intestinal tissue (Fig. 3).

Immunoperoxidase assays employing OW3-biotin. Indirect immunoperoxidase assays employing OW3-biotin successfully demonstrated oocysts in fecal smears (Fig. 4) and infected murine intestinal tissues (Fig. 5). In fecal smears, oocysts were stained brown (DAB) or red (AEC) against a light blue background. Oocysts were most easily identified when numbers were high. Fecal samples with low oocyst numbers required extended time for slide examination. His-

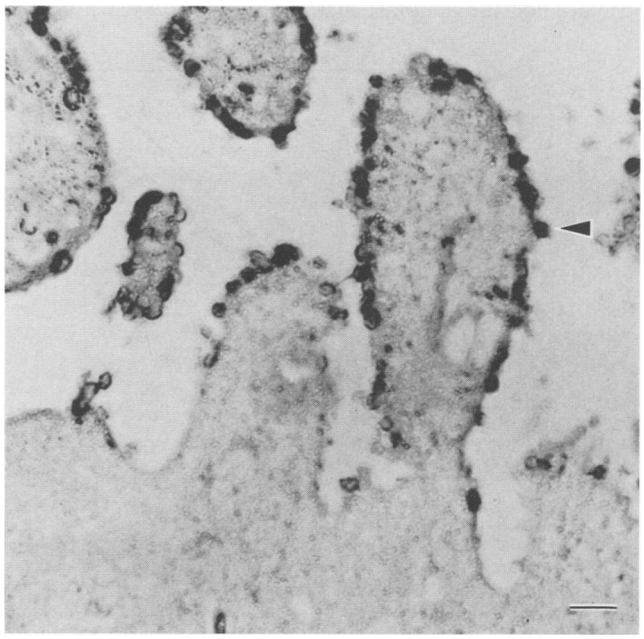

FIG. 5. Immunoperoxidase (DAB)-labeled *C. parvum* oocysts (indicated by the arrowhead) along infected villus surfaces of BALB/c mice using OW3-biotin. Bar = 10 μm.

TABLE 1. Comparison of detection methods for oocyst identification in 311 stool specimens

Method[a]	No. of positives	No. of false-positives	% Positives	% False-positives	Sensitivity[b] (%)	Specificity[c] (%)
AF	13	8	4.2	2.6	40.6	52.0
AR	30	5	9.6	1.6	93.8	85.7
C1B3	30	0	9.6	0.0	93.8	100
DAB	26	0	8.4	0.0	81.3	100
AEC	23	0	7.4	0.0	71.9	100
OW3	32	0	10.3	0.0	100	100
OW3-biotin	32	0	10.3	0.0	100	100

[a] See text for descriptions of individual methods. AF, Acid-fast; AR, auramine-rhodamine.
[b] Sensitivity calculated by dividing the number of confirmed positives identified by each method by the total number of confirmed positives (i.e., 32 positive by the OW3 IIF method) and multiplying by 100.
[c] Specificity calculated by dividing the number of confirmed positives identified by each method by the total number of positives for that method and multiplying by 100.

tologic sections labeled with OW3-biotin were counterstained green, facilitating tissue orientation and oocyst identification.

Chemical oocyst detection methods. Acid-fast-stained fecal smears often contained nonstaining oocysts (oocyst ghosts) as well as unidentified acid-fast structures. The auramine-rhodamine method also stained unidentified fecal structures nonspecifically.

Comparison of oocyst detection methods. Results of applying seven methods for oocyst detection in fecal smears are summarized in Table 1. If the VOLU-SOL cold acid-fast method is considered the standard for oocyst detection (100% specific and sensitive), then by comparison the IIF and immunoperoxidase assays are >100% sensitive and >100% specific. Equivalent sensitivity and specificity were observed for the OW3 and OW3-biotin IIF assays. If the OW3 IIF methods are considered the standard for oocyst detection (100% specific and sensitive), then the DAB method was 81.3% sensitive and 100% specific, the AEC method was 71.9% sensitive and 100% specific, the C1B3 method was 93.8% sensitive and 100% specific, the auramine-rhodamine method was 93.8% sensitive and 85.7% specific, and the acid-fast method was 40.6% sensitive and 52.0% specific. A correlation analysis of oocyst numbers detected in fecal smears for each of the seven methods is illustrated in Fig. 6. The OW3-biotin IIF method and the auramine-rhodamine method were equivalent to the OW3 IIF method, while the DAB, AEC, and C1B3 methods were less sensitive (especially when oocyst numbers were small) and the acid-fast method was much less sensitive. The acid-fast method yielded over half again as many false-positives (8 of 311 samples) as confirmed positives (13 of 311). The auramine-rhodamine method also presented a substantial number of false-positives (5 of 311).

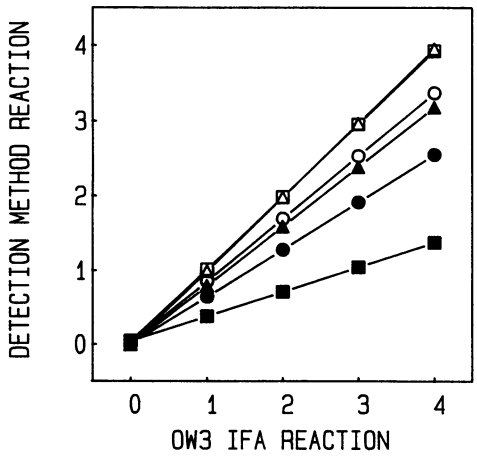

FIG. 6. Correlation analysis of oocyst detection methods. All methods were compared with the OW3 immunofluorescence assay (IFA) which was 100% sensitive and specific. Axes represent the oocyst numbers demonstrated in the fecal smears at ×400 magnification. Scale values in oocysts per field: 1+ for <2 oocysts; 2+ for 3 to 5 oocysts; 3+ for 5 to 7 oocysts; and 4+ for >8 oocysts. The methods tested were acid-fast (■), AEC (●), DAB (▲), C1B3 (○), auramine-rhodamine (□), and OW3-biotin (△).

DISCUSSION

Antioocyst MAb OW3 demonstrated reactivity to *C. parvum* oocysts but not to *C. baileyi* oocysts. Weak oocyst reactivity was observed to the turkey and quail *Cryptosporidium* oocyst isolates (this should not be a problem in a clinical setting, as these isolates should not infect mammals or occur in human stools). Reactivity to *C. muris* oocysts may be of veterinary importance, but no human infections by this agent have been reported (size differences make differentiation of *C. muris* and *C. parvum* possible with the microscope). Additionally, OW3 did not cross-react with any bacteria or yeasts labeled by C1B3 or any other microorganisms or parasites tested to date (8). MAb OW3 reacted with an oocyst wall antigen of 200 kilodaltons in Western blots.

Conjugating OW3 with fluorescent dyes (FITC and fluorescein thiosemicarbazide) was unsuccessful (data not shown). Conjugation of OW3 with biotin hydrazide was successful. Immunoassays employing OW3 or OW3-biotin have been restricted to indirect methods, lengthening assay time to approximately 90 min.

The OW3 immunofluorescence assays were observed to be 100% sensitive and 100% specific compared with the VOLU-SOL cold acid-fast method (40.6% sensitive and 52.0% specific) and the auramine-rhodamine method (93.8% sensitive and 85.7% specific). The nonfluorescence OW3 methods (immunoperoxidase) were 100% specific (no false-positives) though slightly less sensitive than the fluorescent chemical staining method (auramine-rhodamine) and twice as sensitive as the acid-fast method. The performance of the VOLU-SOL acid-fast stain (which we consider very good) may vary to some degree from the performance of acid-fast methods employed in other laboratories.

Antioocyst MAb-based immunofluorescence assays have proven to be valuable in detecting oocysts in fecal samples and water supplies (8, 13, 18, 25–27) and should prove useful in the identification of oocysts in tissue biopsies. Light

microscopy-based immunoperoxidase assays may be of practical value when fluorescence microscopes are not available. Immunoperoxidase methods exhibited the same specificity for oocysts as the fluorescence methods, although with a lower sensitivity, and should have application in generating permanent records of oocyst presence in fecal smears and tissue biopsies. Given the high specificities and sensitivities of the MAb-based assays, it may be expected that immunoassays will replace chemical staining methods now commonly employed for oocyst detection. In this regard, an IIF assay employing OW3 has been commercialized by Meridian Diagnostics Inc., Cincinnati, Ohio.

ACKNOWLEDGMENTS

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