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An In Vitro Method for Detecting Infectious *Cryptosporidium* Oocysts with Cell Culture

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Current assay methods to detect *Cryptosporidium* oocysts in water are generally not able to evaluate viability or infectivity. A method was developed for low-level detection of infective oocysts by using HCT-8 cells in culture as hosts to *C. parvum* reproductive stages. The infective foci were detected by labeling intracellular developmental stages of the parasite in an indirect-antibody assay with a primary antibody specific for reproductive stages and a secondary fluorescein isothiocyanate-conjugated antibody. The complete assay was named the focus detection method (FDM). The infectious foci (indicating that at least one of the four sporozoites released from a viable oocyst had infected a cell) were enumerated by epifluorescence microscopy and confirmed under Nomarski differential interference contrast microscopy. Time series experiments demonstrated that the autoreinfective life cycle in host HCT-8 cells began after 12 h of incubation. Through dilution studies, levels as low as one infectious oocyst were detected. The cell culture FDM compared well to other viability assays. Vital stains and excystation demonstrated that oocyst populations less than 1% viable (by vital dyes) and having a low sporozoite yield following excystation could not infect host cells. Until now, the water industry has relied on an oocyst detection method (under an information collection regulation) that is unable to determine viability. The quantifiable results of the cell culture method described demonstrate two important applications: (i) an infectivity assay that may be used in conjunction with current U.S. Environmental Protection Agency-mandated detection methodologies, and (ii) a method to evaluate oocyst infectivity in survival and disinfection studies.

Waterborne *Cryptosporidium* has become a worldwide concern due to the resistant nature of the oocyst and widespread contamination (5). For water utilities, the U.S. Environmental Protection Agency has mandated the use of an immunofluorescent assay method (IFA) for the detection of *Cryptosporidium* and *Giardia* under an information collection regulation (12). However, among a variety of limitations, this assay does not directly address viability. Oocysts that contain sporozoites or that are full are considered potentially viable. Viability assays involving vital dyes have been developed (6), but they do not measure infectivity. Excystation, a measure of the ability of the oocyst to release the sporozoites, cannot measure infectivity and has limitations regarding the ability to enumerate sporozoites. Until now, the only available infectivity assay requires animal hosts, which is costly and very time-consuming (1).

Cell culture has been used previously to study the *Cryptosporidium* life cycle and metabolic growth requirements in vitro (2, 8, 9, 11). Coccidian development in cultured cells and complete development in vitro had been obtained for only a few species prior to 1991. *Cryptosporidium parvum* was compared with other coccidia by using cells routinely used for studying coccidial growth in vitro. Comparison among cell lines found that HCT-8 cells best supported the life stages of *C. parvum* infection in culture (8). Detection of developmental stages in cell culture began with an enzyme-linked immunosorbent assay that involves a polyclonal antibody developed against *C. parvum* sporozoites. The antibody also reacts with the epitopes on subsequent reproductive stages in culture (3). The cell culture technology has developed into a tool that can be used to study the organism in an environment most similar to the in vivo situation without using animal models (13, 14). This research focused on using HCT-8 cells in culture for development of a detection method (designated the focus detection method [FDM]) to measure the infectivity of oocysts from environmental samples.

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MATERIALS AND METHODS

Culture media. Human illeocecal adenocarcinoma cells (HCT-8 cells) (ATCC CCL-244; American Type Culture Collection) were maintained in RPMI 1640 (Mediatech Cellgro, Herndon, Va.) supplemented with 5% fetal bovine serum (Atlanta Biologicals, Norcross, Ga.), 1% 200 mM L-glutamine (Sigma, St. Louis, Mo.), and 2% 1 M HEPES (pH 7.3; Sigma). During oocyst infection, the FBS was increased to 10% (growth medium). Additional vitamins, sugars, and antibiotics increase parasite numbers in vitro (10); however, that recipe was not used for this procedure.

Cell maintenance. The HCT-8 cells were maintained in 75-cm^2 tissue culture flasks in a 5% CO_2 atmosphere at 37°C and 100% humidity and passaged every 2 or 3 days. The cells were passaged by trypsinization with one part phosphate-buffered saline (PBS)-EDTA and one part 0.25% trypsin. HCT-8 cells required a 5- to 8-min incubation in the trypsin solution at 37°C in the CO_2 atmosphere to assist in the disruption of the monolayer. The cell suspension was pipetted vigorously through a 200-μl pipette tip repetitively until the cells were separated (about 10 times). The cells were pelleted by a 4-min centrifugation (200 × *g*), resuspended in maintenance medium, and split 1:5 at each passage. Supercell (Nalge Nunc, Naperville, Ill.) well chamber slides were plated with 5×10^5 cells per well, grown to 70 to 95% confluence (24 to 48 h), and then infected (see below).

Oocyst preparation. *C. parvum* oocysts were produced by one of us (S.U.) and were a Kansas State isolate originally obtained from a naturally infected calf with diarrhea. The oocyst strain had been perpetuated by manually infecting newborn calves. The oocyst age and percent excystation were recorded for each experiment. The concentration of the oocyst stock suspension was determined by using

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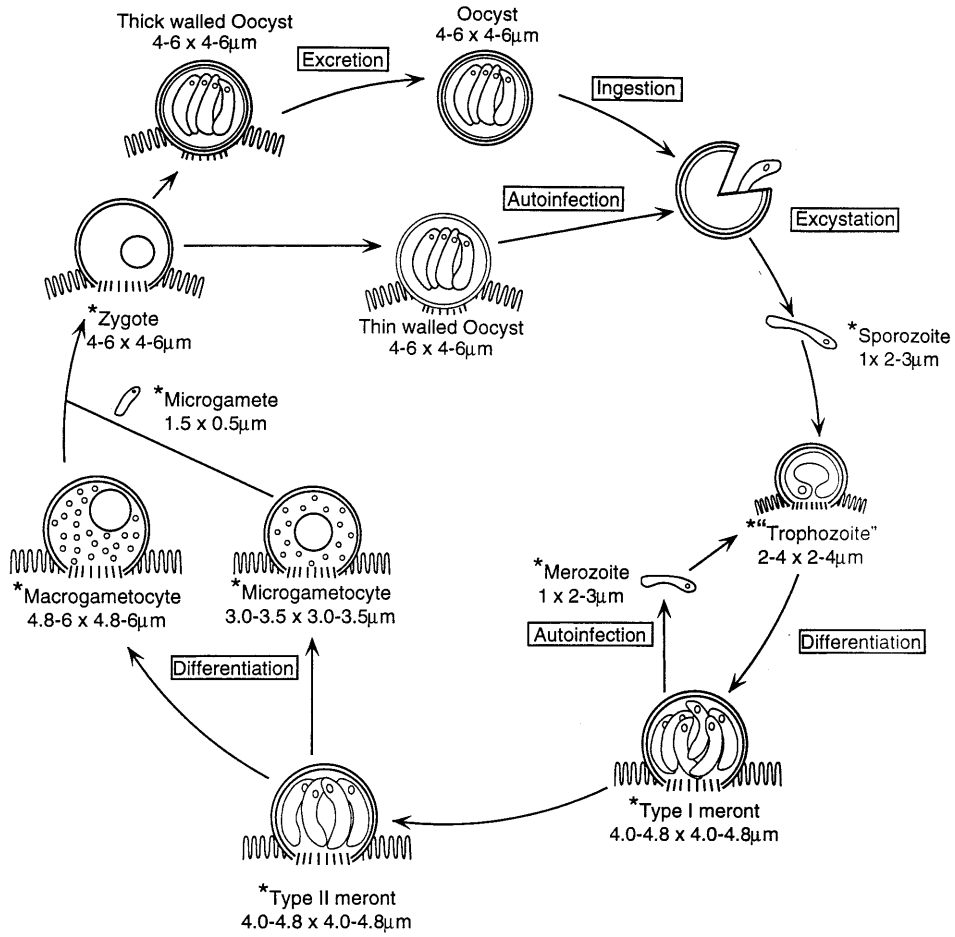

FIG. 1. *Cryptosporidium* life cycle in host cells. Adapted from reference 1a with permission from the publisher.

a direct hemacytometer count, and 10-fold dilutions were made to prepare low concentrations. Negative controls were run concurrently for each experiment and included either oocysts that had been heat inactivated by boiling for 10 min, oocysts that had been subjected to freeze-thaw treatment by immersion in liquid nitrogen for 3 min and then boiling for 10 min, or uninoculated monolayers.

The oocysts were pretreated by suspending 100 μl of stock oocysts (stored in sterile deionized [DI] water) in a 10.5% bleach solution containing 9 parts sterile ultrapure water (800 μl) and 1 part 5.25% sodium hypochlorite (100 μl of 4°C bleach on ice) for 10 min. The preparation was centrifuged at a relative centrifugal force of 10,500 × *g* for 4 min in a tabletop Microfuge 12 (Beckman Instruments, Palo Alto, Calif.), and the supernatant was aspirated off. The oocysts were then washed once in sterile water, centrifuged again, and resuspended in cell culture growth medium warmed to 37°C.

Cell infection. Maintenance medium was aspirated from the cell monolayers, and the cells were washed once with sterile 1× PBS. Oocysts in growth medium were added to the cells and incubated for 90 min in a 5% CO_2 atmosphere at 37°C (to initiate excystation and infection). Oocyst debris and any unexcysted oocysts were washed from the cells with sterile 1× PBS, growth medium was added, and the cells were incubated at 37°C under 5% CO_2 for an additional period ranging from 90 min to 48 h. After incubation, the medium was aspirated and the infected monolayers were washed once with sterile 1× PBS and fixed with 100% methanol (room temperature) for 10 min. The chamber wells were removed from the slides, and the monolayers were rehydrated at room temperature (23°C) for 30 min with blocking buffer containing 1% normal rabbit serum (Sigma) and 0.002% Tween 20 (Fisher, Fair Lawn, N.J.) in 1× PBS. The slides were stained by the indirect antibody method (see below).

Antibody labeling. The monolayers were labeled with primary polyclonal rat anti-sporozoite and -merozoite antiserum, which reacts with all reproductive stages of the organism; this serum was prepared by Steve Upton, Kansas State University. Stock primary antibody (~1:500) was suspended in 15 ml of 1× PBS. The slides were flooded and rocked gently for 30 min at room temperature in a tabletop rocker (Bellco Biotechnology, Vineland, N.J.). The slides were washed four times with 1× PBS. The secondary antibody was goat anti-rat antibody conjugated to fluorescein isothiocyanate (Sigma). The slides were flooded with secondary antibody suspended in 15 ml of 1× PBS (~1:350) and rocked gently for 30 min (in the dark) at room temperature. The slides were washed four times with 1× PBS, then 10 μl of 2% DABCO in glycerol (Sigma) was added to each well, the coverslips were mounted with nail polish, and the cells were viewed under epifluorescence microscopy (Olympus BH2RFCA). Photographs were taken at ×200, ×400, and ×1,000 magnification with an Olympus C-35AD camera.

The specificity of the antibody to the reproductive stages was determined by examining the cell monolayer under IFA and Nomarski differential interference contrast (DIC) microscopy. The infected cells fluoresced an apple-green color under IFA (480-nm excitation and 550-nm emission) against a relatively dark background of uninfected cells, and the number of foci was determined by averaging random fields from separate wells or whole well counts in triplicate. The standard deviation between replicates was determined by Microsoft Excel statistical analysis.

Infection time series. The wells were seeded with either 100 or 1,000 oocysts, incubated for 90 min, and washed once with 1× PBS, and the growth medium was replaced. The infected cells were further incubated and then fixed at time zero, 90 min, 5 h, 12 h, and 18 h. Other experiments compared infection after 24 and 48 h at oocyst concentrations of 1 and 10.

Comparing viability assays. The FDM as a viability assay was compared to two other viability assays commonly used. 4′,6-Diamidino-2-phenylindole (DAPI) and propidium iodide (PI) are fluorogenic vital dyes that indicate the presence of live organisms when DAPI is included with simultaneous PI exclusion (PI exclusion from the nucleus is based on membrane integrity, and organisms are considered dead when PI is included in the nucleus) (3). Excystation measures the ability of a viable organism to release the sporozoites when exposed to bile salts, trypsin, and 37°C (4). Fresh oocysts were suspended in the following solutions: (i) water control (sterile DI water); (ii) high-salt solution in sterile DI water (9% NaCl, 2% dextrose) (pH 6.96); and (iii) high-sugar solution (maple syrup) (pH 4.95).

The oocyst suspensions were incubated at 26.6°C and were analyzed at time zero, 24 h, and 1 week. After incubation, the samples were washed twice by centrifugation as previously described, resuspended in sterile DI water, and

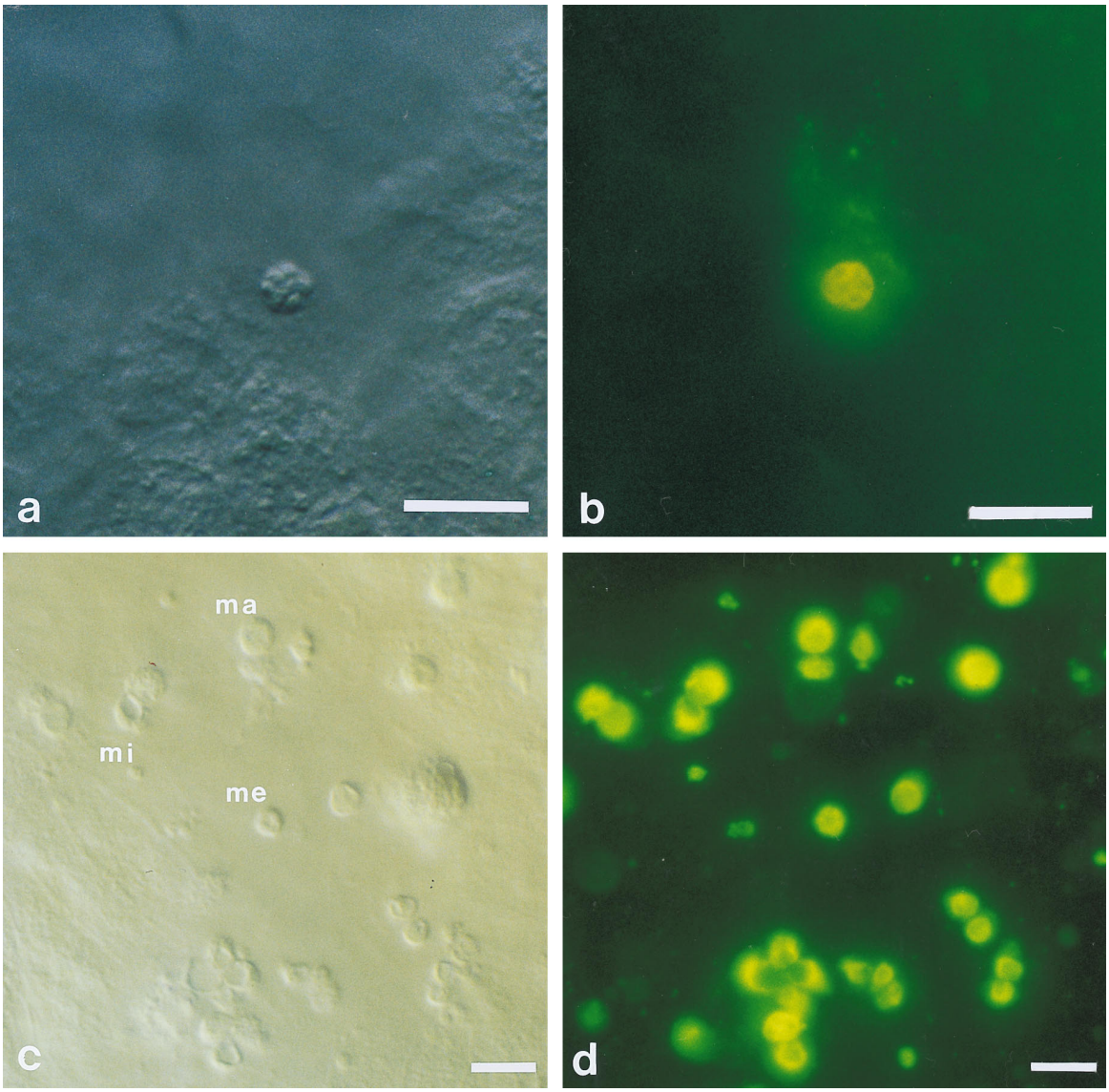

FIG. 2. (a) Nomarski DIC photomicrograph of a meront in HCT-8 cells. (b) Epifluorescence photomicrograph of the meront in panel a. (c) Nomarski DIC photomicrograph of a field of foci showing several life stages after 48 h of incubation, including meronts (Me), microgametocytes (Mi), and macrogametocytes (Ma). (d) Epifluorescence photomicrograph of the field in panel c. Magnifications, ×900. Bars, 10 μm.

prepared for each viability assay. Viability was determined by excystation (4), including the sporozoite yield (number of sporozoites per empty oocyst in 100 oocysts counted), DAPI/PI vital-dye staining (6), and cell culture (FDM). Excystation was determined by measuring the ratio of full to empty oocysts and counting the total number of sporozoites per 100 oocysts counted. DAPI vital-dye inclusion–PI exclusion was used to indicate oocyst viability and potential infectivity. Oocysts which included propidium iodide (PI+) were considered nonviable. The samples were washed once by centrifugation, resuspended in bleach solution, and tested for infectivity by the FDM with a 24-h incubation time as described above.

RESULTS

Infection of HCT-8 cell monolayers with *C. parvum* was monitored by both epifluorescence and Nomarski DIC microscopy. Figure 1 illustrates the life cycle of *Cryptosporidium* and the life stages which were labeled by the primary antibody (indicated by an asterisk), including the sporozoite, trophozoite, type I and II meronts, merozoite, macrogametocyte, microgametocyte, and microgamete. The stages not labeled by the antibody were intact thin- and thick-walled oocysts. Lightly fluorescing oocyst shells were seen sticking to the monolayers. These fluorescing shells are probably the result of residual antigens from sporozoite membranes and residual bodies labeled by the primary antibody. Figure 2a shows an infected cell monolayer as seen under DIC microscopy at ×900 magnification of a type II meront (5.6 μm in diameter) after 48 h of incubation. Figure 2b shows the same field with this meront labeled by the FDM under epifluorescence microscopy. Figures 2c and d show several life stages including meronts, microgametocytes, and macrogametocytes under DIC and epifluorescence microscopy at ×900 magnification. No such foci were detected in any of the negative controls.

Previous studies during the development of the FDM showed that many more infectious foci were counted after 48 h of incubation than the number of oocysts (sporozoites) seeded onto host cells (7). This was presumed to be due to the autoreinfection (secondary replication) of the parasite. Theoret-

TABLE 1. Ratio of foci to oocysts after 24 and 48 h of incubation

No. of oocysts plated per well	No. of replicate wells or fields[a]		No. of foci counted per well after[b]:		Ratio of foci per oocyst after:	
	24 h	48 h	24 h	48 h	24 h	48 h
1,000	2*	8**	512.00 ± 121.62	13,839.50 ± 5,393.50	0.51	13.8
100	2*	8**	151.00 ± 79.20	2,960.20 ± 3,873.50	1.51	29.6
10	2*	4*	5.50 ± 0.71	93.00 ± 80.50	0.55	9.3
1	2*	4*	<1.00 (NA)[c]	18.80 ± 25.70	<1.00	18.8
0[d]	2*	2*	0.00	0.00	≪1.00	≪1.00

[a] *, wells; **, fields.
[b] Results are given as mean ± standard deviation.
[c] NA, not applicable.
[d] Control.

ically, if all oocysts were viable and all sporozoites were infectious, a ratio of foci to oocysts of 4:1 would be seen. Several more experiments were run at low oocyst doses to compare the number of foci developed in 24 h with the number developed 48 h (as shown in Table 1). A 24-h incubation resulted in focus-to-oocyst ratios ranging from 0.5:1 to 1.5:1, and no infection was noted at the one-oocyst inoculum. After 48 h, the focus-to-oocyst ratios ranged from 9.3:1 to 29.6:1. The total number of foci in wells inoculated with one oocyst ranged from 0 to 63 per well, and a single infectious oocyst was detected. For 10 oocysts per well, 5 and 6 foci were counted at 24 h and 6 to 189 were counted at 48 h.

A timed assay was performed to evaluate the secondary replication in culture by using photomicrographs. Figure 3 displays the progression of an infection in the HCT-8 cells with a 100-oocyst inoculum fixed at time zero, 90 min, 5 h, and 12 h. Secondary replication appeared to begin within the first 24 h of incubation.

At time zero (Fig. 3a), a dark monolayer of uninfected cells was observed. No oocysts were seen sticking to the monolayer. After 90 min (Fig. 3b), very small foci were apparent throughout the cell monolayer ("trophozoites"). These foci were all about 2.4 μm in diameter and appeared to have dark spots in the center with the fluorescein isothiocyanate-labeled sporozoite curled up inside a vacuole in the host cell (Fig. 3b). Several empty oocyst shells were seen at this time as well. After 5 h (Fig. 3c), the "hollow"-appearing foci were still obvious in the monolayer. After 12 h (Fig. 3d), the foci were a combination of 2.4- and 4.8-μm-diameter foci. Under Nomarski DIC microscopy (×900), these 4.8-μm foci were the beginning stages of type I meronts. Figure 3e shows an oocyst shell with a fluorescing sporozoite inside, a sporozoite on top of the host cells, and two foci (in different planes of focus), possibly an example of two successful and two unsuccessful infections after 12 h.

Another timed assay was performed at both 100 and 1,000 oocysts per well (by dilution), in triplicate, and foci were counted at time zero, 90 min, 5 h, 12 h, and 18 h. Figure 4 shows the number of foci counted per well over time for the 100-oocyst inoculum (too many foci were present to count in the 1,000-oocyst inoculum). An average of 30 foci were counted per well up to 18 h, when many more foci appeared, and the focus counts varied dramatically from well to well. It was interesting that the focus-to-oocyst ratios were between 0.3:1 and 0.8:1 at these early incubation times.

To compare the FDM with other viability assays, oocysts were evaluated in a survival study. Two food products whose preparation involves water were chosen. Oocysts were stored in sterile DI water (as a control), a high-salt solution, and a high-sugar solution at room temperature for 1 week (Table 2). The sodium dextrose solution simulated salty foods such as raw bread dough, and the maple syrup was used to simulate high-sugar foods. Oocyst survival was determined by excystation, vital-dye staining (with DAPI-PI), and the cell culture FDM.

Initially, the oocyst population had an excystation of between 48 and 78%, with a sporozoite yield of 1.89 to 3.32 (sporozoites per oocyst shell). The DAPI-PI evaluation showed that 93 to 94% of the oocyst population was DAPI positive and PI negative, indicating high viability. These oocysts were infectious by the FDM. Table 2 shows the results of the viability assay after 24 h and 1 week of storage. After 24 h, the oocysts in water had similar excystation, sporozoite yield, DAPI and PI percentages, and infectivity as at time zero; however, the oocyst populations in the salt and sugar solutions had changed dramatically. Excystation was 46% but the sporozoite yield dropped to 0.20 in the salt solution. The oocysts in the sugar solution had only a 0.07 sporozoite yield. The percentage of DAPI-positive and PI-negative oocysts dropped to 24 and 1% for the salt and sugar solutions, respectively. Oocyst infectivity could be seen only with the 1,000-oocyst inoculum by the FDM in the salt solution, and no infectivity was observed in the sugar solution. By 1 week, excystation for the salt and sugar solutions rose to 87 and 99% with sporozoite yields of 0.03 and 0.00, respectively, the percentage of DAPI-positive and PI-negative oocysts was 9 and 3%, respectively, and no infectivity could be seen by the FDM in either solution at up to a 1,000-oocyst inoculum. (This experiment was repeated with cell culture incubation times for up to 72 h, indicating that no infectious oocysts survived in either solution after 1 week.)

DISCUSSION

The viability of *C. parvum* oocysts was detected and quantified microscopically by the FDM developed during this study. The procedure was optimized for use and has the following attributes: (i) infection was measured in as little as 5 h, (ii) ratios of 0.5 to 1.55 foci per oocyst could be demonstrated with 24-h incubations for quantification purposes, and (iii) a single viable oocyst as determined by dilution was detected with a 48-h incubation. Longer incubation periods increase the autoreinfection and the assay sensitivity, and in this case quantification can be made by using a most-probable-number program with presence/absence evaluation. Less than 10 infectious oocysts have routinely been detected by the FDM (reference 7 and unpublished data).

Although ≥3% of the oocysts were potentially viable by DAPI-positive–PI-negative staining, the oocysts were not infective. Procedures that rely on microscopy, such as excystation and vital dyes, make it difficult to assess less than 1% viable organisms (1 viable per 100 oocysts counted); therefore, gen-

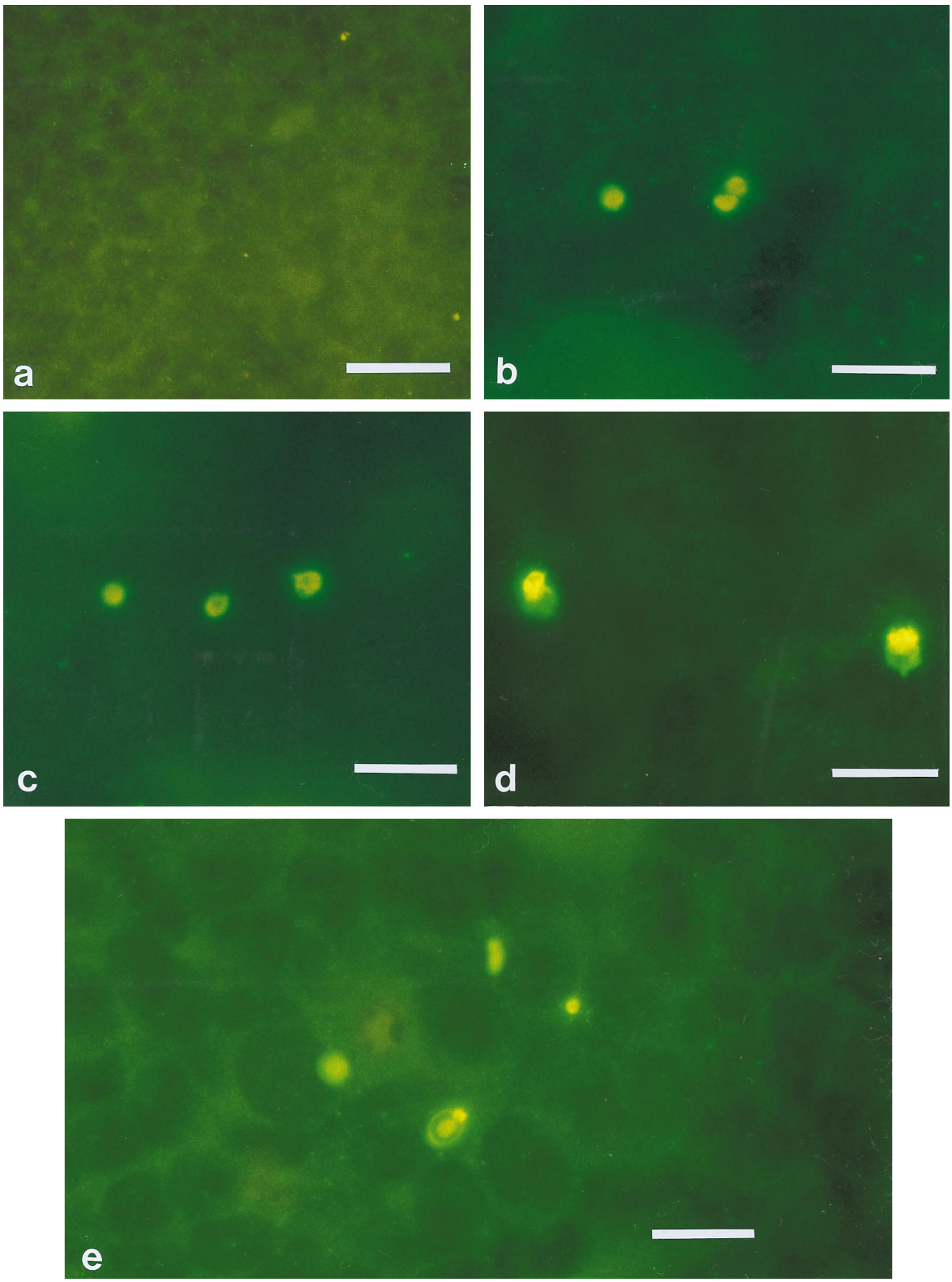

FIG. 3. Photomicrographs of the development of *C. parvum* in HCT-8 cells over time. (a) The uninfected monolayer at time zero after oocysts have been washed off. (b) The early developmental stages called trophozoites after 90 min of incubation. (c) Trophozoites after 5 h of incubation. (d) Two type I meronts beginning to develop after 12 h of incubation. (e) An oocyst shell with a sporozoite partially excysted, two foci (one out of the focal plane), and a sporozoite on the surface of the monolayer. Magnifications, ×900. Bars, 10 μm.

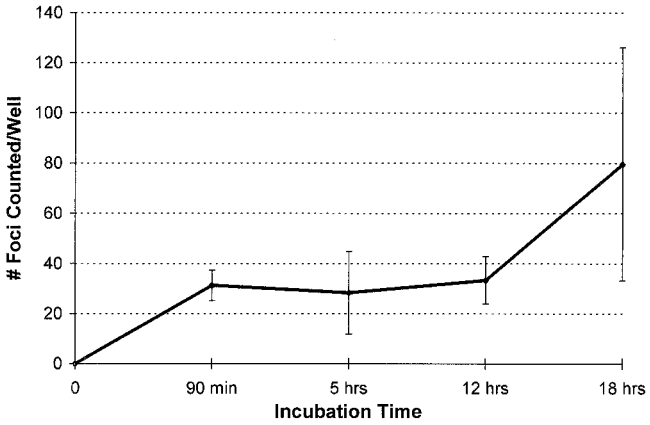

FIG. 4. Timed assay for the quantification of infectious oocysts (100-oocyst inoculum).

erally, only 99% inactivation can be determined. By the FDM, 100 and 1,000 oocysts were easily seeded onto the monolayer and could indicate 99 and 99.9% inactivation. Previously, we have shown that 10,000 and 100,000 oocysts could be inoculated; however, with high infectivity, the foci are difficult to enumerate. After a 24-h incubation, the foci could be quantified and related to the number of infectious oocysts seeded. Although the autoinfectious nature of the life cycle complicates quantification at incubation periods longer than 24 h, this could be an advantage in presence/absence evaluation. It would be logical that by evaluating monolayers after longer incubation periods, one could confirm complete inactivation with the absence of foci. For example, if 1 of 1,000 oocysts was infective, a 48-h incubation would have higher sensitivity (many more foci present) than a 24 h incubation would. During this evaluation, up to 72 h of incubation was tested; however, no greater sensitivity for detecting low-level oocyst viability was achieved compared to the 48-h incubation time.

The oocysts used in the development of this study were less than 1 month old, were stored in sterile DI water, and had excystation rates of >50% (sporozoite yields of 2 to 3). For oocyst populations such as this, the time series indicated that the autoreinfective cycle began after a 12-h incubation. Prior to that, the ratio of foci to oocysts averaged 0.3:1. This indicated that only 7.5% of the sporozoites were successful at initiating infection in this system after 12 h. After 24 h, an average 1 focus per oocyst was detected, indicating that at least 25% of the sporozoites could initiate infection (however, there was a large deviation in counts from well to well). After a 48-h incubation, a single oocyst inoculated was detected and an average of 17.9 foci per oocyst was determined. It is likely that there is a nonsynchronous excystation, infection, and development for the oocysts, sporozoites, and subsequent developmental stages. At present we recommend that the FDM be used with a 48-h incubation with a multiple-well and dilution format. This is easily read, and a most-probable-number program can be used to enumerate viable oocysts.

The measurements of *C. parvum* life stage forms were made under Nomarski DIC and fluorescence microscopy with oil at ×1,000 magnification. It is relevant to mention that these are considered approximate measurements in a two-dimensional field of one strain of oocysts infecting the HCT-8 cells. Higher magnification will be necessary to make accurate measurements; therefore, an electron microscopic evaluation of the size of the life stages will be undertaken in the future to produce more reliable measurements.

The FDM has been used with environmental samples (7). Secondary effluent from a sewage treatment plant was seeded with 2-week-old *C. parvum* oocysts and processed through a bench scale treatment system consisting of high-lime treatment at pH 11.2 and chlorination ranging from 2.2 to 67.0 mg per ml. Pre- and posttreatment oocyst viability was evaluated by an excystation assay (4) and the FDM. A 1-ml sample of posttreatment water was concentrated by centrifugation, bleach treated, washed, resuspended in cell culture growth medium, and inoculated onto a cell monolayer. Infected monolayers were fixed after 48 h, and infectious foci were quantified. No contamination on the monolayers was apparent, indicating effective sterilization by the bleach treatment step.

The FDM has been developed and used as a detection method to quantitatively measure the infectivity of *C. parvum* oocysts. However, *C. muris* was tested by the FDM and did appear to produce a limited infection which did not proceed past the trophozoite stage (unpublished observation). Other strains and species of *Cryptosporidium* must be tested to fully evaluate the specificity of the assay. Molecular techniques currently in development, such as PCR, could possibly be used in combination with the FDM to specifically target *C. parvum.*

Infectivity is an important issue that is currently addressed only by using animal models. Cell culture appears to be a more cost-effective, reproducible, and practical method. Animal models cannot easily be used to assess a single oocyst inoculum or be used with environmental samples. The FDM can be used with environmental samples for the detection of a single oocyst and can also be used as a viability assay for survival studies and disinfection studies. Future work will include further evaluation of the assay with environmental samples (immunomagnetic separation procedures and vortex flow filtration will be tested as ways to clarify environmental samples), additional survival studies, and studies of different strains and species as well as aged oocysts.

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TABLE 2. Viability of oocysts suspended under various conditions over time evaluated by different methods

Storage time	Conditions	% Excystation (sporozoite yield)	% of DAPI+ and PI− oocysts	Infectivity in cell culture by FDM with oocyst inoculation of[a]:	
				100	1,000
24 h	Water control	51 (3.39)	90	++	+++
	9% NaCl–2% dextrose	46 (0.20)	24	−	+
	Maple syrup	82 (0.07)	1	−	−
1 wk	Water control	64 (2.89)	78	++	+++
	9% NaCl–2% dextrose	87 (0.03)	9	−	−
	Maple syrup	99 (0.00)	3	−	−

[a] + and −, degree of presence or absence, respectively, after 24 h of incubation in cell culture. The 1-week samples were also − after 72 h of incubation.

serum. We thank Huw Smith, Scottish Parasite Diagnostic Laboratory, Springburn, Glasgow, Scotland, for his expert technical advice and support. Illustrations and graphics were generated by Chad Edmisten, USF Department of Marine Science, St. Petersburg, Fla.

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