Cytometric Approach for Detection of Encephalitozoon intestinalis, an Emergent Agent

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Encephalitozoon intestinalis is responsible for intestinal disease in patients with AIDS and immunocompetent patients. The infectious form is a small spore that is resistant to water treatment procedures. Its detection is very important, but detection is very cumbersome and time-consuming. Our main objective was to develop and optimize a specific flow cytometric (FC) protocol for the detection of E. intestinalis in hospital tap water and human feces. To determine the optimal specific antibody (Microspor-FA) concentration, a known concentration of E. intestinalis spores (Waterborne, Inc.) was suspended in hospital tap water and stool specimens with different concentrations of Microspor-FA, and the tap water and stool specimens were incubated under different conditions. The sensitivity limit and specificity were also evaluated. To study spore infectivity, double staining with propidium iodide (PI) and Microspor-FA was undertaken. Distinct approaches for filtration and centrifugation of the stool specimens were used. E. intestinalis spores stained with 10 μg/ml of Microspor-FA at 25°C overnight provided the best results. The detection limit was 5 × 10^4 spores/ml, and good specificity was demonstrated. Simultaneous staining with Microspor-FA and PI ensured that the E. intestinalis spores were dead and therefore noninfectious. With the stool specimens, better spore recovery was observed with a saturated solution of NaCl and centrifugation at 1,500 × g for 15 min. A new approach for the detection of E. intestinalis from tap water or human feces that ensures that the spores are not viable is now available and represents an important step for the prevention of this threat to public health.

Microsporidia comprise a diverse group of eukaryotic obligate intracellular parasites (12), including over 140 genera corresponding to more than 1,200 species. Only eight genera have clinical importance and are considered human pathogens (9, 25). Enterocytozoon bieneusi and Encephalitozoon intestinalis, both of which are responsible for gastrointestinal disease, are currently the most prevalent microsporidia identified in humans (6, 16) and among other mammals (12).

The true prevalence of microsporidiosis is not yet defined due to underdiagnosis, especially in immunocompetent individuals (13, 14). Only a small number of cases of Encephalitozoon intestinalis infection have been documented (25); E. intestinalis (reclassified from Septata intestinalis) has been detected in AIDS patients in developed countries and in Africa, as well as in other immunocompromised individuals, including patients who have undergone transplantation (8, 9, 24).

The infectious form is a small spore (1.8 to 5.0 μm), which is the only recognized viable stage of microsporidia outside a host cell. It has two rigid extracellular walls made of chitin, thus suggesting a potential link to a fungal cell (9, 12). The clinical manifestations of E. intestinalis infections in immunocompetent patients range from asymptomatic infections to self-limited diarrhea. However, in immunocompromised patients, it causes chronic diarrhea, which tends to disseminate, with the kidney being the major organ affected (8, 9). Spores may spread to the environment from infected patients via feces, urine, and/or other body fluids and tissues (9, 24). Consequently, the routes of transmission may involve person-to-person contact as well as waterborne or food-borne contamination, especially in developing countries with poor sanitation (11, 25). The microsporidial spores are usually very resistant to environmental conditions (24) and to the usual water treatment procedures (10), and they remain infective for long periods of time, especially when they are protected from desiccation (9). As a result of these characteristics, the U.S. Environmental Protection Agency placed microsporidia in first place as a candidate contaminant for drinking water (10). However, the infectious load needed to cause disease is not yet known.

The detection of spores in human feces or other human body fluids is very cumbersome and difficult. During the last 20 years, several methods for the recovery and detection of microsporidial spores have been developed and improved; these include electron microscopy and histologic examination of tissues samples (25). Light and/or immunofluorescence microscopy with polyclonal or monoclonal antibodies directed against microsporidial spores is nevertheless the most commonly used procedure, especially for the diagnosis of infection in immunocompromised patients (8, 9, 16, 25). Such methods are very time-consuming and are subject to human error (8, 16). Although molecular studies are highly sensitive and specific, they are too complex, too difficult for use for routine analysis, and expensive and are unable to assess spore viability (24). Flow cytometry (FC) allows both morphofunctional evaluation and quantification of individual microorganisms; it additionally

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provides significant advantages and a high degree of specificity, especially when it is combined with specific monoclonal or polyclonal antibodies against the *E. intestinalis* spore wall (8, 9, 16, 25). Our research team has been exploring distinct applications of FC in microbiology in order to increase its diagnostic sensitivity with clinical samples (17, 19) and to evaluate its use for determination of the susceptibility profiles of microorganisms (18, 20, 21, 22). Our main objective was to develop and optimize a specific FC protocol for the detection of *E. intes-
nalis* in hospital tap water and human feces after the simulation of the conditions in environmental and clinical settings.

**MATERIALS AND METHODS**

**Optimization of fluorescent staining.** *Encephalitozoon intestinalis* Didier from culture in rabbit RK-13 cells that had been stored in 5% formalin at a concentration of 1×10^7/ml (Waterborne, Inc., New Orleans, LA) was used. A total of 1×10^7 spores/ml of *E. intestinalis* were suspended in 500 μl of sterile water and stained with serial concentrations (0.5, 1.0, 2.0, 5.0, and 10.0 μg/ml) of an Alexa Fluor 488 mouse monoclonal antibody (Microspor DFA; 20× concentrate; A700AF488; Waterborne, Inc.), followed by incubation in the dark at different temperatures (37°C and 25°C) and for different times (45, 60, 90, and 180 min and overnight). Stool samples were centrifuged at distinct velocities (1,000 x g and 1,500 x g) and times (5 to 15 min). The supernatant was discarded and the pellet was resuspended in 1 ml of sterile H_2O, stained with the monoclonal antibody, and vortexed for 30 s. It was then transferred to a propylene tube and analyzed by FC at FL3 (red fluorescence, 670 nm).

**FC analysis.** The optical characteristics of the spore suspensions were evaluated on a FACS-Calibur cytometer (standard model; BD Biosciences, Sydney, Australia) equipped with three photomultipliers with standard filters (FL1, band pass of 530/30 nm; FL2, band pass of 585/42 nm; FL3, long pass of 680 nm) and a 15-mW 488-nm argon laser. Cell Quest Pro software (version 4.0.2; BD Bio-

**Assessment of sensitivity and specificity.** To assess the sensitivity, serial concentrations of *E. intestinalis* spores (1×10^7 to 5×10^10/ml) were stained with the previously optimized antibody concentration and were analyzed by FC.

To assess the specificity, 1×10^5 spores/ml of *E. intestinalis* were mixed with (i) bacterial suspensions consisting of 1.5×10^5 cells/ml of Escherichia coli ATCC 35218 or 1.5×10^5 cells/ml of *Staphylococcus aureus* ATCC 25923; (ii) fungal suspensions consisting of 5×10^6 *Blastomyces dermatitidis* (M16892; H9262; A700 AF488; Waterborne, Inc) stained with serial concentrations (0.5, 1.0, 2.0, 5.0, and 10.0 μg/ml) of an Alexa Fluor 488 mouse monoclonal antibody (Microspor DFA; 20× concentrate; A700AF488; Waterborne, Inc.) conjugated with Alexa Fluor 488 at the previously optimized concentration and incubated over-night at 25°C in the dark. After incubation, the cell suspensions were vortexed for 30 s, transferred to a propylene vial, and analyzed by FC.

**Staining for live and dead spores.** Propidium iodide (PI; Sigma) at 5.0 μg/ml was used to stain *E. intestinalis* spores with and without the specific fluorescent monoclonal antibody in the dark at different temperatures (37°C and 25°C) and times (45, 60, 90, and 180 min and overnight). Following staining of the spores, the parasite suspensions were vortexed for 30 s, transferred to a propylene tube, and analyzed by FC at FL3 (red fluorescence, 670 nm).

**Evaluation of human stool samples.** Stool samples from healthy volunteers were collected and stored at 4°C until use. One gram of stool sample was transferred to a propylene tube, and analyzed by FC at FL3 (red fluorescence, 670 nm). NaCl flotation solutions (solution A, NaCl at 360 g/liter and specific gravity of 1.21; solution B, saturated NaCl) and ZnSO_4·7H_2O (33%; 705 g/liter; specific gravity, 1.188), and the use of different incubation times before recovery (90 min and 24 h). After flotation, the upper 1 ml of supernatant and the upper 1 ml of sediment were transferred to Eppendorf tubes and stained with the specific monoclonal antibody (Waterborne, Inc.), followed by incubation (at 37°C for 90 min or at 25°C overnight) in the dark. FC analysis was performed by using the previously optimized conditions.

**RESULTS**

**Optimization of FC protocol.** With increasing antibody concentrations, an increase in the mean intensity of fluorescence (MIF) was evident when 10^5 *E. intestinalis* spores/ml were stained and incubated overnight at room temperature and in the dark (Fig. 1). The use of the specific antibody at 10 μg/ml resulted in the highest MIF for the stained spores (10^5 spores/ml). Overnight incubation at room temperature (25°C) in the dark resulted in a higher level of fluorescence than incubation at 37°C for 90 min.

**Assessment of sensitivity and specificity.** A decrease in the MIF was registered with a reduction in the spore concentration. A detection limit of 5×10^3 spores/ml was established, since below that value the fluorescence intensity was not enough to allow discrimination of the spores. No interference with fungi or parasites occurred. Because of the similar sizes of the spores and bacteria, more events on the scatter were found when bacterial suspensions were used, although they were not stained, thus demonstrating the specificity of the staining.

**Staining for live and dead spores.** To confirm that the *E. intestinalis* spores studied were dead, PI staining was used simultaneously with the specific fluorescent antibody. When both fluorescent probes were used, dead *E. intestinalis* spores could be distinguished from other organisms (Fig. 2).

**Evaluation of stool samples.** A better separation of the stained spores from debris was obtained with the use of a saturated NaCl solution rather than a ZnSO_4·7H_2O solution. Regarding the incubation times for recovery, similar results were obtained after 90 min and 24 h. The optimal speed of...
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clinical specimens by light or fluorescence microscopy (26, 27);
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albendazole (1), no treatment is yet available for other species

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tification or determination of the viability of microorganisms.

It also requires a considerable amount of time and equipment,
as well as expertise (15, 25). The development of immunoflu-
orescent reagents (specific monoclonal or polyclonal antibod-
ies directed against microsporidial spores) has contributed to
improvements in the diagnosis of microsporidial infections. 
FC, a method based upon the evaluation of cell fluorescence,
has repeatedly been shown to be specific; to have a high level
of sensitivity, which is 10-fold greater than that of microscopy
(5); and to not be dependent on technician expertise for the
evaluation of samples, as is the case in immunofluorescence
microscopy. In addition, it provides an objective means of
analysis that allows the rapid assessment of clinical specimens
for the presence of opportunistic microsporidial organisms.
Accompanying the increasing complexity of diagnostic labora-
tory techniques, high costs, and the need for human expertise,
the use of more sensitive and automated methods, especially
for biological samples like stool samples, has been recom-
mended (9, 25).

Clinical samples usually contain many different microorgan-
isms, like parasites, fungi, and bacteria. Staining could differ-
entiate microsporidial spores from the debris normally present
in biological samples, especially if a specific monoclonal or
polyclonal antibody is used in combination with a fluoro-
chrome reagent. However, in fecal samples, cross-reactions
with other distinct microorganisms could occur, preventing the
use of polyclonal antibodies in this setting (25). In order to
confirm the specificity of the monoclonal antibody used in this
study, the possibility of cross-reactions with other organisms
was investigated by using both prokaryotic microorganisms
(Escherichia coli, Staphylococcus aureus) and eukaryotic micro-
organisms (Candida albicans, Crypto sporidium parvum, Giardia
lamblia) mixed with an E. intestinalis spore solution. The bac-
teria, fungi, and other parasites present in the mixed suspen-
sions did not interfere with the detection of the microsporidia.
We verified that bacteria showed up in the autofluorescence
zone but did not stain with the microsporidium-specific anti-
body, which was certainly because their size is similar to that of
microsporidial spores. It would be very interesting to evaluate
whether cross-reactions with Enterocytozoon bieneusi, a human
microsporidial species prevalent worldwide, would occur, but
E. bieneusi microorganisms are not commercially available. 
Also, and according to the manufacturer, the antibody used in
this study does not stain E. bieneusi because it is specific to
the genus Encephalitozoon. Specificity is a very relevant topic for
any diagnostic procedure. Our results support the use of the

FIG. 2. Two-dimensional dot plot correlating FL1 (green fluorescence, 535 nm) with FL3 (red fluorescence, 620 nm) of Encephalitozoon
intestinalis spores without staining (autofluorescence) (A), E. intestinalis spores stained with 10.0 μg/ml of specific antibody (Microspor-FA; A488;
Waterborne, Inc.) (B), and E. intestinalis spores stained with 10.0 μg/ml of specific antibody and 5.0 μg/ml of PI (C).

The diagnosis of microsporidiosis in a routine laboratory can
theoretically be performed by direct visualization of spores in
clinical specimens by light or fluorescence microscopy (26, 27);
nevertheless, it is tricky to differentiate the parasite from the
other elements, like debris, usually present in biological sam-
ples (25). Diagnosis based upon PCR assays is emerging. Apart
from being expensive, PCR presents several additional disad-
vantages, particularly because it does not allow the easy quan-
tification or determination of the viability of microorganisms.

The identification of the microsporidial species responsible
for an infection is very important, namely, to define the appro-
priate treatment, as well as to ensure its eradication from the
environment. While E. intestinalis infections are treated with
albendazole (1), no treatment is yet available for other species
(23) except Enterocytozoon bieneusi (1), the treatment of
which requires very specific drugs, like fumagillin.

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protocol described here with clinical samples, as it provides a reliable means of detection of E. intestinalis spores and clearly separates E. intestinalis spores from debris and other microorganisms. The conventional microscopic diagnostic procedure is highly dependent on the experience of the microscopist and the spore concentration in the sample. The protocol described here provides a detection limit (10^3 spores/ml) well bellow the concentration usually detected by conventional procedures (5). Additionally, the simultaneous staining with the specific antibody and PI allowed the clear distinction of dead parasites among the debris and the viable microorganisms that could be present in clinical samples. This is of extreme relevance since it allows the future study of the infectious potential of samples, as this parasite is able to resist common water treatments (9, 10).

The use of saturated NaCl solution improved the detection of the parasite comparing with the use of other solutions. This might result from the fact that spores can easily float, in contrast to other protozoan parasites like Giardia lamblia and Cryptosporidium parvum cysts and oocysts. An improvement in parasite recovery over that in our previous studies (2, 3) was also found by the use of a higher centrifugation velocity.

A new cytometric detection method is now available for the detection of E. intestinalis spores in water and stool samples, as well as for assessment of their viability based upon dye (PI) exclusion. The use of a specific antibody allows the clear discrimination between E. intestinalis spores and the debris or other microorganisms often present in water and human stool samples. We now intend to apply this protocol to the routine analysis of clinical samples, especially those from immunocompromised patients, as well as to the evaluation of environmental water samples.

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