Modified Technique To Recover Microsporidian Spores in Sodium Acetate-Acetic Acid-Formalin-Fixed Fecal Samples by Light Microscopy and Correlation with Transmission Electron Microscopy

PATRICIA L. CARTER,1 DOUGLAS W. MACPHERSON,1,2* AND RICHARD A. McKENZIE3,4

Regional Parasitology Laboratory,1 and Electron Microscopy Facility,4 St. Joseph’s Hospital, and Department of Pathology2 and Electron Microscopy Facility, Faculty of Health Sciences,3 McMaster University, Hamilton, Ontario, Canada

Received 15 March 1996/Returned for modification 3 July 1996/Accepted 31 July 1996

Microsporidia are an emerging cause of significant disease, particularly in the immunocompromised host. Until recently, the diagnosis of enteric infections has required invasive sampling, the use of expensive technology, and considerable technological expertise. The purpose of the present study was to examine three modifications to the processing of fecal specimens for light microscopy (LM) examination for microsporidian spores: the use of pretreatment with potassium hydroxide, modified centrifugation conditions, and a modified staining technique. A sodium acetate-acetic acid-formalin-fixed fecal sample containing numerous microsporidian spores confirmed to be positive by transmission electron microscopy (TEM) was used in all studies performed. A simulation of a heavy to lightly infected individual was used. The results of LM were correlated with those of TEM. Duplicate smears were stained with Weber's modified trichrome and Giemsa (GS) stains. The stained slides were randomized and examined blindly by LM at ×625 and ×1,250 magnifications. A portion of the dilutions after centrifugation were fixed for TEM. The Weber modified trichrome stain performance rating was higher than the Giemsa stain rating because of ease of interpretation, and material stained with Weber modified trichrome stain required less examination time at a lower magnification. The number of positive smears and the quantity of spores detected were significantly higher following pretreatment of the sample with KOH. TEM was positive only when numerous spores were present, but the quality of the photomicrographs was superior after pretreatment with KOH. Pretreatment of sodium acetate–acetic acid–formalin-fixed fecal samples with 10% KOH and then a 5-min centrifugation time and staining with Weber modified trichrome stain provide for the excellent recovery of microsporidia in the routine diagnostic parasitology laboratory.

Microsporidia are obligate intracellular spor-forming protozoa which infect invertebrates and vertebrates, including humans. Their presence has been reported with increasing frequency, and some species, Enterocytozoon bieneusi and Septata intestinalis, have become important causes of chronic malabsorptive diarrhea, especially in human immunodeficiency virus (HIV)-infected patients (2, 3, 5, 8, 9, 11, 19, 21, 26, 29).

Five genera of microsporidia are known to infect humans: Encephalitozoon, Enterocytozoon, Nosema, Pleistophora, and Septata (6, 14). The most commonly reported parasites are E. bieneusi (3, 5, 6, 8, 14, 19–21, 24, 26, 27, 31) and S. intestinalis (4, 28, 31), both of which parasitize the gastrointestinal tract and can be found extraintestinally (E. bieneusi infects the gastrointestinal tract only rarely, however) (31). A recent study has also described finding Encephalitozoon cuniculi as a cause of intestinal microsporidiosis, confirmed by transmission electron microscopy (TEM) and PCR (13). The small size (1 to 2 by 0.5 to 1.0 μm) and poor staining qualities of E. cuniculi organisms have impeded detection by usual parasitological and histological techniques (19). Light microscopy (LM) with either Weber’s modified trichrome (WMT) staining (30) or Giemsa staining (19, 22, 26, 27) is the standard technique for detecting microsporidiosis in clinical parasitology laboratories (6).

TEM has long been considered the “gold standard” for the diagnosis of microsporidia in tissue biopsy specimens and aspirates of body fluids (5, 14, 19, 24, 27). The invasive techniques required to obtain the specimens (22) and the decreased ability of very ill patients to withstand the procedures may have resulted in the underreporting of the organisms as a cause of chronic malabsorptive diarrhea, chronic sinusitis, interstitial nephritis, keratoconjunctivitis, hepatitis, peritonitis, and disseminated microsporidiosis. Fecal samples have since been shown to be as good as or in some cases better than biopsy or duodenal aspirates (7, 27).

The purpose of the investigation described here was to optimize the conditions of processing and staining of fecal specimens for the examination of microsporidian spores. It was hypothesized that reducing the mucus, which is often present in chronic diarrhea stool specimens, would increase the recovery of spores from infected individuals shedding low numbers of spores. Potassium hydroxide was chosen as the mucolytic agent. Dilutions were prepared to simulate heavily to lightly infected patients.

The effect of centrifugation on the recovery and yield of microsporidian spores was also examined. Some investigators have suggested that no concentration procedure is necessary to detect spores (15, 30). The results of centrifugation sedimen-
tation and LM were compared with those of TEM to determine the breakpoint of positivity by each technique and the agreement between the methods.

MATERIALS AND METHODS

A sodium acetate-acetic acid-formalin (SAF)-preserved (32) fecal sample containing mucus and numerous microsporidial spores detected by LM and WMT staining and confirmed by TEM as *S. intestinalis* spores was chosen for testing in the study. The specimen was obtained from an HIV-infected patient.

Laboratory investigations. (i) Stool specimen. Two aliquots were prepared. The first aliquot of the specimen was diluted with normal saline with manual mixing in four sets of 10-fold serial dilutions of 10⁵ to 10⁻¹. Set 1 represented a SAF-fixed unconcentrated fecal sample with decreasing quantities of microsporidial spores and mucous. No centrifugation was used. The remaining three sets were processed and concentrated by centrifugation in a Beckman AccuSpin centrifuge, as follows: set 2, 5 min at 700 × g (2,000 rpm), with the supernatant discarded; set 3, 10 min at 700 × g, with the supernatant discarded; and set 4, 2 min at 400 × g (1,500 rpm), with supernatant decanted into a second tube and centrifuged for an additional 10 min at 700 × g and then the supernatant was decanted and discarded.

A second aliquot of the SAF-preserved specimen was diluted with a mucolytic agent (10% [wt/vol] to 50% [vol/vol] KOH) and allowed to stand for a minimum of 10 min or until mucous threads were macroscopically dissolved and then processed as described above for sets 1 to 4. Preliminary studies were done with KOH-treated feces to ensure that the KOH did not adversely affect or interfere with the WMT staining or Giemsa processed as described above for sets 1 to 4.

(ii) Smears. Smears were prepared in duplicate from the sediment with a Medical Laboratory Automation Inc. (Mount Vernon, N.Y.) micropipettor and by applying 20 μl of sediment to the area adjacent to the frosted end of each slide. The suspension was spread as thinly as possible if any sedimental material was visible. If the specimen was very dilute, the spot was allowed to dry without spreading. Smears were dried at 50°C and then methanol fixed for 5 min and air dried. Two of the four smears prepared from each dilution at various centrifugation times were stained by either WMT staining (15) or Giemsa staining (1/10 dilution). The stained smears (a total of 272 smears plus positive and negative controls) were coded and randomized by an independent technologist for blind interpretation by the microscopist with a Leitz light microscope with a 625 magnification. Suspected spores were confirmed at ×625 magnification. Giemsa-stained smears were examined at ×1,250 magnification.

The diagnostic yield results for set 1 (no centrifugation) were inconsistent across all dilutions within the set. No predictable comparisons could be made with any of the centrifuged samples. No further presentation of results for set 1 will be made. Sets 3 and 4 produced results comparable to those for set 2, but the use of sets 3 and 4 entailed considerable additional processing costs. For these reasons, only the results for sets 2 are presented and discussed here. A summary of the results for set 2 are presented in Table 1.

Although use of Weber’s modification of the chroomatoprobe-based stain required lengthy processing (approximately 2 h), it fit easily into the routine of the laboratory and was preferred over use of Giemsa stain for several reasons, the first being ease of interpretation of the results. Very little background material stained similarly to the microsporidial spores, because most of the background was pale green to almost colorless. The dark pink to nearly red spores were readily recognizable at ×625 magnification and were quickly confirmed at ×1,250 magnification. Smear examination time, which ranged from 30 s to 3 min for most observations at the lower magnification, required decreased light intensity with the WMT stain compared with that required with the Giemsa stain. This lessened the eyestrain for the microscopist.

The results for the Giemsa-stained smears were difficult to

### TABLE 1. Comparison of staining and KOH pretreatment in the detection of microsporidial spores

<table>
<thead>
<tr>
<th>Stain and KOH pretreatment</th>
<th>Cost (Canadian $)</th>
<th>Processing time (min)</th>
<th>Examination time (min)</th>
<th>Total cost</th>
<th>Ease of examination</th>
</tr>
</thead>
<tbody>
<tr>
<td>WMT stain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>0.2092</td>
<td>1.808</td>
<td>11</td>
<td>2</td>
<td>6.71</td>
</tr>
<tr>
<td>No pretreatment</td>
<td>0.2092</td>
<td>1.3424</td>
<td>8</td>
<td>6</td>
<td>7.62</td>
</tr>
<tr>
<td>Giemsa stain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
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<td>1.4581</td>
<td>18</td>
<td>22</td>
<td>17.67</td>
</tr>
<tr>
<td>No pretreatment</td>
<td>0.2092</td>
<td>1.5418</td>
<td>15</td>
<td>13</td>
<td>8.75</td>
</tr>
</tbody>
</table>

* Data are for set 2 only. On the basis of the diagnostic yield scale of the Laboratory Proficiency Testing Program (18) of from rare to numerous, all samples yielded rare numbers of organisms (all were detected at a 10⁻¹ dilution).

* Cost (in 1995 Canadian dollars) to examine two stained slides. Technologist’s time calculated at $Can24.00/h. Costs are rounded to the nearest cent.

* Scale, 1 (easiest) to 5 (most difficult). WMT-stained smears were examined at ×625 magnification; Giemsa-stained smears were examined at ×1,250 magnification.

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### RESULTS

The diagnostic yield results for set 1 (no centrifugation) were inconsistent across all dilutions within the set. No predictable comparisons could be made with any of the centrifuged samples. No further presentation of results for set 1 will be made. Sets 3 and 4 produced results comparable to those for set 2, but the use of sets 3 and 4 entailed considerable additional processing costs. For these reasons, only the results for set 2 are presented and discussed here. A summary of the results for set 2 are presented in Table 1.
interpret because the colors of the spores and all background were similar shades of blue, gray, and blue-gray. Microsporidian spores in KOH-treated specimens stained inconsistently as a bright robin’s egg blue. Because there was very little difference between the spores and the background, the amount of magnification (×1,250), the level of illumination, and the examination time increased significantly. Examination of Giemsa-stained smears requires a high level of intensity and experience for reliable interpretation. This has also been noted in other studies (7, 27). Although the staining results obtained with Giemsa stain and WMT stain agreed 86% of the time, the results for Giemsa-stained smears were more difficult to interpret, and lengthy examination times and higher magnifications and illuminations were required for Giemsa-stained smears than for WMT-stained smears.

Centrifugation improved the capacity to detect small numbers of spores in fecal samples. The addition of KOH as a mucolytic agent prior to smear preparation eliminated the need to select a mucus fleck in sampling and increased sensitivity and yield. In KOH-treated smears, spores were evenly dispersed in the fluid and across the slide compared with spores in untreated specimen.

Giemsa-stained smears were considered as a comparison, but results for the Giemsa-stained smears were not used in the following results and statistics.

(i) Concentration, no added KOH, and WMT staining. The results for the sets that were concentrated but that did not contain KOH were marginally improved compared with results for sets which were concentrated and had KOH added, but the results were unpredictable and did not correlate well with the expected results. Spore quantification could not be reliably reproduced when a mucus strand was not obtained in the sampling. In addition, little agreement was found between the duplicate smears of each dilution, regardless of the centrifugation time and the relative centrifugal force used.

(ii) Concentration, KOH added, and WMT staining. The positive and negative results for the duplicate smears were in very close agreement (99.5%). The centrifugation times by the techniques studied gave excellent results, with a detection limit of 10^{-3} dilution in all cases. The treatment of specimens with KOH prior to any of the three specified centrifugation times served to increase the recovery of spores over that from unconcentrated direct smears.

The incremental costs of the reagents (KOH) and processing time (centrifugation) for the KOH-treated, spun, WMT-stained specimens were more than offset by ease of examination, the reduced examination time, and the greater yield of microsporidian spores, especially at low concentrations.

(iii) Correlation of LM to TEM. The smears containing numerous to rare quantities of spores were detectable with little difficulty by LM with WMT stain. The recorded LM examination time per slide varied from less than 30 s for numerous quantities of spores to up to 10 min for rare quantities of spores. Most of the positive observations with WMT staining were made within 30 s for slides containing few to numerous spores. The smears had considerably less debris when KOH treatment was used, allowing for a shorter examination time by LM, with easy visualization of spores and less background material for rapid scanning.

A total of 46 observations of specimens containing numerous to rare spores were made by TEM. Dilutions which did not contain a visible pellet after centrifugation were unable to be processed further for TEM and were considered negative. TEM detected spores of many to numerous quantities. In the present investigation, rare to moderate quantities of spores could not be detected by TEM. In a random sample it is technically possible to detect small numbers of spores (19), provided that the specimen contains other usual material such as debris, yeasts, and bacteria. Less than half of the positive LM samples prepared for TEM were positive for microsporidia, showing a decrease in sensitivity from the LM technique (samples treated with KOH, concentrated, and stained with WMT stain). The observation that TEM is less sensitive than LM for the detection of spores in fecal samples containing low numbers of spores because of the small volume of specimen examined has been reported by other investigators (6, 30). It has been noted that TEM is often required for species identification (6, 20, 24, 30). New procedures that use serodiagnosis and PCR are under development but are not readily available.

KOH treatment did not increase the sensitivity of TEM, since the rate of spore recovery for both KOH-treated and untreated feces was the same. KOH treatment did intensely boost the ease of infiltration of the fixative and resin, which enhanced the definition of the characteristic ultrastructures.

DISCUSSION

The addition of the mucolytic agent 10% (wt/vol) KOH as a first step in the processing of SAF-fixed fecal samples resulted in the even dispersal of microsporidian spores in specimens used directly and in concentrated specimens. After the addition of KOH and in combination with centrifugal sedimentation for 5 min at 700 × g, the recovery and yield of spores were significantly increased. It was clearly demonstrated that the value of placing specimens directly on microscope slides is significant only when the number of spores present is high and the sample includes a mucus thread.

Other investigators have suggested that centrifugation is unlikely to result in increased recovery of microsporidian spores (19, 30). The present study indicates that treatment of fixed fecal samples with KOH prior to centrifugation does produce a higher yield of spores than is possible from an unconcentrated specimen. Serial dilutions of a specimen confirmed to be positive for microsporidia show a predictable curve with declining numbers of spores that was unachievable with the same dilutions of uncentrifuged specimen used directly after sampling.

The examination time per slide required to exclude a diagnosis of microsporidian infection is 10 min. It was found that beyond this limit of time and after meticulous interpretation of the slide, any additional spores could rarely be found. Testing of a second sample may be necessary if the first specimen is found to contain no detectable spores because of the uneven distribution of spores (17).

Prolonged storage (up to 6 months) of the WMT-stained slides was done in the laboratory. Prolonged storage at room temperature and in darkness had little effect on the quality and intensity of staining of the stained slides, enabling long-term records and teaching sets to be kept available. The results of the present study indicate that fecal samples can be quickly and easily concentrated in a routine parasitology laboratory and that regardless of the centrifugation time used, the addition of KOH before the concentration step will give reliable and reproducible results in the diagnostic laboratory, with minimal disruption to the workflow and other standard testing procedures.

It is not recommended that the portion of specimen treated with KOH be used in the formalin-ether concentration technique (23) because further study is necessary to determine if there is any effect on the cysts, trophozoites, and ova of other parasites that may be present.

TEM of fecal samples is less sensitive than the LM tech-
nique with or without KOH treatment and staining with WMT stain when the numbers of spores present is low. The results of TEM correlates well with those of LM for microsporidian spore-rich specimens. The addition of KOH enhances spore fixation in glutaraldehyde and the quality of the photomicrographs of microsporidian species. Definitive diagnosis is possible by LM even with the presence of only rare spores. Although TEM remains the gold standard for the diagnosis of this organism in biopsy specimens (10, 12, 19, 30), its value as a confirmatory tool or stand-alone test is questionalbe in cases of very low numbers of spores or for specimens from HIV-negative immunocompromised patients. HIV-positive patients infected with microsporidia may benefit from treatment with albendazole, which may result in a shortened duration of symptoms and the prevention and delay of microsporidian dissemination, thereby providing an increased quality of life for the patient (4, 10, 16).

The results of the present studies indicate that fecal specimens preserved in SAF should be treated with KOH and concentrated by centrifugal sedimentation before being stained with WMT stain in investigations for microsporidian spores. Examination of the thin smears must be meticulous, and the presence of any suspicious structures should initiate further examination or sampling. If no spores are found, an additional sample should be examined. For a positive finding, the species of the organism should be determined by TEM if the number of spores present exceeds 25 per coverslip; otherwise, because of the small sample size, spores may be undetectable by this technique.

The method of KOH treatment with centrifugation has significant advantages over the standard technique of directly staining the specimen without concentrating it when multiple attributes of the test system are considered: reagent cost, processing time, observer fatigue, technologist examination time, and yield of spores. Additional evaluation of these modifications with other stool preservatives, such as polyvinyl alcohol and 10% formalin, is warranted.

ACKNOWLEDGMENTS

We express our appreciation to colleagues who advised and assisted with the study: K. Lafferty, W. MacQueen, R. McQueen, and Y. Sooan.

REFERENCES