Detection and identification of gastrointestinal microsporidia using non-invasive techniques

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Abstract

Aims—To detect enteric microsporidia in faecal specimens from patients with the acquired immunodeficiency syndrome (AIDS), and to identify the spores to species level without using invasive procedures.

Methods—Formalised faecal preparations were examined using a modification of the strong trichrome staining method to demonstrate microsporidian spores. Six positive specimens were prepared for electron microscopy by emulsification and separation using a 9% Ficoll gradient.

Results—The modified staining technique readily identified microsporidian spores. Spores of different species showed variation in size. Identification using electron microscopy was successful for five of the six positive specimens examined. It was unsuccessful for one specimen in which spores were less abundant on initial staining.

Conclusions—The modified strong trichrome staining method is a useful way of detecting spores of intestinal microsporidia in faecal specimens. Variation in spore size may permit provisional identification by light microscopy. Electron microscopic examination of faecal preparations is useful for identifying spores to species level.

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Microsporidia are primitive eukaryotic intracellular protozoan parasites with a characteristic spore mechanism. They have recently come to attention as opportunistic pathogens in the acquired immunodeficiency syndrome (AIDS). Three species of microsporidia are felt to cause most cases of clinical infection in this patient group: *Enterocytozoon bienersi*, *Septata intestinalis* and *Encephalitozoon hellem*.¹ Of these, the first two infect the enterocytes of the small intestine and are associated with chronic diarrhoea. *S. intestinalis* tends to cause disseminated disease more often than *E. bienersi* but the latter has been found in the biliary tract in cases of sclerosing cholangitis.¹²

In the past detection of enteric microsporidia has required examination of small intestinal biopsy material by light and electron microscopy.¹ Recently, alternative methods for detecting microsporidian spores in faecal specimens have been investigated to avoid the

need for invasive procedures. Giemsa staining is not satisfactory for routine use on faecal specimens as it is insensitive.³ Fluorescence microscopy is more sensitive,¹³ but adequate facilities are necessary. The chromotrope based modified trichrome staining method described by Weber *et al*⁴ is very useful. However, it is a lengthy technique and detection of spores by this method is difficult when they are scanty.¹

Identification of microsporidia to species level by electron microscopy has been mainly of taxonomic interest. However, the advent of albendazole as a therapeutic option and an increased awareness of the natural history of microsporidiosis caused by different species highlights its importance, but electron microscopy of faecal specimens is both difficult and time consuming.

This study examined ways of making the detection and identification of microsporidia without using invasive techniques, more practicable in the routine laboratory.

Methods

SLIDE PREPARATION AND STAINING

A drop of formalised faecal homogenate (about two volumes 10% formalin to one volume faeces) was placed at one end of a microscope slide and spread over the entire surface of the slide using an orange stick, making a very thin smear. The slides were dried in air and fixed for five minutes in methanol before staining. A modification of Weber's staining method⁴ described by Kokoskin *et al* was used.⁵ At least 100 high power fields were examined before a specimen was declared negative, as recommended by Bendall and Chiodini.⁶

PREPARATION OF FAECAL SPECIMENS FOR ELECTRON MICROSCOPY

Six positive faecal specimens were prepared for electron microscopy by a simple method which helps to remove faecal debris. Approximately equal volumes (1-2 ml) of formalised stool and detergent solution (0.1% Triton) were emulsified until the mixture was fluid enough to be taken up in a disposable Pasteur pipette. A 10 ml centrifuge tube was one third filled with 9% Ficoll solution and about 2 ml of the test faecal solution was carefully layered onto the Ficoll so that it remained on top. The test tube was left to stand in an upright position for six to seven hours, after which time four distinct layers could be identified, two above and one below the Ficoll solution. The topmost layer and interface with the next layer was removed

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Figure 1 (A) Spores of *S. intestinalis*. (B) Spores of *E. bienewsi*. Transverse banding, characteristic of microsporidia, is clearly visible in one spore (modified strong trichrome stain). (Measuring bar = 4.0 μ m.)

and centrifuged at 6500 rpm for five minutes to pellet the spores present. The pellet was washed with distilled water and, after a further centrifugation, resuspended in 0.25–0.50 ml

Figure 2 Electrophotomicrograph of a spore of *S. intestinalis*, showing the coiled filament arranged in a single row.

distilled water. A drop was placed on a microscope slide and stained as described above to confirm the presence of microsporidian spores before the preparation was examined by electron microscopy.

ELECTRON MICROSCOPY

The method used was similar to that described by McCaul *et al.*⁷ The faecal preparation was pelleted, fixed in buffered 3% glutaraldehyde, embedded in 2% Nobel agar, and postfixed in buffered 1% osmium tetroxide. Dehydration of the preparation using methanol solutions of increasing concentration was followed by embedding in resin and polymerisation at 60°C for 24 hours. Ultrathin sections were mounted on copper grids, stained with Reynolds lead citrate and examined on a JEOL 100 CX electron microscope.

Results

Staining using the modified Weber's method gave better definition with less uptake of stain by faecal elements other than spores. This made the microsporidian spores, which appear pink, more distinct and easier to detect (figs 1A and 1B) and confirms the experience of Kokoskin *et al.*⁵ who introduced this modification. Electron microscopy of the positive faecal specimens was successful in five of six cases. Three contained spores of *S. intestinalis* (fig 2), two contained spores of *E. bienewsi* (fig 3), while no spores were seen in one preparation. In this last case it had been noted that the microsporidian spores were scanty when the faecal specimen was examined by light microscopy.

Although many bacteria were evident in the electron microscopy preparations, the spores of microsporidia were easily distinguished. Identification to species level was achieved by examination of the arrangement of the coiled filament, *S. intestinalis* having a single row (fig 2) and *E. bienewsi* a double row (fig 3). On light microscopy, the spores subsequently identified as *S. intestinalis* (fig 1A) tended to be larger than those confirmed as *E. bienewsi* (fig 1B) (1.5–2.0 $\times$ 0.5–1.0 μ m for *S. intestinalis* compared with 1.0–1.5 $\times$ 0.5–1.0 μ m for *E. bienewsi*).

Discussion

It is now possible to detect microsporidia in the gastrointestinal tract and identify them to species level, without recourse to invasive endoscopic procedures and biopsy, an advantage in cases where patients are too ill to undergo endoscopy. As microsporidiosis usually occurs at an advanced stage of infection with HIV—that is, when the CD4 lymphocyte count is below 100/ μ l,¹ patients are often ill with other AIDS associated conditions and invasive procedures may not be feasible. The modification of the strong trichrome staining technique, as described by Kokoskin *et al.*, has been adopted for routine use at the Hospital for Tropical Diseases. It is more rapid than the original technique, and the clearer background and more intensely staining spores makes detection of microsporidia easier.

Figure 3 Electrophotomicrograph of a spore of *E. bienewisi*, showing the coiled filament arranged in a double row.

Identification of microsporidian spores is likely to be of increasing clinical importance. *S. intestinalis* is felt to be more susceptible than *E. bienewisi* to albendazole, currently the most promising therapeutic option in microsporidiosis.¹ In addition, the spectrum of infections caused by each species differs; those infections due to *S. intestinalis* are more likely to become disseminated. Identification of microsporidian spores from faecal specimens using electron microscopy is now practicable. If spores are scanty in the initial faecal examination, electron microscopy may not be successful, presumably because preparation of the specimen for electron microscopy results in some spore loss and because very fine sections are examined which may not contain spores. Spore loss during preparation does not appear to be significant when spores are initially abundant but may be so when they are scanty. The method devised in this paper for preparation of a faecal specimen for electron

microscopy is simple and easy to perform in a non-specialist laboratory. Other workers have found that concentration procedures such as centrifugal sedimentation, flotation based stool concentration and centrifugation through discontinuous gradients fail to concentrate microsporidian spores and do not adequately separate spores from bacteria.⁴ This finding is probably because of the small size of the spores. The method described here, however, involves an initial step which does not necessarily concentrate spores but removes larger faecal debris without too great a spore loss. Centrifugation is then used to concentrate the spores. Separation of spores from bacteria remains a problem because of their similar size, but this has not been a major obstacle to identification of microsporidia in the preparations obtained in this study.

Although there is an overlap in their size range, the spores of *E. bienewisi* (fig 1B) are consistently smaller and more rounded than those of *S. intestinalis* (fig 1B), a fact which should afford an experienced light microscopist the opportunity to identify microsporidian spores provisionally while electron microscopy is awaited, or in situations where electron microscopy is unavailable.

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