317

The development and performance of a simple, sensitive method for the detection of *Cryptosporidium* oocysts in faeces

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SUMMARY

Features are described of a new staining method for the detection of *Cryptosporidium* oocysts in faeces. Smears, fixed in acid-methanol, are stained with heated safranin and counterstained with methylene blue. Oocysts stain a vivid orange-pink and are easily recognized. The method is rapid and simple with little source of error. The method is more sensitive than currently recommended Ziehl-Neelson methods; of 26 cases of cryptosporidiosis diagnosed by the new method only 19 were detected by acid-fast and only 11 by acid-alcohol-fast methods. Oocysts can be stained by safranin-methylene blue after concentration by various methods, in paraffin-embedded material, and after storage for months at various temperatures.

INTRODUCTION

The protozoan *Cryptosporidium* is a cause of enterocolitis in various animal species (Angus, 1983; Tzipori, 1983), and increasing interest is being shown in the parasite as a cause of human infection (Schultz, 1983; Editorial, 1984). In immunodeficient or immunosuppressed patients the infection is severe and the prognosis poor, but in otherwise healthy patients the infection runs a less serious, self-limiting course (Current *et al.* 1983; Tzipori *et al.* 1983; Pitlik *et al.* 1983). Laboratory diagnosis is made by recognition of *Cryptosporidium* oocysts in faecal smears stained by Giemsa (Tzipori *et al.* 1980) or modified Ziehl-Neelson methods (Henriksen & Pohlenz, 1981; Garcia *et al.* 1983).

We find the Giemsa technique inconvenient because of the lengthy staining period, the critical decolorization stage, and poor colour contrast which necessitates oil-immersion microscopy. Ziehl-Neelson methods also have a critical decolorization stage, and are still rather slow, although colour contrast is good.

We used these methods to detect cryptosporidiosis in a children's hospital (Baxby, Hart & Taylor, 1983), but felt that improved methods might be devised. Our chosen method, heated safranin counterstained with methylene blue (S-MB) has been outlined very briefly elsewhere (Baxby & Blundell, 1983). Here we provide more details of the method, partly in response to correspondents who asked for amplification of our original short account. In addition we present evidence that the S-MB method is more sensitive than currently used methods. Also included is information on the staining of oocysts prepared by various concentration techniques, and also stored under various conditions.

MATERIALS AND METHODS

Oocysts

Faecal samples containing *Cryptosporidium* oocysts were obtained during a survey of enterocolitis in a children's hospital. In all samples became available from 36 cases. Samples were stored at $+4$ °C in tightly sealed plastic bottles until used.

Safranin-methylene blue staining technique

The method is described here in detail, but various points will be dealt with below under 'Results'.

(1) Smear the sample, diluted in saline if necessary, on a microscope slide to a thickness slightly greater than necessary for routine bacteriological examination.

(2) Air dry.

(3) Fix briefly, by one pass through the bunsen flame.

(4) Fix in 3% HCl in 100% methanol, 3–5 min.

(5) Wash with water.

(6) Stain with 1% aqueous safranin, 60 sec (Paramount Reagents, Bootle, Merseyside). Heat thoroughly, preferably until boiling occurs. Add more stain and continue heating if necessary.

(7) Wash with water.

(8) Counterstain with 1% methylene blue, 30 sec (Paramount Reagents).

(9) Wash with water, blot dry.

(10) Fix coverslip with suitable mountant, e.g. D.P.X. (B.D.H.).

Examine using X20 objectives.

Modified Ziehl-Neelson staining methods

Modified Ziehl-Neelson techniques are currently recommended for the diagnosis of cryptosporidiosis. We used three variations: (a) a slow, cold stain decolorized by 0·25–10% H_2SO_4 (Henriksen & Pohlenz, 1981), (b) a short, hot method decolorized by 5% H_2SO_4 (Garcia *et al.* 1983), (c) a short, cold method decolorized by 3% HCl in 95% ethanol (D. R. Snodgrass, pers. comm.).

Concentration of oocysts

The methods tested were the sucrose-phenol flotation method (Current *et al.* 1983), formalin–ether sedimentation, and zinc sulphate flotation (Adam, Paul & Zaman, 1971). With the flotation methods the S-MB technique was tested on smears made from the meniscus, and on material which had been washed and deposited by dilution and centrifugation of the upper layer of the flotation stage.

Histology

The S-MB stain was tested on faecal material containing oocysts which had been variously fixed (see Results) and embedded in paraffin wax.


EXPLANATION OF PLATE 1

A   Smear of faeces from a case of cryptosporidiosis stained with safranin-methylene blue. This particular specimen and field was chosen to show *Cryptosporidium* oocysts (orange-pink) and yeasts (blue) ($\times 1500$).

B   Smear of faeces from a case of cryptosporidiosis stained by a hot Ziehl-Neelson method and decolorized by acid-alcohol. A different specimen from that in (A) and selected to show the poor retention of carbol fuchsin ($\times 1500$).
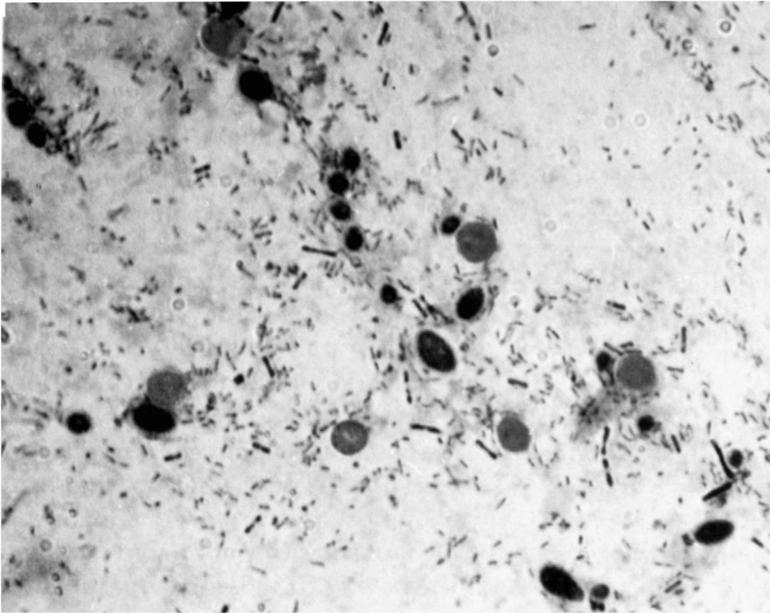
1A
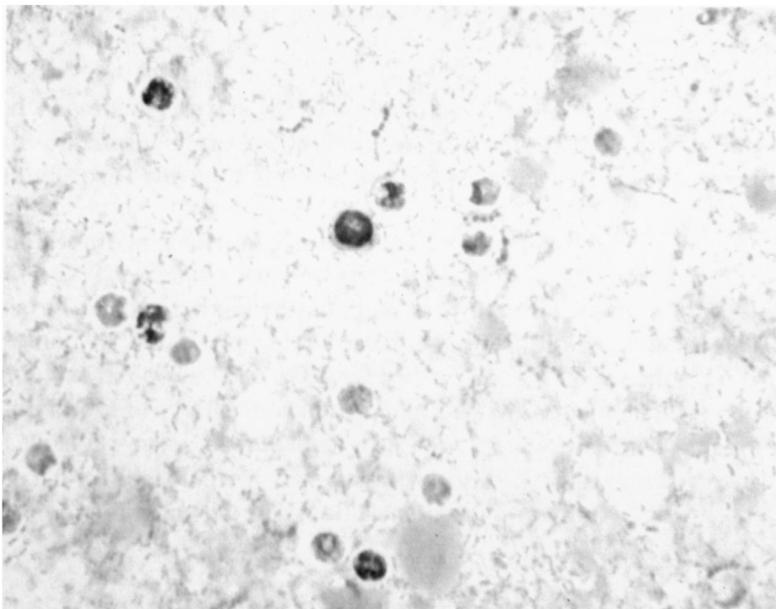
1B

D. BAXBY AND OTHERS

Storage of oocysts

Aliquots of faeces containing large numbers of oocysts were stored at -70, -20, $+4$ or $+22$ °C. Smears were stained after increasing periods of storage.

RESULTS

Safranin-methylene blue staining technique

In spite of the detailed and lengthy description given above, the S-MB staining technique is extremely simple and rapid.

When stained as described above, *Cryptosporidium* oocysts are seen as vivid orange-pink bodies approximately 5 μm diam., usually spherical or slightly ovoid (Plate 1A). The sporozoites within the oocyst stain slightly darker and are sometimes arranged around the periphery. This, which may be a fixation artifact, produces a roughly doughnut or horseshoe-shaped effect.

In our experience only *Cryptosporidium* oocysts have the combination of size, shape, internal structure and staining characteristics described above. *Giardia* and *Entamoeba*, for example, are larger and take up the counterstain, as do yeasts. Most faecal debris and bacteria also stain with methylene blue, but bacterial spores and occasional unidentified rods stain with safranin. Faecal debris occasionally retains safranin, and a small proportion of samples contain 'acid-fast bodies'. These are usually 3–4 μm diam. but occasionally larger, stain with safranin but are quite different from oocysts and have not been a source of confusion. They stain an even red-purple with no evidence of internal structure and have a thick rim of counterstain. Originally thought to be a type of yeast, they could not be cultured and, as they are not present in ether-extracted material, are probably fat droplets.

Fixation

Smears fixed chemically, without heat, had fewer stained oocysts and correspondingly more unstained 'holes'. These were caused by oocysts becoming detached. A single pass through the bunsen flame helps to attach oocysts more firmly to the slide.

Formol saline, Bouin and formol-Zenker fixation only worked if, after treatment with these fixatives, the smears were washed with acid-methanol before staining. Similarly, smears fully heat-fixed required this additional wash, and in this case the oocysts stained rather darker. Hydrochloric or sulphuric acid at 1–10 % in 100 % methanol was effective. The staining method could be speeded up by using full heat fixation and a brief acid-methanol wash. In practice we prefer to use 3 % HCl-methanol without prior chemical or full heat-fixation. Fixed smears which had been stored for more than a few days required a further brief wash with acid-methanol before staining to give full satisfactory results.

Heated safranin

Attempts were made with acidic, alkaline, alcoholic and carbol safranin, with various fixatives and mordants, to circumvent the necessity for heat during the safranin stage. None was successful.

Oocysts heated so that no steam appeared did not retain the stain, and the best results were obtained if the stain was allowed to boil on the slide; this would also inactivate oocysts not killed by acid-methanol treatment.

Counterstain

Because no separate decolorization stage is used, and the counterstain performs this function, the relative concentrations of primary and counterstain are important. One per cent methylene blue gave the best results; 0·1 % crystal violet was less successful, and malachite green, alcian blue and aniline blue were unsatisfactory.

Diagnosis of cryptosporidiosis

As with any method which relies on direct microscopy rather than isolation of the pathogen, care should be taken to avoid transfer of the agent from strongly positive to negative slides. In practice we process up to eight slides at a time, spaced on a rack over the sink, heated from below by a bunsen burner.

We initially investigated cryptosporidiosis using Ziehl-Neelson stain decolorized by 3 % HCl in 95 % ethanol (D. R. Snodgrass, pers. comm.). Using this method we diagnosed ten cases. By that time we had developed the S-MB method and divergent views were available on the sensitivity of acid-fast stains (Miller, Holmberg & Clausen, 1983; R. A. Miller, pers. comm.; Garcia *et al.* 1983). Consequently we examined smears from the original diagnostic specimens by S-MB and Ziehl-Neelson techniques (Table 1). The principal factor affecting the results obtained with Ziehl-Neelson techniques was the choice of decolorizer. Consistently more oocysts were detected when acid only was used. However, the S-MB technique proved superior to the acid-fast method. In only two samples did acid-fast staining detect as many oocysts as S-MB. Plate 1B shows the result of acid-alcohol-fast staining on a fresh sample of faeces containing many oocysts, which retained the carbol fuchsin poorly.

Subsequently we diagnosed 26 more cases (23 human, three feline) using the S-MB method. In 20 human cases it was possible to make a semi-quantitative assessment of the number of oocysts detected by the different staining techniques (Table 2). Again the S-MB proved superior and consistently more oocysts were stained by S-MB than by the Ziehl-Neelson methods. Of the 20 cases only 15 would have been diagnosed by acid-fast and only nine by acid-alcohol fast methods. In the remaining six cases oocyst numbers were too low to permit semi-quantitative assessment. However, two of these were recorded as positive by acid-alcohol-fast and four by acid-fast techniques.

Staining of concentrated oocysts

Oocysts concentrated by zinc sulphate flotation or formalin-ether sedimentation methods stained well by S-MB. When sucrose-phenol flotation was used, oocysts smeared directly from the meniscus did not retain safranin, but did so when diluted in saline and washed and deposited by centrifugation.

Staining of paraffin-embedded oocysts

Oocysts could be stained by S-MB in faecal material which had been suitably fixed and embedded; an acid-methanol wash was necessary immediately before the safranin stage. Oocysts retained the safranin if sections were air-dried and mounted after staining. If stained sections were dehydrated through graded alcohols and cleared in xylene before mounting, safranin retention was poor.

Table 1. *Staining of oocysts in faeces from patients in whom cryptosporidiosis was originally diagnosed by acid-alcohol-fast stain*

	Number of cases, and assessment of oocyst staining*					
Stain ...	−	+	+ +	+ + +	+ + + +	Total†
Acid-alcohol-fast	0	4	4	2	0	10/10
Acid-fast	0	1	3	3	2	9/9†
S-MB	0	0	0	0	9	9/9†

* −, All oocysts stained by counterstain. +, Occasional oocyst stained by primary stain (<10%). + +, Moderate numbers stained by primary stain (10–50%). + + +, Substantial numbers stained by primary stain (50–90%). + + + +, Virtually all stained by primary stain (>90%)

† One sample, rated + + by acid-alcohol-fast stain, was not available for test with other stains

Table 2. *Staining of oocysts in faeces from 20 patients in whom cryptosporidiosis was originally diagnosed by SM-B stain*

	Number of cases, and assessment of oocyst staining*					
Stain ...	−	+	+ +	+ + +	+ + + +	Total†
Acid-alcohol-fast	11	3	4	2	0	9/20
Acid-fast	5	2	5	5	3	15/20
S-MB	0	0	0	0	20	20/20

* See key to Table 1

† Figure gives number of cases in which any oocysts were stained by primary stain.

Storage of oocysts

Oocysts in some samples of faeces, although acid-fast when fresh, lost their acid fastness to some extent when stored at +4 °C. This loss was unpredictable; in one sample *c.* 50% were still acid-fast after 8 months storage, but in another < 10% were acid fast after 4 months. Oocysts in faeces were fully stained by S–MB after storage at −70, −20, +4, and 22 °C for at least 4 months. We also have samples of faeces in which 90–100% oocysts can be stained after storage for 8 months at +4 °C.

Frozen oocysts could be fully stained with S-MB after being thawed once. After two cycles of freeze-thaw only *c.* 30% could be stained, and *c.* 5% after three cycles. After a fourth cycle only the occasional oocyst would retain safranin.

DISCUSSION

At present, laboratory diagnosis of cryptosporidiosis can most conveniently be made by examination of faeces for oocysts. This can be a slow and labour-intensive exercise. In starting the work described here our aim was to develop a method that was quicker and simpler than the ones currently in use.

When diagnosis is made by direct microscopy of faeces, sensitivity is important. Consequently it is of interest to note that oocysts which do not retain carbol fuchsin in modified Ziehl-Neelson methods can be stained with S-MB. Since introducing

the S-MB method we have diagnosed 26 cases of cryptosporidiosis, only 19 of which would have been recognized by acid-fast and only 11 by acid-alcohol-fast methods.

Since this paper was first written, further attention has been drawn to the failure of Ziehl-Neelson modifications to stain oocysts and phenol-auramine methods have been suggested as alternatives (Casemore, Armstrong & Jackson, 1984; Nichols & Thom, 1984). These techniques are relatively slow and require access to a fluorescence microscope. In addition Casemore *et al.* (1984) use their method to screen samples and recommend that separate smears from 'positive' material be checked by other methods. Yeasts are present in some samples of faeces and the importance of distinguishing between them and oocysts has been stressed (Angus *et al.* 1981; Ma & Soave, 1983). This has not been a problem with the S-MB method which has given unequivocal results. The method is rapid and simple, providing attention is paid to the necessity for acid-methanol treatment before and vigorous heating during the safranin stage. Some old safranin powders may give unsatisfactory results but the liquid concentrates produced by Paramount Reagents have performed consistently well.

Cryptosporidiosis is relatively uncommon and the parasite is proving difficult to cultivate. The fact that oocysts can still be stained after long term storage will help to ensure a constant supply of suitable reference material for teaching and diagnosis.

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