A comparative assessment of commonly employed staining procedures for the diagnosis of cryptosporidiosis

D. MOODLEY, T. F. H. G. JACKSON, V. GATHIRAM, J. VAN DEN ENDE

Summary

Following an increase in the number of reports of *Cryptosporidium* infections and the problems encountered in detecting these organisms in faecal smears, a comparative assessment of a modification of the Sheather's flotation technique and other commonly employed staining procedures proved the modified Sheather's technique to be most useful in identifying *Cryptosporidium* oocysts in diarrhoeal stools. This technique not only detected the parasite in the highest number of stools but also proved to be cost-effective and the least time-consuming. Other staining techniques assessed were the modified Ziehl-Neelsen, safranin-methylene blue and auramine-phenol fluorescence. Both the modified Ziehl-Neelsen and the auramine-phenol fluorescence procedures produced nonspecific staining, while the safranin-methylene blue method was found to be the least sensitive technique.

S Afr Med J 1991; **79:** 314-317.

Since the description of *Cryptosporidium* as an aetiological agent in diarrhoeal illness in 1976,[1] the parasite has been found in both immunocompromised and immunocompetent individuals in most countries. The prevalence of cryptosporidiosis has been found to vary from 1% in Manitoba, Canada,[2] to 18,4% in a recent survey carried out in the RSA.[3] However, it is difficult to compare different epidemiological results on cryptosporidiosis, since the rates of stool positivity may vary according to the diagnostic techniques employed.[4,5] The most commonly employed procedures involve the demonstration of *Cryptosporidium* oocysts in stained faecal specimens and include the following techniques: acid-fast staining (modified Ziehl-Neelsen[6] and Kinyoun acid-fast[7]); safranin methylene blue;[8] fluorescence (auramine-phenol[9] and direct immunofluorescence[10,11]); negative staining (periodic acid-Schiff[12]); and flotation (Sheather's sucrose flotation[13]). Cross *et al.*[14] used Giemsa and Ziehl-Neelsen stains on the same faecal specimens and found that Giemsa stain failed to detect *Cryptosporidium*, while 2,6% of smears (19/735) were positive using Ziehl-Neelsen stain. Bogaerts *et al.*[5] compared modified Ziehl-Neelsen with the safranin methylene-blue method and found that they were equally specific, with the safranin-methylene blue technique proving to be more sensitive. The latter technique was the first to be used for the diagnosis of cryptosporidiosis in Natal.[15] Since *Cryptosporidium* oocysts have similar sizes, shapes and staining characteristics to yeasts and other coccidia, diagnostic difficulties can be expected.

The four most commonly used procedures in South African clinical laboratories include modified Ziehl-Neelsen,[6] safranin-methylene blue,[8] auramine-phenol fluorescence[9] and Sheather's sucrose flotation[13] techniques. They were comparatively assessed for their relative efficacies in detecting *Cryptosporidium* oocysts in diarrhoeal stools in order to decide on the most suitable routine technique for local use.

Research Institute for Diseases in a Tropical Environment of the South African Medical Research Council, Durban
D. MOODLEY, B.SC. HONS
T. F. H. G. JACKSON, PH.D
V. GATHIRAM, F.C.P. (S.A.)
Department of Medical Microbiology, University of Natal, Durban
J. VAN DEN ENDE, F.F. PATH. (S.A.)

Accepted 30 Mar 1990.

Materials and methods

Stool specimens were collected from 93 children admitted to King Edward VIII Hospital, Durban, with diarrhoea. All patients were < 2 years old. Each specimen was examined for *Cryptosporidium* oocysts using the following four techniques:

Modified Ziehl-Neelsen.[6] In this method thin faecal smears are first heat-fixed and then stained with concentrated carbolfuchsin (E & O Reagents, Durban) for 10 minutes at room temperature. This step is followed by decolorisation in 10% sulphuric acid until the background is free of excess stain, and thereafter the smears are counter-stained with 1% aqueous methylene blue for 1 minute. Stained preparations were examined by light microscopy at 400 × magnification.

Safranin-methylene blue.[8] Heat-fixed thin faecal smears are stained in 1% aqueous safranin for 5 minutes, washed briefly in water and counter-stained in 1% methylene blue for 30 - 60 seconds. The stained smears are air-dried and examined microscopically, as above.

Auramine-phenol fluorescence.[9] Thin faecal smears are fixed in methanol for 5 minutes and stained in auramine-phenol (auramine-O 0,03 g, phenol 3 g and distilled water 100 ml) for 10 minutes. This is followed by quick rinses in tap water and decolorisation in 3% HCl-ethanol for 5 minutes. The smears are again rinsed in water and counter-stained with 0,1% potassium permanganate for 30 seconds. Stained slides are air-dried and examined with a fluorescence microscope at 400 × magnification.

Sheather's flotation. A modification of this method was developed for screening diarrhoeal stools. Previous experience indicated that the original technique described by Current *et al.*[13] and our modification thereof were equally effective in the detection of *Cryptosporidium* oocysts in diarrhoeal stools. Since this modified Sheather's flotation method is directly comparable to the other three techniques in terms of volume of stool screened, it was used in this study.

Sheather's solution was prepared by dissolving 500 g sucrose and 6,5 g phenol in 320 ml distilled water. The original Sheather's flotation method involves the concentration of oocysts by centrifugation of stools in Sheather's solution at 500 *g* for 5 minutes; with the use of a wire-loop the buoyant oocysts are removed from the surface, placed on a slide, coverslipped and examined microscopically at 400 × magnification. In our modification a small quantity of faeces (equivalent in volume to a faecal smear) was first emulsified in a drop of Sheather's solution on a slide by use of a disposable

applicator stick. The preparation was mounted directly with a glass coverslip and examined by bright-field microscopy at 400 × magnification.

Results

Cryptosporidium oocysts measure 4 - 6 μm in diameter and are spherical to slightly oval in shape.

Modified Ziehl-Neelsen method

The acid-fast *Cryptosporidium* oocysts stained red and the internal structures, which comprise four sporozoites and the residual bodies, were not clearly defined but stained more strongly than the oocyst wall (Fig. 1). Yeasts, bacteria, faecal debris and other non-acid-fast organisms failed to retain carbolfuchsin and thus took up the counterstain and appeared blue. However, certain bacterial spores and other unidentified spherical organisms (Fig. 2), which were also acid-fast, could easily be confused with *Cryptosporidium* oocysts.

Safranin-methylene blue method

Cryptosporidium oocysts took up the safranin stain and appeared reddish-orange, while the faecal debris stained blue. However, only a small proportion of oocysts stained uniformly, while others stained weakly or not at all (Fig. 3). Poor definition of structural details within the oocysts was noted with this technique.

Auramine-phenol fluorescence

With fluorescence microscopy *Cryptosporidium* oocysts were visualised as bright yellow discs (Fig. 4) surrounded by a pale halo; no characteristic structural features were visible. However nonspecific staining of other organisms and certain faecal debris occurred and these could be mistaken for *Cryptosporidium* oocysts (Fig. 5).

Sheather's flotation method

When examined microscopically *Cryptosporidium* oocysts appeared as pink, refractile round to ovoid bodies, with one or two residual granules that appeared green. Oocysts were buoyant in Sheather's solution and were detected in higher planes of focus immediately beneath the coverslip, while non-refractile yeasts and other faecal debris were found lower down (Fig. 6). Although most coccidia are refractile in Sheather's solution (Figs 7 and 8), the characteristic morphology of *Cryptosporidium* oocysts (containing one or two residual bodies) shape (spherical) and size (4 - 6 μm) allowed them to be easily distinguished from other coccidia.

General observations

A notable observation with all of the techniques employed was that a larger number of oocysts occurred in faecal smears made from the mucoid portion of stool specimens. Table I shows that the modification of Sheather's flotation test detected more *Cryptosporidium*-positive faecal smears and was the least time-consuming.

Discussion

Using light microscopy, unstained *Cryptosporidium* oocysts are not readily detected and appear to be morphologically similar to the spores of yeasts and oocysts of other coccidia. Thus the differentiation of these organisms in standard or wet faecal smears is difficult. Problems have also been encountered with staining procedures for the detection of *Cryptosporidium* oocysts. Since the original Sheather's flotation technique concentrates *Cryptosporidium* oocysts, it makes this technique more efficient and enables oocysts to be more readily detected and identified, which therefore makes it a suitable 'gold standard'. Only those techniques that approach this level of efficiency are worth employing.

In this study the modified Sheather's flotation procedure proved to be superior to the other staining techniques assessed for the detection of *Cryptosporidium* oocysts. The advantages of Sheather's flotation technique over modified Ziehl-Neelsen, safranin methylene-blue and auramine-phenol fluorescence methods were: (*i*) this method detected *Cryptosporidium* oocysts most easily in the largest number of stool specimens; (*ii*) the reagents are cheap and easy to prepare; and (*iii*) the procedure is quick to perform. Thus in our hands the modified Sheather's flotation technique is the most effective of the four smear-based methods described for screening diarrhoeal stools. In studies that include asymptomatic (i.e. non-diarrhoeic) persons, we would strongly recommend use of the conventional Sheather's technique, since it is both a concentration technique and allows definitive identification; furthermore, it is the best method for formed stools.

Disadvantages of the modified Sheather's flotation technique are: (*i*) that permanent preparations cannot be made, since oocysts are osmotically sensitive and tend to disintegrate in the sucrose solution; (*ii*) the refractile oocysts are best detected using 400 × magnification and thus only a small field can be examined; and (*iii*) although it is more effective than any of the other smear techniques described, it is not appropriate for screening formed stools.

Faecal specimens stored in 2,5% potassium dichromate solution for up to 5 months can be used with both the Sheather's techniques discussed when diagnostic difficulties arise.

Some authors[16-18] claim that the modified Ziehl-Neelsen method is both easy to interpret and reliable because the acid-fast *Cryptosporidium* oocysts stain strongly, while yeasts and

TABLE I. COMPARATIVE ANALYSIS OF 4 STAINING TECHNIQUES FOR THE DETECTION OF *CRYPTOSPORIDIUM* IN 93 FAECAL SPECIMENS

	Mod. Z/N	Safr/meth	Aur/phenol	Sheath/flot
No. positive (%)	**32 (34,4)**	**29 (31,2)**	**25 (26,9)**	**38 (40,9)**
False-positive	**1**	**0**	**1**	**0**
Preparation time (min)	**15**	**8**	**20**	**2**

Mod. Z/N = modified Ziehl-Neelsen technique; Safr/meth = Safranin-methylene blue technique; Aur/phenol = auramine-phenol fluorescence; Sheath/flot = Sheather's sucrose flotation.

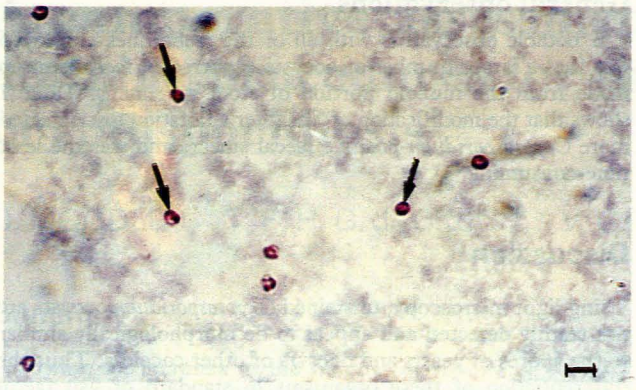


Fig. 1. Modified Ziehl-Neelsen: acid-fast *Cryptosporidium* oocysts appear as red-stained spherical bodies (arroes). Blue background includes bacteria and faecal debris (bar = 8 μm).

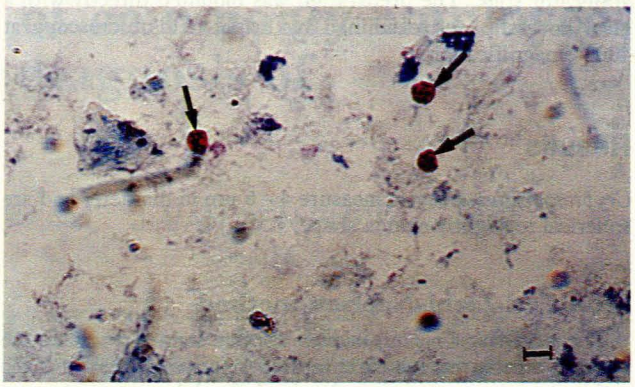


Fig. 2. Modified Ziehl-Neelsen: nonspecific staining of unidentified coccidia (arrows) that differ from *Cryptosporidium* in size and morphology (bar = 8 μm).

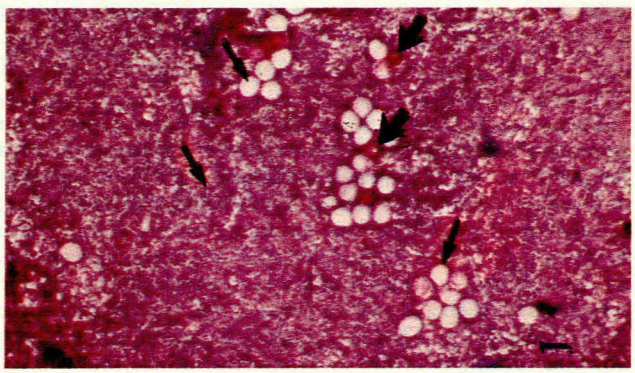


Fig. 3. Safranin-methylene blue stains only a small proportion of *Cryptosporidium* reddish-orange (large arrows), while others stain weakly or not at all (small arrows) (bar = 8 μm).

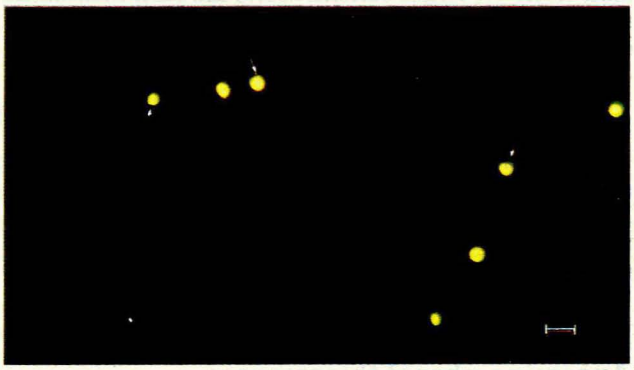


Fig. 4. Auramine-phenol fluorescence: *Cryptosporidium* oocysts can be recognised as yellow discs usually with a bright centre and a pale halo (arrows) (bar = 8 μm).

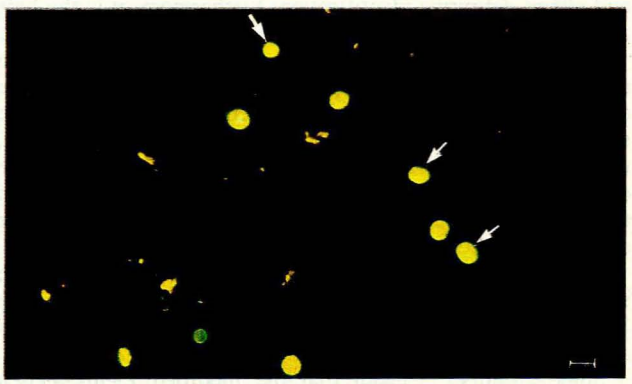


Fig. 5. Auramine-phenol fluorescence: nonspecific staining of unidentified coccidia (arrows) that resemble *Cryptosporidium* but differ in size (bar = 8 μm).

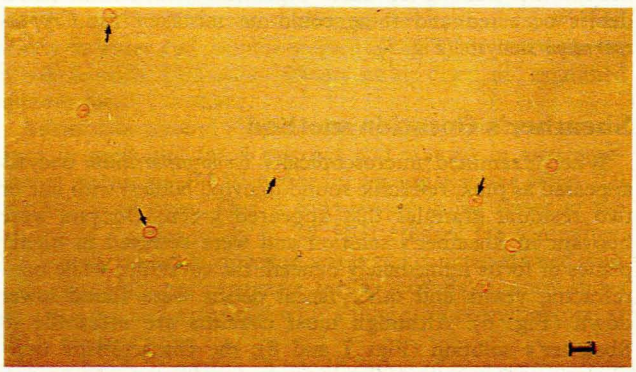
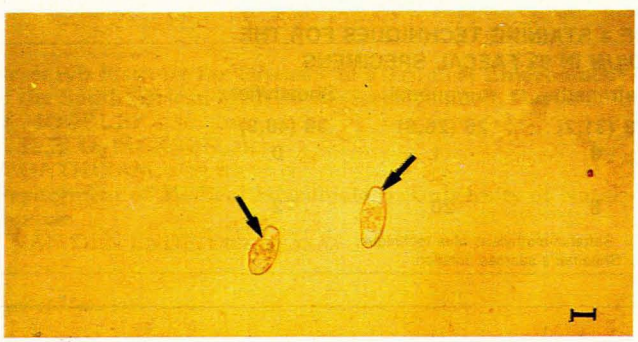
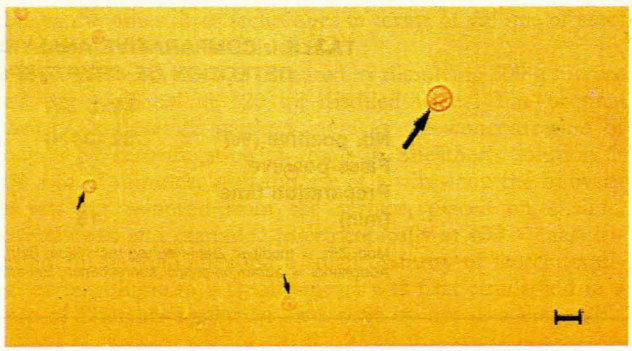


Fig. 6. Sheather's flotation: *Cryptosporidium* oocysts can be observed as small spherical refractile structures (arrows) on a higher plane of focus (bar = 8 μm).





Figs. 7 and 8. Sheather's flotation — although *Isospora* (Fig. 7) and other coccidia (large arrow, Fig. 8) are also refractile in Sheather's solutions, *Cryptosporidium* oocysts (small arrows, Fig. 8) can be differentiated on the basis of size and morphology (bar = 8 μm).

faecal debris do not; originally described by Henricksen and Pohlenz,[6] this is the most popularly used technique. However, variability in carbolfuchsin uptake by *Cryptosporidium* oocysts and the loss of acid-fast properties with time[19–21] may lead to errors in the identification of the parasite. Nonspecific staining of faecal debris and other micro-organisms may lead to diagnostic inaccuracy, as has been reported with both safranin methylene-blue[11,22,23] and auramine-phenol fluorescence[24,25] techniques. Furthermore the auramine-phenol fluorescence technique necessitates the use of a fluorescence microscope, a facility not available in many peripheral laboratories.

Direct fluorescent antibody tests[10,11] have been developed that are reportedly sensitive as well as specific; the use of high quality monoclonal antibodies will further enhance these tests. Unfortunately, the cost of commercially preparing antisera against *Cryptosporidium* is likely to make these tests expensive for routine use.

With the increasing implication of *Cryptosporidium* in diarrhoeal disease, it is expected that a greater burden will be placed on microbiology laboratories for the detection and identification of this parasite. Consequently, since it is reliable, fast and cost-effective, we recommend the modified Sheather's flotation technique for screening loose stools. However, we believe that the conventional Sheather's flotation method should be used for community studies.

We wish to thank the South African Medical Research Council for permission to publish.

REFERENCES

1. Nime FA, Burek JD, Page DL, Holscher MA, Yardley JH. Acute enterocolitis in a human being with the protozoan *Cryptosporidium*. *Gastroenterology* 1976; **70**: 592-598.
2. Mann ED, Sekla H, Nayar GP, Koschik C. Infection with *Cryptosporidium* spp. in humans and cattle in Manitoba. *Can J Vet Res* 1986; **50**: 174-178.
3. Berkowitz FE, Vallabh W, Buqwana A, Heney C. *Cryptosporidium* in black South African children. *S Afr Med J* 1988; **74**: 272-273.
4. Crawford FG, Vermund SH. Human cryptosporidiosis. *CRC Crit Rev Microbiol* 1988; **16**: 113-159.
5. Bogaerts J, Lepage P, Rouvroy D, Vandepitte J. *Cryptosporidium* spp; a frequent cause of diarrhoea in Central Africa. *J Clin Microbiol* 1984; **20**: 874.
6. Henricksen SA, Pohlenz JFL. Staining of *Cryptosporidium* by a modified Ziehl-Neelsen technique. *Act Vet Scand* 1981; **22**: 594-596.
7. Garcia LS, Bruckner DA, Brewer TC, Shimuzu Y. Techniques for the recovery and identification of *Cryptosporidium* oocysts from stool specimens. *J Clin Microbiol* 1983; **18**: 185-190.
8. Mall N, Sehgal R, Genguly NK, Mahajan RC. Cryptosporidiosis in children in Chandigarh. *Ind J Med Res* 1987; **86**: 722-725.
9. Nichols G, Thom BT. Screening for *Cryptosporidium* in stools. *Lancet* 1984; **1**: 735.
10. Stibbs HH, Ongerth JE. Immunofluorescence detection of *Cryptosporidium* oocysts in fecal smears. *J Clin Microbiol* 1986; **24**: 517-52.
11. Sterling CR, Arrowood MJ. Detection of *Cryptosporidium* sp. infections using a direct immunofluorescence assay. *Pediatr Infect Dis* 1986; **5**: 139-142.
12. Horen WP. Detection of *Cryptosporidium* in human faecal specimens. *J Parasitol* 1983; **69**: 622-624.
13. Current WL, Reese NC, Ernst V, Bailey WS, Heyman MB, Weinstein WM. Human cryptosporidiosis in immunocompetent and immunodeficient persons. *N Engl J Med* 1983; **308**: 1252-1257.
14. Cross JH, Alcantara A, Alquiza L, Zaraspe G, Ranoa C. Cryptosporidiosis in Phillipine children. *Southeast Asian J Trop Med Public Health* 1985; **16**: 257.
15. Bush JB, Marcus MB. Misdiagnosis of sarcocystosis as giardiasis. *S Afr Med J* 1986; **70**: 436.
16. Fripp PJ, Bothma MT. *Cryptosporidium* associated with diarrhoea at Ga-Rankuwa Hospital. *S Afr J Epidemiol Infect* 1987; **2**: 45-47.
17. Anderson BC. Patterns of shedding of cryptosporidial oocysts in Idaho calves. *J Am Vet Med Assoc* 1981; **178**: 982.
18. Jokipii L, Pohjola S, Jokipii AMM. *Cryptosporidium*: a frequent finding in patients wth gastrointestinal symptoms. *Lancet* 1983; **1**: 358-360.
19. Collignon P. Staining of atypical oocysts from patients with cryptosporidiosis. *Lancet* 1987; **2**: 1494-1495.
20. Baxby D, Blundell N, Hart CA. Excretion of atypical oocysts by patients with cryptosporidiosis. *Lancet* 1987; **1**: 974.
21. Casemore DP, Armstrong M, Sands RL. Laboratory diagnosis of cryptosporidiosis. *J Clin Pathol* 1985; **38**: 1337-1341.
22. Jackson B, Casemore DP. *Cryptosporidium* and diarrhoea. *Br Med J* 1986; **293**: 631-632.
23. Cartensen H, Hansen HL, Kristiansen HO, Gomme G. The epidemiology of cryptosporidiosis and other intestinal parasitoses in children in southern Guinea-Bissau. *Trans R Soc Trop Med Hyg* 1987; **81**: 860.
24. Ma P, Soave R. Three-step stool examination for cryptosporidiosis in 10 homosexual men with protracted watery diarrhoea. *J Infect Dis* 1983; **147**: 824-828.
25. Angus KW. Staining of faecal yeasts and *Cryptosporidium* oocysts. *Vet Res Commun* 1981; **108**: 173.