Short Communication

Improved staining method for detecting *Cryptosporidium* oocysts in stools using malachite green

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The coccidian protozoan parasite *Cryptosporidium* is recognized as a significant pathogen of vertebrate hosts, including humans and many other mammals, birds, reptiles, and fishes (Fayer et al., 1997). Current laboratory methods for the diagnosis of cryptosporidial infections generally rely on microscopic examination of fecal samples for the presence of *Cryptosporidium* oocysts. These oocysts are similar in size and shape to other fecal components (especially some yeasts), and therefore staining techniques that differentiate between the two are very desirable to avoid confusion (O'Donoghue, 1995). A safranin-methylene blue stain has been described by Baxby et al. (1984), which stains oocysts red and yeasts and other fecal debris blue. Acid-fast stains such as Kinyoun (Ma and Soave, 1983), Ziehl-Neelsen (Henriksen and Pohlenz, 1981), and DMSO-carbol fuchsin (Pohjola et al., 1984) stain oocysts red with the background counterstained and are commonly used in many laboratories.

Differential staining techniques, however, are time-consuming and vary in their sensitivity and specificity (Baxby et al., 1984; Moodley et al., 1991; Smith et al., 1989). Furthermore, research in our laboratory has shown that unless samples have been fixed overnight in formalin, oocysts may frequently take up the counterstain (unpublished observations).

Negative staining techniques using nigrosin (Pohjola, 1984), light green, and merbromide (Chichino et al., 1991), which stain background yeasts and bacteria but not the oocysts, have also been developed.

Negative staining methods are faster but are considered by some to be less sensitive than conventional staining techniques (Casemore et al., 1985; Garcia et al., 1983). In this study, 60 fecal samples from human and animal hosts that were microscopically negative for *Cryptosporidium* were spiked with known concentrations of *Cryptosporidium* oocysts and examined blind by a microscopist experienced at diagnosing cryptosporidial infections. An additional 20 samples containing several known naturally infected *Cryptosporidium* positive samples were also screened blind by the microscopist. The details of the samples screened are listed in Table 1. The 80 samples were examined by using 4 different staining techniques outlined below, including a negative malachite green stain, which we have found to be fast and reliable and should improve the microscopic diagnoses of cryptosporidial infections in clinical laboratories.

Fecal samples microscopically negative for *Cryptosporidium* were divided into 1 g aliquots and emulsified in 8 ml of distilled water. Fresh *Cryptosporidium* oocysts were obtained by passaging oocysts from a naturally infected calf through mice as previously described (Meloni and Thompson, 1996). Fecal samples were seeded at random with 10^4 or 10^2 oocysts and tested blind by the microscopist. Known *Cryptosporidium* positive samples were obtained from a local pathology laboratory and also tested blind by the microscopist.

For the malachite green negative stain, feces were spun at $800\times g$ for 5 min and the supernatant removed. A drop of 5% malachite green in distilled water was then placed on a slide and mixed with one drop of the fecal pellet collected from its surface. Slides were examined for the presence of *Cryp-*

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Table 1. Stool specimens examined.

Source of feces	Spiked stool samples		Naturally infected stool samples	
	No. positive	No. negative	No. positive	No. negative
Human	17	23	9	11
Cat	4	6		
Dog	5	2		
Horse	1	2		
Total	27	33		

cryptosporidium oocysts under 40× and 100× objectives.

Light green staining of slides was carried out for malachite green, except that a 2% light green stain was used.

For the safranin-methylene blue stain, fecal samples were resuspended in 8 ml of 10% buffered formalin and fixed overnight at 4°C. Samples were spun for 5 min at 800×g and a drop of the pellet smeared on a slide and allowed to air-dry. Slides were fixed by being passed through a blue flame and allowed to cool. Slides were then stained for a minimum of 90 min with 3% filtered safranin, washed and counterstained with 1% methylene blue for 45–60 min, washed again, air-dried, and examined at 20× and 40× objectives.

For the cold Ziehl-Neelsen stain, samples were prepared in the same manner as above. Slides were fixed in absolute alcohol for 10 min, then flooded with strong carbol fuchsin for 1 h. Following washing, slides were decolorized in 3% acid/alcohol from 15 s to 1 min, depending on the film thickness. Slides were then washed, counterstained with 1% methylene blue for 4 min, washed and air-dried, and examined at 20× and 40× objectives. Slides were examined for 3 min per sample for all four stains tested.

The results of the test are listed in Table 2. Malachite green negative staining proved to be the most reliable and fastest method of detecting *Cryptosporidium* oocysts, which appeared plump and bright against a dark green background. Malachite green detected a total of 25 of 27 positives among the experimentally infected samples, and 9 of the 9 positive naturally infected samples, an overall sensitivity of 94%. No false positives were detected. The malachite green failed to detect *Cryptosporidium* in two of the spiked samples; one sample was from a dog, and oocysts are generally more difficult to detect in canine feces (unpublished observations). The second sample, from a human, contained large quantities of yeasts that made diagnosis difficult. Moreover, the samples were spiked with relatively low numbers of oocysts (10⁴ or 10² oocysts/g feces), and only a drop of this was examined per slide. The light green stain, though fast,

Table 2. Comparison of 4 different staining techniques for the detection of *Cryptosporidium*.

A. Spiked fecal samples

Staining method	No. positive	No. negative	No. false positives	No. false negatives
Malachite green	25	35	0	2
2–5% light green	0	0	0	27
Carbol fuchsin	6	54	0	21
Safranin	4	56	0	23

B. Naturally infected fecal samples

Staining method	No. positive ^a	No. negative ^a	No. false positives ^a	No. false negatives ^a
Malachite green	9	11	0	0
2–5% light green	8	12	0	1
Carbol fuchsin	6	14	0	3
Safranin	10	10	1	0

^a All samples confirmed by PCR screening.

failed to detect *Cryptosporidium* in any of the experimentally spiked samples tested because of the low numbers of oocysts and because the background debris failed to take up the stain very effectively. The background was lighter; therefore the oocysts did not stand out as clearly as those with malachite green did. The superiority of the malachite green stain also appears to be due substantially to the effect the stain has in flocculating fecal particles, thus rendering the background less cluttered than with light green. The light green stain, along with the other stains, however, did perform much better on naturally infected fecal samples compared with the spiked samples, largely because the samples from clinical cases had much higher numbers of oocysts. Light green staining detected 8 of 9 naturally infected fecal samples (Table 2). Figure 1 shows light green and malachite green staining of the same naturally infected fecal sample.

Diagnoses of *Cryptosporidium* oocysts, using carbol fuchsin staining, were very time-consuming and unreliable. Variable background coloration often resulted from the critical decolorization step. Frequently the background was pale pink and not blue, which made the pink oocysts extremely difficult to see. This method detected only 6 of 27 experimentally spiked fecal samples and 6 of 9 naturally infected samples, a sensitivity of only 33%. No false positives were detected. These results are of concern because carbol fuchsin is a widely used technique for the detection of *Cryptosporidium* oocysts in clinical samples.

Oocysts stained by the use of safranin and carbol fuchsin stain exhibited a great deal of variation in stain uptake. Incubation, at least overnight in formalin, was required for the oocysts to take up the stain at all, and

Fig. 1. Staining of *Cryptosporidium* oocysts in the same naturally infected stool sample by using two different negative stains: (a) 2% light green stain and (b) 5% malachite green stain ($\times 200$ magnification).

Note that (1) the *Cryptosporidium* oocysts are much easier to visualize against the dark malachite green background than against the light green background and (2) the less cluttered background in the malachite green stain from the effect the stain has in flocculating fecal particles.

frequently fat droplets and other organisms in the feces took up the stain, which made diagnosis more difficult. The safranin stain detected 4 positives of 27 experimentally infected fecal samples and 9 of 9 naturally infected samples, an overall sensitivity of 36%, and also gave 1 false positive result (Table 2).

Effective diagnosis of cryptosporidial infections requires diagnostic tools to be fast, cost-effective, accurate, and sensitive. Counterstaining methods using carbol fuchsin and safranin are time-consuming, frequently stain for yeasts, and lack sensitivity (Baxby et al., 1984; Moodley et al., 1991; Smith et al., 1989). Immunological methods are expensive and can cross-react with other organisms (Clancy et al., 1994). Microscopy provides the advantage of direct visual confirmation of the presence of *Cryptosporidium* oocysts.

The malachite green method described here is a practical, safe, and sensitive method of detecting *Cryptosporidium* oocysts in stool specimens and was significantly more sensitive than the other three staining methods tested by use of a multiple comparison for proportion test ($p < 0.001$; Zar, 1984). When the light green stain was used, the yeasts did not always take up the stain, greatly confusing diagnoses. Similarly, negative staining with nigrosin also results in yeasts remaining unstained (Pohjola, 1984). With the malachite green stain, yeasts were clearly differentiated from oocysts as they took up the stain. Because of the ease with which oocysts can be differentiated from yeasts, less experienced microscopists can still accurately diagnose cryptosporidial infections. Be-

cause pathology laboratories can vary widely in their expertise in diagnosing *Cryptosporidium* and because variable laboratory expertise can result in a large variation in results (Clancy et al., 1994), the malachite green staining procedure described here should greatly improve microscopic diagnoses and the detection of cryptosporidial infections.

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