Laboratory diagnosis of cryptosporidiosis

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SUMMARY *Cryptosporidium* spp is now widely accepted as a cause of gastroenteritis. Various methods have been applied to detect oocysts in faeces, but the difficulties of discriminating between non-cryptosporidial bodies, acid fast bodies like cryptosporidia, and cryptosporidia remain. A simple examination in two stages, suitable for routine use is described, using auramine phenol and carbol fuchsin for screening and a modified Ziehl-Neelsen staining method for confirmation. A further method, using Jenner and Giemsa stains, is of value for confirmation of identity, especially where fluorescence microscopy is unavailable. A modification of the formol-ether method of concentration is also described. Immunofluorescence and thin section electron microscopy provide definitive identification. Vomiting can be an important clinical feature of gastroenteritis, and the first description of oocysts in vomit is reported. Preliminary findings, after more than two years of study show that *Cryptosporidium* is an important pathogenic agent in gastroenteritis, confirm the increased incidence in children, and suggest a possible seasonal trend.

The coccidian protozoan parasite *Cryptosporidium* sp is now widely accepted as a cause of acute gastroenteritis. In some normal subjects the symptoms may be both severe and persistent, while in immunocompromised patients the infection may be life threatening.¹ Various methods have been recommended for studying cryptosporidia using light and transmission electron microscopy. These vary from the examination of wet films to negative staining by transmission electron microscopy. Since beginning our study early in 1983 we have supplied many laboratories with positive material and guidance, and in this paper we make recommendations for the routine diagnostic examination of faecal samples. A comparison of the methods studied has shown that the fluorescence stain described, together with modified formol-ether concentrations when appropriate, are significantly more sensitive than other methods ($p < 0.001$).

Material and methods

WET FILM: DIRECT EXAMINATION OF FAECAL MATERIAL

Jokipii *et al*² and Ma and Soave³ recommended preliminary screening by the use of wet preparations. We found this method to be insensitive and not particularly helpful, although it may be helpful for detecting cysts or ova of other parasitic species that may also be present.

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STAINING

The use of Romanowsky stain to show endogenous stages in gut mucosa smears was first described by Tyzzer.⁴ This was extended to the detection of oocysts in smears of cattle faeces by Pohlenz *et al*⁵ and in man by Tzipori *et al*.⁶ The method is relatively insensitive but still retains a useful role as an additional technique in cases of doubtful morphology. We prefer a combination of Jenner and Giemsa stains, which we use in an automatic slide staining apparatus (Shandon). Smears are placed in racks in the following reagents for five minutes for each stage: methanol 64 over proof $\times 2$; Jenner stain (BDH: diluted 1:6 in Sorensen's buffer pH 6.8) $\times 2$; Giemsa stain (BDH: diluted 1:10 in Sorensen's buffer) $\times 2$; Sorensen's buffer pH 6.8.

Cryptosporidium oocysts stain blue to azure, often with a crescentic pattern; about four to six red or purple dots may be seen, and there may be a clear halo surrounding the oocyst resulting from shrinkage.

ZIEHL-NEELSEN METHOD

A modified cold Ziehl-Neelsen method was first described by Henriksen and Pohlenz.⁷ Further modifications have now been described.⁸⁻¹⁰ The method is widely used, and we recommend the following procedure. Moderately thick faecal smears dried in air placed in multislide carriers for fixation and staining in batches of up to 16 slides in troughs as

follows: methanol fixation, three minutes; strong carbol fuchsin¹¹ (Paramount Reagents, Liverpool), 15 minutes, followed by thorough rinsing; minimal decolourisation in 1% hydrochloric acid in alcohol for 10–15 seconds, followed by rinsing; counter-stain in 0.4% malachite green for 30 seconds; rinse well and dry in air.

Oocysts present as round objects, usually 4–5 μm in diameter, with some degree of red staining of the internal structure, varying from an amorphous red mass filling the oocyst to obvious, multiple, crescentic, sporulated forms. In addition, highly refractile thick walled bodies may sometimes be seen, often unstained, which are possibly empty zygote cases rather than oocysts. Although these are often empty, they may sometimes contain an oocyst. The degree and proportion of staining of the oocysts varies, and holes may sometimes be apparent in the smear where oocysts have detached themselves. The appearance of unstained oocysts is, however, characteristic, and in practice this does not present a problem. A much higher proportion of oocysts appear to stain by auramine phenol using fluorescence microscopy.

Fluorescence staining

This has been reported by several workers.^{3,8,12} Most diagnostic microbiological laboratories have access to fluorescence microscopy, and the following novel modification first described by Casemore *et al.*¹⁰ combines auramine phenol staining as previously described,¹¹ with negative staining by strong carbol fuchsin as described by Heine¹³ in a simple two step procedure suitable for rapid screening of large numbers of samples.

AURAMINE AND CARBOL FUCHSINE

1 Thick faecal smears dried in air were placed in multislide carriers into a trough of auramine-phenol, stained for five minutes, then rinsed in tap water.

2 The smears were immersed briefly (five to 10 seconds) in strong carbol fuchsin, rinsed, and dried.

Total handling time for staining 16 slides was two to three minutes. Slides were examined using fluorescence microscopy at low magnification. Cryptosporidia appear as very characteristic brightly fluorescent discs against a dark red background. Positive specimens may be re-examined at high power by immersion in oil or in doubtful cases the object may be located by vernier reading and the slide re-examined after overstaining by the modified Ziehl-Neelsen technique. Doubtful scanty positive results may be checked after concentration of the specimen.

Concentration methods

Faeces received from acutely ill patients usually contain sufficient numbers of cysts, obviating the neces-

sity for concentration methods. However, the examination of specimens from close family contacts, cases in which diagnosis has been delayed, and follow up specimens from patients who have recovered merits the use of such techniques. A widely reported method is that of sucrose flotation as described by Sheather in 1923¹⁴: this method has several drawbacks, including interference with staining properties and adhesion of samples to the slide. We prefer a modification of the method of Allen and Ridley.¹⁵ Some workers have failed to obtain satisfactory results with the formol-ether method, which may have resulted from a failure to realise that the characteristics of cryptosporidial oocysts differ from those of the parasite forms for which the method was originally devised. We would advocate the following new modification:

1 Fluid specimen of faeces, 0.5 ml, (or equivalent of solid stool) is emulsified in about 3 ml of 10% formalin in water in a glass universal container using a vortex mixer (with initial breaking up with an applicator stick if necessary). Add 3 ml ether and shake vigorously for 30–40 seconds.

2 Make up to about 15 ml with 10% formalin, remix, and pour through a 40 mesh gauze sieve (Endecotts Ltd, London: certified test sieve 3 inches ASTM, 425 μm) into a glass conical centrifuge tube.

3 Centrifuge by setting the regulator to 1000 rpm (450 RCF) and spin for half to one minute at this speed. Using a plastic disposable bulb pipette, carefully pass this down the side of the ether or debris layer and remove the column fluid to a second tube. Top up with formol water. (The deposit may be used to prepare wet films for examination for other ova and cysts if required.)

4 Centrifuge at 3000 rpm (1000 RCF) for five to 10 minutes depending on the viscosity of the specimen. The deposit is used to make a smear stained in the usual way.

Electron microscopy

The appearance of oocysts after negative staining of faecal material for the detection of viruses is quite characteristic, although the insensitivity of the method in terms of sample size makes the technique inappropriate for routine use, even where electron microscopy facilities are available. Thin sections of possible oocysts in faeces may be useful in providing definitive identification (Fig. 1).

Results

Fig. 2 shows a comparison of concentration methods: the modified formol ether method is the most sensitive. A comparison of the same methods, but staining by two different procedures (Ziehl-Neelsen and auramine and carbol fuchsin) showed

Fig. 1 Fresh faeces were suspended in agarose, fixed, and sectioned using a diamond knife. Oocyst contains four partially developed sporozoites and residual cytoplasmic mass. $\times 40\,000$.

Fig. 2 Standardised smears prepared from concentrates. Statistical analysis was carried out (Dec 20 computer) using χ^2 test with the null hypothesis and Yates's correction where appropriate. Results showed a significantly better performance by the modified ether method described ($p < 0.001$). Sodium chloride gave the best results for flotation methods.

a significantly better result with the auramine or carbol fuchsin stain (Fig. 3). Results may vary according to the consistency of the stool, a finding also supported by P Ma (personal communication).

Fig. 3 Same specimens were subjected to identical tests, but duplicate smears were prepared and stained by modified Ziehl-Neelsen method; and auramine/carbol fuchsin. Results showed that the auramine/carbol fuchsin method performed significantly better than the Ziehl-Neelsen method ($p < 0.001$).

During two years of study over 3000 specimens were examined from a diverse population of patients, yielding more than 60 positive cases. Examination covered the most commonly recognised pathogens. Tables 1 and 2 show the results of investigations covering the first 50 positive cases. Overall, reports show that *Cryptosporidium* is found

Table 1 First 50 cases of infection with *cryptosporidium* obtained (March 1983 to June 1984)

Date	Total no of cases (n = 2573)	No positive	Age and Sex
1983:			
March	70 (selected)	0	M 0 F 9/12
April			
May			
June	181 (unselected)	0	M 0 F 0
July	154	5	M 1, 5, 12 F 8, 8
August	183	1	M 0 F 32
September	238	7	M 1, 2, 9, 26 F 1, 30, 44
October	201	4	M 1½, 13, 35 F 35
November	183	0	M 0 F 0
December	139	1	M 0 F 7
1984:			
January	149	0	M 0 F 0
February	204	3	M 1, 1, 18 F 0
March	214	9	M 2, 4, 6, 28, 36, 37 F 1, 35, 89
April	218	12	M 1½, 2, 4, 9, 10 F 8/12, 3, 6, 8, 19, 21, 28
May	210	3	M 7, 16 F 30
June	229	4	M 7, 28 F 4, 9
Totals	2573	50	28 M : 22 F

1.94%

No for 0-15 (969), ≥16 (1333).
Age not known (271).

Table 2 League table of faecal pathogens (May 1983 to June 1984)

Pathogen	No positive (%)	Total No of positives expressed as %
<i>Campylobacter</i>	117 (27.7)	4.5
Rotavirus	84 (19.9)	3.3
<i>Cryptosporidium</i>	50 (11.8)	1.9
<i>Salmonella</i>	48 (11.4)	1.9
Adenovirus (electron microscopy)	38 (9.0)	1.5
Enteropathogenic <i>Escherichia coli</i>	26 (6.2)	1.0
<i>Giardia lamblia</i>	25 (6.0)	1.0
<i>Shigella sonnei</i>	15 (3.2)	0.6
Other bacteria	12 (2.8)	
Other viruses	8 (1.9)	
Total positive	423	16.4
Total examined	2573	

Low level of positives overall reflects the inclusion of samples from non-infective diarrhoeas, some routine samples, and a high proportion of samples from adults.

in about 2% of all specimens examined, although among children the incidence appears to be about 4% (Table 3). In our laboratory it is the third most

Table 3 Occurrence of *cryptosporidium* by age 1983-4 (published reports)

Source	No examined	No positive (%)
World wide*	11 067	272 (2.46)
Children†	2531	124 (4.9)
Adults†	2026	41 (2.0)
United Kingdom*	6580	140 (2.13)
Children†	1363	59 (4.32)
Adults†	1739	35 (2.01)

*All patients including those whose ages were not specified.

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frequently encountered pathogen after rotavirus and *Campylobacter*.

Vomiting is often a predominant symptom. *Cryptosporidium* was found in the vomit of one patient from whom we examined specimens (a child admitted to hospital as a result of severe vomiting and diarrhoea). In addition, we found cryptosporidia in the stool of one child and one adult patient who complained of vomiting with anorexia and abdominal pain but without diarrhoea. Mixed infections were found in some cases, principally with *Campylobacter* and *Giardia*. Similar findings have been reported elsewhere.^{16 17}

No particular seasonality has been noted, although an outbreak occurred in this area during March and April 1984 in which campylobacter was also often isolated.¹⁶ During both winters there was a low prevalence with an increase again in the spring of 1985.

Discussion

No staining method for cryptosporidia is completely effective, a view shared by P Ma (personal communication). The appearance of cryptosporidial oocysts in faeces, however, is generally sufficiently typical for there to be little difficulty in identifying the organism in most cases. On the other hand, the inexperienced may be confused by a variety of objects resembling oocysts in general appearance, whichever staining method is applied. In addition, cryptosporidia have been described that are larger than the usual size range of 3-6 µm. Triffitt,¹⁸ Bearup,¹⁹ and Dubey and Pande²⁰ described what were claimed to be cryptosporidia in snakes, dingoes, and jungle cats, respectively, which were considerably larger (7-17 µm). Although not yet reported from other species, including man, we found similar large objects like cryptosporidia in both environmental and occasionally human specimens.

Objects comparable in size to cryptosporidia may also be found, but they have different internal structures or staining features. In any doubtful case we apply immunofluorescence staining by a method to be described in detail elsewhere, together with

Fig. 4 *Cryptosporidium* oocysts stained by immunofluorescence using convalescent human serum and an antihuman globulin/fluorescein isothiocyanate conjugate. $\times 2000$.

details of serological diagnosis. Briefly, the specimen is dispensed on to the wells of a microscope slide coated with telfon, air dried, and fixed in cold acetone for five minutes. A pool of high titre IgA positive convalescent human sera is then used at a predetermined titre in a sandwich technique using an antihuman fluorescein isothiocyanate conjugate against the IgA fraction (Wellcome Reagents) used at optimal dilution (Fig. 4).

Our own continuing study, together with reports from elsewhere²¹⁻²³ confirm earlier observations from this laboratory^{24,25} that the infection is not uncommon, especially in children, and should not be assumed to be a zoonosis as it occurs in the urban environment, especially in children in day care centres. It may also be found as a cause of travellers' diarrhoea.^{2,26} In addition to probable person to person spread, local studies^{16,28} and evidence from elsewhere²⁷ suggest that raw milk, sausage, and environmental sources may all have a role in transmission.

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References

- 1 Anonymous. Cryptosporidiosis [editorial]. *Lancet* 1984;ii:492-3.
- 2 Jokipii L, Pohjola S, Jokipii AMM. Cryptosporidium: a frequent finding in patients with gastrointestinal symptoms. *Lancet* 1983;ii:358-61.
- 3 Ma P, Soave R. Three-step stool examination for cryptosporidiosis in 10 homosexual men with protracted watery diarrhea. *J Infect Dis* 1983;5:824-8.
- 4 Tyzzer EE. Extracellular coccidium, *Cryptosporidium muris* (gen et sp nov), of the gastric glands of the common mouse. *J Med Res* 1910;23:487-509.
- 5 Pohlenz J, Moon HW, Cheville NF, Bemrick WJ. Cryptosporidiosis as a probable factor in neonatal diarrhea of calves. *J Am Vet Med Assoc* 1978;172:452-7.
- 6 Tzipori S, Angus KW, Gray EW, Campbell I. Vomiting and diarrhea associated with cryptosporidial infection. *N Engl J Med* 1980;303:818.
- 7 Henriksen SA, Pohlenz JFL. Staining of cryptosporidia by a modified Ziehl-Neelsen technique. *Acta Vet Scand* 1981;22:594-6.
- 8 Garcia LS, Bruckner DA, Brewer TC, Shimizu RY. Techniques for the recovery and identification of *Cryptosporidium* oocysts from stool specimens. *J Clin Microbiol* 1983;18:185-90.
- 9 Bronsdon MA. Rapid dimethyl-sulfoxide-modified acid-fast stain of *Cryptosporidium* oocysts in stool specimens. *J Clin Microbiol* 1984;19:952-3.
- 10 Casemore DP, Armstrong M, Jackson B. Screening for *Cryptosporidium* in stools. *Lancet* 1984;ii:734-5.
- 11 Cruickshank R, Duguid JP, Marmion BP, Swain RH. *Medical microbiology*. Vol II. *The practice of medical microbiology*. London: Churchill Livingstone, 1975.
- 12 Nichols G, Thom BT. Screening for *Cryptosporidium* in stools. *Lancet* 1984;ii:735.
- 13 Heine J. Eine einfache Nachweismethode für Kryptosporidien im Kot. *Zentralbl Veterinärmed (A)* 1982;29:324-7.
- 14 Sheather AL. The detection of intestinal protozoa and mange parasites by a flotation technique. *Journal of Comparative Pathology and Therapeutics* 1923;36:266-75.
- 15 Allen AVH, Ridley DS. Further observations on the formol-ether concentration technique for faecal parasites. *Technical Methods* 1970;23:545-6.
- 16 Casemore DP, Jackson FB, Douce D, Jessop EG. *Cryptosporidium* plus campylobacter: an outbreak in a semi-rural population. *J Hyg (in press)*.
- 17 Wolfson JS, Hopkins CC, McCarth DM. An association between *Cryptosporidium* and *Giardia* in stools. *N Engl J Med* 1984;311:788.
- 18 Triffitt MJ. Observations on two new species of coccidia parasitic in snakes. *Protozoology* 1925;1:19-26.
- 19 Bearup AJ. The coccidia of carnivores in Sydney. *Aust Vet J* 1954;30:185-7.
- 20 Dubey JP, Pande BP. Observations on the coccidian oocysts from Indian jungle cat (*Felis chaus*). *Ind J Microbiol* 1963;13:103-8.
- 21 Hunt DA, Shannon R, Palmer SR, Jephcott AE. Cryptosporidiosis in an urban community. *Br Med J* 1984;289:814-6.
- 22 Wright PA, Harrison JM, Byrom I. Cryptosporidiosis. *Br Med J* 1984;289:1148.
- 23 Alpert G, Bell LM, Kirkpatrick CE, et al. Cryptosporidiosis in a day care center. *N Engl J Med* 1984;311:860-1.
- 24 Casemore DP, Jackson B. Sporadic cryptosporidiosis in children. *Lancet* 1983;ii:679.
- 25 Casemore DP, Jackson FB. Hypothesis: cryptosporidiosis in human beings is not primarily a zoonosis. *Journal of Infection* 1984;9:153-6.
- 26 Soave R, Ma P. Cryptosporidiosis—travellers' diarrhoea in two families. *Arch Intern Med* 1985;145:70-2.
- 27 Isaacs D, Hunt GH, Philips AD, Price EH, Raafat F, Walker-Smith JA. Cryptosporidiosis in immunocompetent children. *J Clin Pathol* 1985;38:76-81.
- 28 Casemore DP. Cryptosporidiosis. *J Environ Health* 1985;93:5.

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