An enzyme immunoassay for detecting *Cryptosporidium* in faecal and environmental samples

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Summary. An enzyme immunoassay (EIA) with a monoclonal antibody to cryptosporidium oocysts was developed and evaluated for the examination of faecal and environmental samples. The EIA was as sensitive as microscopy for detecting *Cryptosporidium* but also produced some positive results which could not be confirmed by a modified Ziehl-Neelsen technique or by immunofluorescence microscopy. These specimens reacted also with heterologous antibodies in EIA, suggesting false positive results.

Introduction

Cryptosporidium is now recognised worldwide as an important intestinal pathogen causing diarrhoeal disease in man and animals.¹ The source of infection for man is diverse. Infections in domestic and farm animals are common^{2–4} and infection of man may result from contact with infected animals or ingestion of contaminated food.^{5–7} Person-to-person transmission of *Cryptosporidium* may also occur,^{8–11} and possible outbreaks of cryptosporidiosis associated with contaminated drinking water supplies^{12–15} and a municipal swimming pool have been a recent cause for concern to public health authorities. Microscopic confirmation of the presence of *Cryptosporidium* in water samples is laborious and costly.¹⁶ The aim of this study was to develop an enzyme immunoassay (EIA) based on monoclonal antibody (MAb) to cryptosporidium oocysts, and to compare this with conventional methods of detecting *Cryptosporidium* by microscopy.

Materials and methods

Selection and preparation of samples

Faecal samples were taken at random from those submitted to Sheffield Public Health Laboratory (PHL) during 1987 from patients with acute diarrhoea of presumed infectious origin. Samples of reservoir water and the intestinal contents of brown trout (*Salmo trutta*) were collected by Sheffield Environmental Health Department (EHD) during investigation of a possible

outbreak of waterborne cryptosporidiosis in Sheffield.¹⁴ Cattle faeces were collected randomly from an abattoir by Sheffield EHD during another investigation.¹⁷ Samples of water and filter-sand from a swimming pool were collected by Doncaster EHD and Sheffield EHD during investigation of an outbreak of cryptosporidiosis associated with a swimming pool in Doncaster (Dr P. Fenton, personal communication).

For faeces or intestinal contents, a suspension (c. 25% v/v) was made in phosphate-buffered saline pH 7.2 (PBS). This was tested by EIA as a crude suspension, and also as a supernate after centrifugation at 3000 g at 4°C for 20 min.

Water samples (20 L) were filtered through a 0.45-µm membrane filter (Pall Process Filtration Ltd). The membranes were then cut into small pieces and agitated in 100 ml of PBS with Tween 20 0.1% v/v, to release any deposit. The resulting suspension was centrifuged at 3000 g at 4°C for 20 min, and the deposit was resuspended in 200 µl of PBS and used directly in the EIA.

Sand samples (100 g) were added to 100 ml of PBS with Tween 20 1% v/v in a plastic jar, and shaken vigorously for 5 min. The sand was allowed to settle for 5 min and the supernate was then removed and centrifuged at 3000 g at 4°C for 20 min. The supernate was discarded and the deposit was resuspended in 1 ml of PBS.

Microscopic examination on all samples, and EIA on human faecal samples, were performed within 24 h of receipt. Other samples were stored at 4°C for up to 1 month before examination by EIA.

Microscopy

Samples were examined by a modified Ziehl-Neelsen (ZN) method¹⁸ and those containing bodies resembling cryptosporidium oocysts were tested by a direct immunofluorescence (IF) method with a mouse IgM MAb

conjugated to fluorescein isothiocyanate (FITC).¹⁹ The reagents were supplied by the Division of Microbiological Reagents and Quality Control (DMRQC).

Enzyme immunoassay (EIA)

Monoclonal antibodies and conjugates. A mouse MAb to cryptosporidium oocysts (MAb-C1), and MAb-C1 conjugated to FITC, have been described by McLauchlin *et al.*¹⁹ A MAb to FITC described by Samuel *et al.*²⁰ has been conjugated with horseradish peroxidase (EC 1.11.1.7) by Wilson and Nakane.²¹ For the present study, these antibodies and conjugates were prepared at the DMRQC and were used at their optimal dilutions.

Coating of EIA plates. Polystyrene 96-well EIA plates (Costar, Northumbria Biologicals) were coated with unconjugated MAb-C1 (whole ascitic fluid): 100 μ l of the MAb-C1 diluted in PBS was placed in each well and the plate was shaken at ambient temperature for 10 min. After refrigeration overnight, plates were washed three times with PBS containing Tween 20 0.5% v/v. Any uncoated sites were blocked by adding 100 μ l of blanking reagent (RPMI 1640 medium [Flow Laboratories] with fetal bovine serum 10% v/v and bovine serum albumin 1% v/v), shaking, and incubating at 37°C for 1 h. Blanking reagent was then discarded and the plates were washed three times as above. If they were not used immediately, they were stored in a heat-sealed polythene bag at -20°C for up to 1 month, and were then allowed to reach ambient temperature before use.

EIA test procedure. On each plate were included a faecal sample known to contain cryptosporidium oocysts and a negative control sample (washing buffer alone). For the test, 50 μ l of sample was added to each of two wells, and the plates were shaken at ambient temperature for 90 min and washed three times as above; 100 μ l of MAb-C1 conjugated with FITC, diluted in blanking reagent, was then added to each well. After shaking at ambient temperature for a further 90 min, plates were washed three times as above; 100 μ l of anti-FITC conjugated with horseradish peroxidase, diluted in blanking reagent, was then added to each well and the plate was shaken and washed as above. Freshly prepared substrate solution (chromogenic substrate 3:3',5',5'-tetramethylbenzidine dissolved in dimethyl sulphoxide) was diluted for use in citrate acetate buffer as described by Bos *et al.*;²² 100 μ l was added to each well, and the plate was shaken at ambient temperature for 10 min. The colour reaction was stopped by adding 50 μ l of sulphuric acid 3% to each well, and the results were then read within 1 h. Optical density (OD) at 450 nm was read against an untreated well containing 150 μ l of PBS as zero. Results were recorded as positive if the mean of the OD values for the two wells of the sample exceeded the mean + 3 SD of the OD values for the two wells of the negative control.

Further tests on EIA-positive microscopy-negative specimens

Faecal samples that gave positive results by EIA but

negative results by microscopy were, where possible, investigated further as follows: (1) concentration by a formalin-ether procedure²³ and re-testing by modified ZN and IF techniques; (2) examination for rheumatoid factor with a latex kit (Rheuma-Wellcotest, Wellcome Diagnostics); (3) examination for salmonellae, shigellae, campylobacters, parasites, and pathogenic *Escherichia coli* by methods described previously,^{17,24} and examination for viruses by a rotavirus EIA (DMRQC) and electronmicroscopy; (4) examination by EIA with other mouse MAbs (DMRQC) raised against *Bartonella bacilliformis*, *Listeria monocytogenes*, and *Legionella pneumophila*.

Titration of EIA-positive specimens and an oocyst suspension

Cryptosporidium oocysts were purified from a positive human faecal sample by sucrose flotation concentration²⁵ followed by Percoll (Pharmacia/LKB) discontinuous density gradient centrifugation.²⁶ Purified oocysts were then washed three times in PBS and resuspended to give a concentration of *c.* 10⁶ oocysts/ml. Purified oocyst suspension, faecal samples that gave positive results by both EIA and microscopy, and faecal samples that were EIA-positive and microscopy-negative, were then diluted serially to 1 in 64 in liquid faeces that gave negative results by both EIA and microscopy. Each dilution was tested in triplicate by the cryptosporidium EIA, and the mean OD was plotted against the dilution.

Results

The results of initial EIA and microscopic examination for *Cryptosporidium* are summarised in table I. Of 494 human faecal samples examined, 17 (3%) gave positive results by both microscopy and EIA, 28 (6%) were positive by EIA but negative by microscopy, and 1 (0.2%) was positive by microscopy but negative by EIA. Of 81 environmental samples examined, 30 (37%) gave positive results by both microscopy and EIA, 11 (14%) were positive by EIA but negative by microscopy, and 5 (6%) were positive by microscopy but negative by EIA. All samples that gave positive results when stained by a modified ZN method also gave positive results by IF. No differences in reactivity in EIA were observed between tests with faecal suspensions and faecal supernates.

The results of some further examinations on specimens which were positive by EIA but negative by microscopy are shown in table II. All 28 samples examined gave negative results by microscopy for cryptosporidium oocysts after concentration. Five of 28 contained other recognised intestinal pathogens (*Campylobacter jejuni* 3, *Salmonella enteritidis* 1, and rotavirus 1). All 19 samples tested gave negative results for rheumatoid factor. Of nine

Table I. Detection of *Cryptosporidium* by microscopy and EIA

Type of specimen (number examined)	Number of specimens that were positive by			
	both microscopy and EIA	microscopy alone	EIA alone	neither microscopy nor EIA
Human faeces (494)	17	1	28	448
Surface water (14)	10	0	4	0
Treated drinking water (2)	0	0	0	2
Cattle faeces (12)	9	3	0	0
Brown trout (18)	7	2	5	4
Swimming pool water (32)	3	0	0	29
Filter-sand (3)	1	0	2	0

samples examined in other EIA systems, nine gave positive results with MAb to *B. bacilliformis*, nine and seven gave positive results with two different MAbs to *List. monocytogenes* and eight gave positive results with MAb to *Legion. pneumophila*.

The results of the titrations are shown in the figure. Curves obtained with purified oocysts, and with faecal samples that gave positive results by both microscopy and EIA, are similar and show a steady decrease in OD with dilution of the specimen. With faecal samples that were positive by EIA but negative by microscopy, there was no such simple relationship between the OD and the dilution of the specimen.

Discussion

The laboratory diagnosis of cryptosporidiosis, as with other protozoal diarrhoeal diseases, presently relies on the recognition by conventional light microscopy of morphological features specific to the parasite. Unfortunately these techniques may be laborious, insensitive, and prone to error with inexperienced staff.^{27,28} Staining procedures for

oocysts of *Cryptosporidium* have been reviewed by Garza *et al.*²⁹ and Casemore *et al.*²³ and difficulties in distinguishing cryptosporidium oocysts from non-cryptosporidial bodies have been reported.^{29,30} Immunofluorescent microscopy based upon fluorescein-labelled MAb to cryptosporidium oocysts has been described;^{19,31} this indicated the

Table II. Further tests on 28 faecal samples that were positive for *Cryptosporidium* by EIA but negative by direct microscopy

Further test	Number of specimens	
	tested	positive
Concentration and microscopy	28	0
Examination for other pathogens	28	5
Examination for rheumatoid factor	19	0
EIA for <i>B. bacilliformis</i>	9	9
EIA for <i>List. monocytogenes</i>	9	9
EIA for <i>Legion. pneumophila</i>	9	8

Figure. Titration, by EIA for *Cryptosporidium*, of a suspension of purified oocysts (○), and of faecal samples that were EIA-positive but by microscopy were either positive (▲) or negative (●). Each point represents the mean of three assays.

presence of oocysts in human and animal faeces, but did not include detection of cryptosporidium oocysts in environmental samples.

Confirmation of the presence of *Cryptosporidium* in environmental samples is laborious and costly³² and is often difficult because oocysts from such samples may stain atypically in conventional and IF techniques.^{16,33} Separation of oocysts from other particulate material in water, to permit microscopy, may require sequences of filtration, chemical treatment, flotation and density gradient centrifugation.^{16,32-35} According to Ongerth and Stibbs,¹⁶ microscopic examination of the material collected from 20 L of water may take up to 6 h. It is possible that waterborne spread of *Cryptosporidium* may be suspected more often in future, and the ability to detect this parasite in drinking water supplies will be a serious problem with present detection methods.^{32,35}

In a review of parasite-related diarrhoeas, the World Health Organization³⁶ advocated the development of methods less subjective than microscopy for detecting protozoa and their cysts. Early enzyme immunoassays to detect cysts^{37,38} were based on polyclonal antibodies and were of unacceptably low sensitivity. Other assays³⁹ based on MAb appeared to offer improved sensitivity and specificity.

We describe here an EIA based on a MAb to *Cryptosporidium* (MAb-C1) for detecting the organism in faecal and environmental samples. The assay gave positive results with both faecal suspensions and faecal supernates, suggesting that the antigen detected may be either soluble or particulate. It also gave positive results when cysts were not recognisable by microscopy, possibly because they had disintegrated. This assay is as sensitive as, but less subjective than, microscopic examination

of stained smears. However, the assay did produce a positive result in 28 (6%) of 476 faecal specimens that gave negative results by microscopy. Microscopy of all 28 samples remained negative after concentration; others⁴⁰ have reported that such concentration techniques offer little increase in diagnostic efficiency. It is possible that the EIA may offer a genuine increase in sensitivity compared with microscopy. However, the observed parallel between titration curves of EIA-positive microscopy-positive samples and purified oocysts, and the lack of parallel between either of these curves and that obtained with EIA-positive microscopy-negative samples suggests that something other than cryptosporidium oocysts was causing positive EIA results in the latter.^{41,42} This may be soluble antigen(s) or other stages in the life cycle of *Cryptosporidium*, or merely a non-specific background effect. Rheumatoid factor, which may be present in body fluids during inflammation and which is a common cause of false positive EIA results,^{43,44} was not detected in 19 samples examined with a latex kit. The finding that many of the EIA-positive microscopy-negative specimens reacted in other EIA systems with MAbs raised against unrelated organisms suggests that these may be false positive results. Although potentially useful as a screening test, further development and refinement of the method is required before it could replace microscopy in the diagnosis of cryptosporidiosis.

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