INTER-LABORATORY ROUND-ROBIN TESTING OF IMMUNO-MAGNETISABLE SEPARATION (IMS) FOR THE CONCENTRATION AND SEPARATION OF *CRYPTOSPORIDIUM* OOCYSTS FROM WATER SAMPLES

Contractor: Scottish Parasite Diagnostic Laboratory

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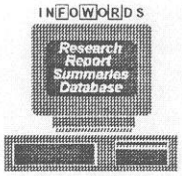

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FR/DW 0001

APRIL 1996

PREPARED FOR
THE DEPARTMENT OF
THE ENVIRONMENT - DRINKING
WATER INSPECTORATE

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1. INTER-LABORATORY ROUND-ROBIN TESTMG OF IMMUNOMAGNETISABLE SEPARATION (IMS) FOR THE CONCENTRATION AND SEPARATION OF *CRYPTOSPORIDIUM* OOCYSTS FROM WATER SAMPLES: EXECUTIVE SUMMARY

Following inter-laboratory trials of an immunomagnetisable separation (IMS) technique developed and optimised at the Scottish Parasite Diagnostic Laboratory, this method is considered to be better than the other ho techniques in current use (the "Blue book" Standing Committee of Analysts (SCA) method and flow cytometry (FCM)) for the concentration and separation of *Cryptosporidium* oocysts from clean water concentrates (<60 nephlometric turbidity units (NTU)). In certain turbid water concentrates >600 NTU, the recovery efficiency of the IMS technique and, to a lesser extent the SCA method, were reduced. The recovery efficiency, in these waters, using FCM was least affected, nevertheless, no significant **difference** in recovery efficiency could be detected between the SCA and FCM methods. However, in clean waters FCM was significantly more likely to fail to detect low numbers of oocysts in oocyst-positive samples than IMS or SCA. Inter-laboratory variation in recovery efficiencies of the techniques under investigation was extensive, but comparison between the performances of the laboratories was not considered to be the major remit of this report.

The IMS technique was not considered to affect the viability of oocysts, but was found to affect some characteristics used in the identification of oocysts by microscopy. In particular, the morphology of "old" oocysts was found to be affected by the IMS technique, the fluorescence-antibody staining of oocysts was found to be improved following the IMS technique and the uptake of 4'6 diamidino-2-phenylindole (DAPI) into the sporozoite nuclei was found to be impaired by the IMS technique.

The participating laboratories found the IMS technique simple and user-friendly and were keen to incorporate it into their armouries of analytical techniques. Additional research has the potential to improve the utility and application of this technique further.

2. INTER-LABORATORYROUND-ROBTNTESTTNGOF IMMUNOMAGNETISABLE SEPARATION (IMS) FOR THE CONCENTRATION ANDSEPARATIONOF *CRYPTOSPORIDIUM* OOCYSTS FROM WATER SAMPLES: SUMMARY

Immunomagnetisable separation (IMS) technology for the separation and concentration of target cells has been of increasing application within the biomedical field, both for routine diagnostic and measurement use and also for application as a research tool, in recent years. Whilst the use of this technology for the concentration of *Giardia lamblia* cysts from water samples has been published (Bifulco and Schaefer, 1993) and the potential for the use of this technique for the separation and concentration of *Cryptosporidium* oocysts from water has been recognised, (Robertson and Smith, 1992; Smith *et* al, 1993; Parker and Smith, 1994), no full-scale testing of the actual practical application of this technique for the separation and concentration of parasites from water has been previously conducted.

In the work undertaken for this report, the use of this technique was tested in five laboratories which undertake routine analyses of water samples for *Cryptosporidium* oocysts, by comparing the recovery efficiency of a carefully designed IMS technique with those techniques in current use (the "Blue Book" Standing Committee of Analysts (SCA) method and flow cytometry). The parameters investigated included the use of a range of target seeds of oocysts (3.3, 13 and 33 oocysts), two different volumes of water (1 ml and 10 ml) and a range of different turbidities (clean water, 40-60 nephlometric turbidity units (NTU) and greater than 600 NTU). Furthermore, as well as allowing comparison between the recovery efficiencies of these three techniques, under the constraints of the various parameters summarised above, work was undertaken to identify whether or not the IMS technique affected the viability of oocysts and also to compare the morphology, fluorescence and uptake of 4'6 diamidino-2-phenylindole (DAPI) by the oocysts following this technique.

Whilst inter-laboratory variation occurred (with some laboratories consistently finding higher or lower numbers of oocysts with the different techniques), comparison of the performance of the analytical laboratories was not the subject of this study. The laboratories were anonymised by the use of code letters and in the results section of this report the results from the laboratories are combined to allow comparison between methods and other variables without being influenced by the relative recovery efficiencies of the laboratories at the different techniques.

In very low turbidity samples (clean water), the IMS technique appeared to be significantly better than both SCA and FCM methods at recovering oocysts both from 1 and 10 ml samples. Not only were higher recovery efficiencies reported. but variation in recovery efficiency was reduced and fewer negative results were reported from oocyst-positive samples than with the other two techniques. Furthermore: the simple acid desorption stsp for dissociating the oocysts from the beads was considered to be successful, with >90% of the oocysts dissociated from the beads.

However, when the water sample is turbid: the recovery efficiency of the IMS technique may be reduced. In one trial with turbid 1 ml samples, significantly less oocysts were recovered using the IMS technique than either of the other methods and in another trial with a 1 ml turbid sample the IMS technique recovered significantly less oocysts than the FCM technique. Assessment of all the results from 1 ml turbid samples indicates that whilst the recovery efficiency of the IMS technique may be reduced by suspended matter, when the turbidity is relatively low (between 40-60 NTU), all 3 techniques performed with similar efficiency. However, when the turbidity is high (>600 NTU), the efficiency of the IMS technique is significantly affected in some water types. These results suggest that the IMS technique is affected to different extents by different material constituents in water concentrates and that FCM is apparently least affected by interfering particulate matter. However, it should be noted that in trials with clean water or low turbidity water this technique was the one which consistently reported negative results in oocyst-positive seeded samples (for clean water, this difference was found to be statistically significant).

Attempts were made to address the problems experienced in the IMS technique in samples of high turbidity, by introducing blocking agents into the method protocol. Whilst some of the blocking agents showed promise, insufficient time was available for development of this improved methodology and subsequent testing by the participating laboratories.

Whilst the IMS technique was found not to have any detectable effect on the viability of oocysts, it did appear to result in significant differences in the morphology of the oocysts (if the oocysts were "old"), fluorescent antibody staining characteristics and uptake of DAPI into the sporozoite nuclei as compared to the SCA method. Following IMS of "old" oocysts, more broken, misshapen and 'pac man' shaped oocysts were noted, however this did not appear to hinder the operators' identification of the oocysts. Following the IMS technique the fluorescence antibody staining was reported to be improved; this could be because acidification of the oocysts increases the number of epitopes available for antibody binding. The use of DAPI to assist in identification of oocysts was considered to be more useful following the SCA method than following IMS; this might be due to the acidification during IMS, hydrochloric acid is known to affect nucleic acids. However, it should also be noted that these differences were also, in part, due to characteristics of the oocysts themselves and not necessarily due to the techniques per se.

Despite the potential difficulties with the IMS in turbid water samples, the results from these trials indicate that this technique would be a very useful addition to the armoury of methods for the concentration of oocysts from water samples and was considered by the participants to be simple and user-friendly; all the participating laboratories indicated that they would be eager to use the IMS technique in routine analysis. Furthermore, with further research to address problems which may be encountered in specific water types, the potential for this technique may be realised to an even greater extent.

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4. INTRODUCTION

Background information on *Cryptosporidium*, the disease and its transmission, with emphasis on the waterborne route

Since the mid-1970's when *Cryptosporidium parvum* became recognised as an important pathogenic protozoan of man, with the potential to cause diarrhoeal disease, considerable attention and resources have been directed at investigating the epidemiology of the disease and limiting the spread of the parasite.

Cryptosporidium has a life-cycle which is completed within an individual host, with transmission by the faecal-oral route, and the potential exists for transmission by the waterborne route. Whilst many infections are, probably transmitted directly from person-to-person; transmission by the waterborne route can result in large numbers of consumers being infected by ingestion of contaminated potable water, resulting in outbreaks of waterborne disease. Detection of *Cryptosporidium* oocysts in water, and the development of technologies to remove and/or inactivate these organisms in water treatment has thus been of concern to all individuals involved with the supply of potable water and public health.

Of the *various* species of *Cryptosporidium*, one, *C.parvum*, is considered to be of particular importance to public health. This parasite also known to be infectious to over 40 species of mammal.

The life-cycle of *Cryptosporidium* is complex, involving both asexual and sexual reproductive cycles, and transmission is *via* environmentally robust oocysts excreted in the faeces of the infected host. Following ingestion and excystation of infective oocysts, sporozoites are released (four per oocyst) which infect the epithelial cells. Subsequent developmental stages have an unique intracellular, extracytoplasmic location. Here both asexual and sexual development occurs resulting in the production of large numbers of oocysts which are released into the gut lumen. Furthermore, some oocysts can release their sporozoites as they pass down the intestine, causing auto-infection within the life-cycle, which results in vast numbers of infective oocysts being excreted in faeces. For example, Blewett (1989) stated that infected calves can excrete up to 10^{10} oocysts daily, for up to 13 days.

In most individuals *Cryptosporidium* infections are self-limiting. Symptoms commence on average 3 to 6 days post-infection, and oocyst excretion generally occurs from less than 3 to 30 days, (mean: 12 days). Oocyst shedding usually coincides with the presence of clinical symptoms. However, oocyst shedding can be intermittent and can continue for up to 50 days after the cessation of symptoms (mean: 7 days).

In immunocompetent people, the clinical symptoms of cryptosporidiosis diarrhoea, malaise, abdominal pain, anorexia, nausea, flatulence, malabsorption, vomiting, mild fever and n-eight loss (Fayer and Ungar, 1986). Illness and oocyst excretion patterns may vary due to factors such as immune status: infective dose: host age, and possible variations in the virulence of the organism. In some immunocompromised individuals: (e.g. those with Acquired Immune Deficiency Syndrome (AIDS)), cryptosporidiosis can be a life-threatening condition with profuse, intractable diarrhoea, severe dehydration, malabsorption and wasting, and spread to other organs (Crawford and Vermund, 1988). Although there is evidence to indicate that infection with *Cryptosporidium* can *be* asymptomatic, neither the prevalence nor the importance of asymptomatic infection is fully understood.

Laboratory diagnosis of infection is usually dependent upon the detection of intact parasites in faecal samples or concentrates; a number of staining methods are commonly used (e.g. modified Ziehl-Neelsen, auramine-phenol) and are considered to be generally reliable although of low sensitivity (Weber *et* al., 1991; Webster et al., 1996). The relative insensitivity of conventional detection methods has encouraged the development of alternative diagnostic methods with progress in the development of sensitive techniques for the detection of parasite products by immunoassay and/or parasite DNA in faeces, and serology. At present there is no effective specific drug therapy for

osporidiosis and in cases where severe dehydration occurs. oral or intravenous rehydration therapy can be offered.

Cryptosporidium infections occur world-wide (it is common in industrialised nations and almost ubiquitous in developing countries where sanitation is frequently minimal) and the extent of occurrence of this parasite indicates its adaptation to numerous environments; the relative robustness of oocysts, the low infectious dose [median infectious dose of 132 oocysts (du Pont *et* al., 1995),] and the variable state of immunity elicited by infection contributes to the success of this infection.

Infection with *Cryptosporidium* may be transmitted either directly (person-to-person), possibly from animals (animal-to-person): and from contamination of the environment, with rhe recognised potential for waterborne transmission. Person-to-person transmission has been documented between family/household members, sexual partners, health workers and their patients, and children in day-care centres and other institutions. The last of these routes of transmission is particularly common (Ungar, 1990) probably due to the lower standards of persona1 hygiene exhibited by pre-school children and their tendency to put almost all objects that they handle in their mouths. Zoonotic, or animal-to-person, transmission has also been documented, particularly in children on farm visits, but also from laboratory animals and household pets.

Whilst transmission of *Cryptosporidium* via the food-borne route has been reported (Millard et *al*, 1994), the most important route of environmental transmission is indubitably through the contamination of water by human or animal faecal material from infected individuals. *Cryptosporidium* oocysts excreted by infected humans and other animals can contaminate surface waters either in faeces, in sewage effluent, in slurry discharges or in run-off from land. Analysis of raw waters in USA and USA has indicated that *Cryptosporidium* has a widespread occurrence (Rose *et* al., 1991; LeChevallier *et al.*, 1991; The National Cryptosporidium Survey Group, 1992) although the concentrations detected are generally low and will depend upon the variety of contributors and their associated activities performed in the catchment area. *The* occurrence of *Cryptosporidium* oocysts in potable water n-ill be dependent upon a number of factors including the size and duration of the exposure to oocysts at the treatment plant. the treatments in place within the plant, the combined **removal** efficiency of those treatments and the integrity cfthe distribution system. The few documented analyses of final, treated waters in the UK and USA have indicated that these organisms do occur in potable waters, even in the absence of epidemiological evidence of disease within the community (Smith et *al.* 1993).

Where outbreaks of waterborne cryptosporidiosis have occurred there seems often to have been an unusual occurrence in the water supply, either due to an irregularity in procedure or treatment, a greater than usual exposure to oocysts at the treatment plant, or from post-treatment contamination.

Detection of *Cryptosporidium* oocysts in water

Outbreaks of waterborne cryptosporidiosis ha\-e led to considerable interest in monitoring of water for the presence of oocysts. However, environmental monitoring for these parasites is made problematic by their small size, their relatively low concentrations in most waters, the inability to augment their numbers by in vitro culture and the difficulty in identifying them amongst other particles and debris. Currently recognised detection techniques consist of a *variety* of methods geared to extracting oocysts from complex media such as water concentrates: sand, sludges, effluents etc., according to nationally recommended procedures **(Anon,** 1990; Anon, 1994). These procedures include filtering a large volume of water through either a membrane or cartridge filter, releasing and eluting the trapped oocysts from rhe filter matrix, concentrating rhe eluate to a small volume, possibly with a clarification procedure for dirty samples, and analysing the final concentrate, or a proportion thereof, by fluorescence microscopy. Laboratory experiments reveal that within every step of the procedure the possibility for losses occurring exists, and that the more steps that are included the greater are the losses that can be expected. As well as being inefficient, this methodology is time-consuming, labour-intensive and tedious and the microscopy, in particular, requires full-time, well-

trained, patient personnel. The need to improve the methodology for detecting these parasites in water is, therefore, a very real concern and has been the focus of a large amount of research in recent years. Amongst the variety of different techniques which have been suggested for improving both the efficiency and ease of monitoring water samples for these parasites, some in particular seem to have value and have attracted the attention of laboratory staff who routinely undertake this work. Calcium carbonate flocculation has been recommended (Vesey *et al.*, 1993) for recovery of oocysts from water, although Campbell *et* al. (1994) state that this method can reduce oocyst viability, if that parameter is also of interest. Use of immunomagnetisable separation, in which the parasite of interest is bound, either directly or indirectly, to antibody-coated magnetic beads, has also been used for concentrating *Cryptosporidium* oocysts from environmental samples, and evaluation of this technique in UK Water Company laboratories for concentration of *C.parvum* oocysts is the subject of this report.

Immunomagnetisable separation technology and its application to the concentration of parasites from water

In recent years the use of immunomagnetisable particles for the separation and concentration of a variety of target cells from various fluids has been a technology of increasingly wide application and acceptance; one of its major uses is in the immunomagnetic separation of sub-populations of cells from a mixed population within the biomedical field. Within microbiology, immunomagnetisable particles have been used for the rapid selection. separation and concentration of bacteria such as *Salmonella* (Tuley, 1992) and *Escherichia coli* 0157 (Chapman et *al.*, 1994).

The first documented use of immunomagnetisable panicle technology for the separation of parasites from water samples, involved the concentration of *Giardia lamblia* cysts from water samples using magnetite particles (Bifulco and Schaefer: 1993). In this work, an indirect antibody technique was utilised, with a mouse *anti-Giardia* IgG as the primary antibody and an anti-mouse IgG, coated onto the magnetite particle, as the secondary antibody. *Giardia* cysts (500/ml) were seeded into waters of varying turbidities (target turbidities: 6, 60, 600, 6000 nephlometric turbidity units (NTU); actual turbidities: 70, 568, 1260 and 6400 NTU) and the recovery efficiency from 1 ml aliquots assessed. The overall recovery of the cysts from waters of all turbidities was found to be 82%, but excessively high turbidities (>600 NTU) were considered to interfere with cyst recovery by their method.

The first publication referring to the use of immunomagnetisable separation for the concentration of *Cryptosporidium* oocysts from water is a review article (Robertson and Smith, 1992) in which the results of preliminary studies are quoted: *anti-Cryptosporidium* monoclonal antibody coated magnetisable panicles were reported to bind 75% of oocysts seeded into water and *anti*-fluorescein isothiocyanate (FITC) monoclonal antibody coated magnetisable particles were reported to bind between 75100% of oocysts already labelled with FITC-*anti-Cryptosporidium* monoclonal antibody. The next publication referring to this work is another review article (Smith *et al.*, 1993) which reports that iron-cored latex beads coated with *anti*-FITC monoclonal antibody used to separate oocysts coated with FITC-*anti-Cryptosporidium* monoclonal antibody achieved recoveries ranging from 74 - 100% in sewage effluent seeded nith between 10^2 and 10^4 oocysts, and that up to 66% recovery could be achieved in a raw water concentrate containing 16 oocysts. Whilst the advantage of this approach is that the amplification step, the interaction of bead-bound anti-FITC monoclonal antibody with oocyst-bound FITC-anti-*Cryptosporidium* monoclonal antibody, allows more beads to adhere to oocysts, the disadvantage is that in 'real' environmental samples oocysts will not be ready-labelled with FITC-anti-*Cryptosporidium* monoclonal antibody. Further details of this work are described in a PhD thesis and a presentation abstract (Parker, 1993; Parker and Smith, 1994) in which greater recovery efficiencies are reported from cleaner water samples (comparisons between phosphate buffered saline, pond water and diluted faeces), with larger numbers of beads (comparisons between 10^4 beads and 10^6 beads), and with different antibodies. However, whether this research refers to the direct or indirect technique is not identified and the recovery efficiencies quoted show a wide variation which had not been indicated in the previous review articles (e.g. 10^6 beads

gave recovery efficiencies ranging from 9.2-107.2% and 10^4 beads gave recovery efficiencies ranging from 6.3-70.9%).

Nevertheless, results of the work available were considered to be sufficiently encouraging to merit further research and the Department of the Environment, through their Water Quality and Health interests, decided to appoint a contractor to supervise and co-ordinate a programme of inter-laboratory testing to compare the performance of the SCA "Blue Book" Method with that of an IMS technique. This work was undertaken under contract to the Department of the Environment and was managed by the Drinking Water Inspectorate.

5. MATERIAL AND METHODS

I. Paramagnetic anti-*Cryptosporidium* beads;

DYNABEADS'" M-450 paramagnetic polystyrene beads were coated, by collaborators at Dynal Research and Development (R&D), Oslo: Norway, with a monoclonal antibody raised against *Cryptosporidium* sp. oocysts and reactive to epitopes on the outer wall of oocysts. Initial research at the Scottish Parasite Diagnostic Laboratory (SPDL) and Dynal R&D indicated that this bead (M-450) was appropriate for the trials, whereas previous research (Parker, 1993) had suggested that the streptavidin M-280/anti-fluorescein isothiocyanate (FITC) bead might be most suitable.
The coated M-450 beads were supplied at a concentration of 10 mg/ml (~10^8 beads/ml).

II. Oocvst isolates

Two different isolates of *Cryptosporidium* oocysts were used in these trials. Both were purified by the trials co-ordinator (ATC) at the SPDL using the ether/sucrose purification techniques detailed in Campbell et al. (1992). The first isolate (used in trials l-3) was from a bovine source and was obtained and purified in November 1994. The viability of this "old" isolate was assessed by both the fluorogenic dye method of Campbell *et al.* (1992) and by the in *vitro* escystation method of Robertson *et* al. (1993) and considered to be less than 5%. The second isolate (used in trials 4-20) was from a human source and was obtained and purified in April 1995. The viability of this "new" isolate was approximately 85% as assessed by both the fluorogenic dye method of Campbell *et al.* (1992) and the in *vitro* excystation method of Robertson *et al.* (1993).

III. Fluorescent (FITC) anti-*Cryptosporidium* antibodies

All FITC-conjugated anti-*Cryptosporidium* monoclonal antibodies used for analysis and screening of samples in these trials were purchased by the participating laboratories from either CellLabs Diagnostics PTY Ltd, (Dale Street. Brookvale, NSW, Australia), Waterborne Inc. (Hurst Street, New Orleans: LA, USA) or Shield Diagnostics (Technology Park: Dundee, Scotland, UK).

IV. Automatic repeating dispenser

An automatic repeating dispenser (Alpha Laboratories, Eastleigh, Hampshire, UK) fitted with disposable dispenser tips was used for the preparation of seeded samples for distribution to the panicipating laboratories.

V. Consumables and specialist items of equipment

Details of other materials used in these trials including consumables and specialist items of equipment are described in detail in the protocols distributed to the participating laboratories (see appendix 1 and 2).

VI. Participating laboratories;

Four laboratories were contracted for this Round Robin test of immunomagnetisable separation (IMS) by the Drinking Water Inspectorate (DWI); SPDL, Thames Water Utilities, Yorkshire Environmental and Southern Science.
One other laboratory (Strathclyde Water Services) also asked to be included in the trials although not officially contracted to be so by the DWI. In all cases, the laboratories were treated identically, with no notice of target seed level, or any other information, given by the trials co-ordiiator and all trials were performed by trained staff regularly involved with the detection of *Cryptosporidium* oocysts in various water samples. Throughout rhis report, the laboratories are anonymised by being randomly assigned code-letters, A-E. For each trial, every laboratory retains its ovn code letter.

VII. Immunomagnetisable separation (IMS) method.

The protocol for this method (see appendix 1) was drafted in the style of the "Blue book" (UK Standing Committee of Analysts (SCA) book) and sent to the participating laboratories for initial comments. Originally the procedure did not require detergents (Tween-20) as the microfuge tubes available made additional detergents unnecessary. However, due to changes in the manufacturing of this product outwith our control, the use of detergents became necessary and, from trial 6, 0.005% Tween-20 was added at the same time as the phosphate buffered saline (PBS; see appendix 2).

The IMS system was tested by the participating laboratories in these trials as follows; the trials co-ordinator at SPDL seeded *Cryptosporidium* oocysts, of accurately estimated number, into either 1 or 10 ml volumes of water of varying turbidity and distributed the samples for analysis. Details of preparation of the seeded samples for distribution are given below. Distribution to the laboratories was by Royal Mail Special Delivery (guaranteed next working day delivery). For the two volumes of sample to be analysed, separate, detailed methods were sent to the participating laboratories (see appendix 2). A summary of the protocols used is given below. A protocol for the microscopic examination of slides for *Cryptosporidium* oocysts was also supplied (see appendix 3).

VIII. Summary of IMS protocol.

Tubes containing beads and oocyst sample are mixed, and PBS and beads added to the sample, The sample tube is rotated for 30 min. The tube is placed in a magnetic particle concentrator (MPC-M) and gently rocked for 1 min. The beads and oocysts form a 'dot' on the back wall of the tube. All fluid is carefully aspirated. The tube is removed from the MPC-M and the sample re-suspended in 100µl water. 5µl 1N hydrochloric acid (HCl) is added and the tube shaken then allowed to stand twice. 5µl 1N sodium hydroxide (NaOH) is then added and the sample again shaken. The tube is placed in a magnetic particle concentrator (MPC-E) and allowed to stand. Fluid is carefully removed in two 55µl aliquots and placed on the wells of the slide. The tube is removed from MPC-E and 100µl water added and shaken. The fluid (containing the beads) is removed in two 50µl aliquots and placed on two wells of the slide. The slides are then dried.

The slides are fixed by addition of 50µl methanol to each well and evaporation to dryness. 25µl monoclonal antibody are added to each well and incubated in an humid chamber at 37°C for 30 min. Monoclonal is aspirated from each well. 50µl PBS are added to each well, left for 2 min and then aspirated off. The wash with PBS is repeated. A third wash is conducted in the same manner but with 4'6 diamidino-2-phenylindole (DAPI) in PBS. One drop of water is added to each well, left for 2-3 seconds and then aspirated off. Following addition of mounting medium and a cover-slip, slides can be screened by microscopy.

IX. Modified Standing Committee of Analysts "Blue Book" (SCA) method.

This method was drafted in the style of the "Blue book" (see appendix 1, section 2) and sent to the participating laboratories for initial comments. As all the participating laboratories perform *Cryptosporidium* analyses of water samples on a routine basis, and were thus considered to be fully competent in this technique, no detailed method of this technique was distributed nor requested by the participating laboratories.

X. Flow Cytometry with cell sorting (FCM) methods.

All the participating laboratories, except the SPDL, have a flow cytometer set up for the analysis of water samples for *Cryptosporidium* oocysts and for three of these laboratories it is the method which is routinely used. Each laboratory used either their own "in house" or the manufacturers' method for analysis by flow cytometry (see appendix 4).

XI. Trial preparation and distribution

A set of calibrated and tested pipettes was used for the enumeration of oocysts from stock suspensions. For the duration of the trials these pipettes were not used for any other routine or experimental work within SPDL. Oocyst stock concentrations were initially estimated by enumeration of aliquots by light microscopy using a haemocytometer. Following these counts, dilutions were performed in order to obtain oocyst suspensions containing between l-2 oocysts per µl in 30 ml volumes of deionised sterile (0.2~) filtered water in sterile 50 ml centrifuge tubes (Bibby, Corning). These suspensions were used within 1 month from the date of dilution. Confirmation of the numbers in a given volume of a stock was conducted prior to distribution of samples to the participating laboratories by conducting between X-30 analyses and using the median result to calculate the target figure. These confirmatory counts were performed by directly fixing a known volume (typically 50µl) from the stock oocyst suspension onto one well of a multi-well slide, labelling with FITC-anti-*Cryptosporidium* monoclonal antibody and examination using fluorescence microscopy. At all times, before removing any aliquot of oocysts from the suspension, the stock was thoroughly mixed by vortexing for 60 sec.

Initially, three concentrations of oocysts, as follows, were set as the target oocyst seeds; 33, 13 and 3.3 oocysts per replicate in 1 ml volumes using both “new” and “old” oocysts.

1 ml seeded samples, non-turbid volumes: Trials 1-4, 6 & 7.

Seeds were prepared with the target seed (33. 13 and 3.3) per ml by aliquoting the required volume (based upon the direct count) from rhe stock into a clean 250 ml Pyrex beaker and adding deionised sterile (0.2µm) filtered water to make a final volume of 100 ml. This was mixed thoroughly using a magnetic stirrer with a Teflon-coated magnetic follower. The automatic repeating dispenser was used to distribute 1 ml volumes into 100 individual microfuge tubes. Of these microfuge tubes, 90 were randomly grouped into 3 groups of 30 tubes, with each group of tubes further subdivided into 5 sets of 6 tubes, by the trials co-ordinator. A second member of staff at the SPDL assigned the 3 groups as either IMS, SCA or FCM. The remaining 10 tubes were used as controls (see below).

Each group was assigned an unique code number using the system below:

IMS ..... Series starting from number 106.
SCA ..... Series starting from number 1020.
FCM Series starting from number 10001.

The 5 sets of 6 tubes within each group was rhen numbered so that all 6 tubes within each set had a different (sequential) number. Each of the 5 sets was numbered identically and the 6 tubes with their unique numbers placed together in labelled polythene bags which were then sealed and sorted by group. Once all tubes were labelled using this system each participating laboratory was sent a sealed bag from each group. Thus each participating laboratory would receive three sealed bags each containing 6 tubes with a unique code number. Each bag of samples would be analysed by one of the three methods. Also the participating laboratories would be sent anti-*Cryptosporidium* Dynal beads, a specification sheet (see appendix 5 for a typical example) and protocols as required.

The samples, sent by Royal Mail Special Delivery in sealed envelopes, were timed to arrive at the participating laboratories on the day prior to that time-tabled for the processing of the samples. Laboratories were instructed to store the samples and beads at 4°C. The participating laboratories examined at least 5 of the 6 tubes of each group and all the sample was analysed. For SCA and FCM analyses the samples had to be further concentrated to approximately 100 µl by microfuging at between 11000-13000 revolutions per minute (rpm) for 30 sec.

10 ml seeded samples, non-turbid volumes: Trials 13 & 14.

Seeds were prepared with the target seed (33 and 3.3) per 10 ml by aliquoting the required volume (based upon the direct count) from the stock into a clean 2 L Pyrex beaker and adding deionised sterile (0.2µm) filtered water to make a final volume of 1000 ml. This was mixed thoroughly using a magnetic stirrer and a Teflon-coated magnetic follower. The automatic repeating dispenser, was used

to distribute 10 ml volumes into 100 centrifuge tubes. Of these centrifuge tubes, 90 were randomly grouped into 3 groups of 30 tubes, with each group of tubes further sub-divided into 5 sets of 6 tubes, by the trials co-ordinator. A second member of staff at the SPDL assigned the 3 groups as either IMS, SCA or FCM. These were labelled, numbered and distributed as described for 1 ml samples above. For SCA and FCM the sample had to be further concentrated by centrifugation at 1,500 *g* for 10 min before analysis.

Turbid seeded samples (1 ml and 10 ml volumes): Trials 5, 8 - 12, 15 & 16.
Seeds were prepared with the target seed (33 and 3.3) per replicate (either in 1 ml or 10 ml volumes) as above. However, instead of seeding into deionised sterile filtered water as previously described, this water was supplemented with pooled raw-water concentrates to known turbidity. The turbidity was set to approximately 60 nephlometric turbidity units (NTU) and approximately 600 NTU using a nephlometer calibrated with formazan standards.
The raw-water concentrates were obtained from the participating laboratories and consisted of water sample pellets which had all been screened in routine analytical work for *Cryptosporidium* oocysts and which were considered to be negative for *Cryptosporidium* oocysts. These *Cryptosporidium*-negative raw-water concentrates covered a wide range of water types, including raw highland and lowland river water, filter back-flush water containing alum and ferric salts and borehole water. All trials with defined turbidity water were made using rhe same pooled water concentrates, except trials 12, 15 and 16 which used rhe same pooled concentrate. but without the inclusion of filter back-flush concentrate.

1 ml seeded samples including blocking agents (non-turbid and turbid water): Trials 17 - 20
Seeds were prepared, as above, with the target seed of 33 oocysts per ml, seeded into deionised sterile filtered water and deionised sterile filtered water supplemented with pooled water concentrates to known turbidity (-600 and ~ 6000 NTU).
Experimental blocking agents were assessed for their ability to inhibit the non-specific binding of material which previous results had indicated as reducing the recovery efficiency by the IMS procedure. Various blocking agents of different formulations including dispersants and deaggregants mere assessed and were substituted in place of the PBS/Tween 20 used in step 4 of the detailed IMS method (see appendix 2.).

Controls

Immediately after removing the required seed aliquot to prepare the target seeds for distribution to the participating laboratories (as described above), at least 4 direct controls were prepared by aliquoting the equivalent unit sample volume onto a multi-well slide, drying and fixing the sample, staining with monoclonal antibody and screening under immunofluorescence with enumeration of all oocysts detected.
The tubes remaining following the random allocation to the participants were used as either IMS or SCA controls. At least 4 were processed on the day of preparation by the trials co-ordinator using the method described for IMS. Initially (for trials 1-4 & 7) a number of the remaining tubes (at least 4) were also analysed using the SCA method.
Negative control samples were supplied trials 6 (non-turbid) and 12 ("high" turbidity) and processed using all three methods.
Randomly selected slides were requested to be returned by the participating laboratories to the trials co-ordinator at SPDL for quality control checks on numbers of oocysts reported.

XII. Reporting of trial results (method and time scale)

All samples were received by the participating laboratories according to schedule and the samples processed, up to the stage of fixing of samples onto slides, on the day designated (± 24 h). The samples were then examined and reported to the trials co-ordinator as soon as could be accomplished

by staff at the participating laboratories. This was typically within 2-4 weeks, but was frequently longer and largely depended upon other commitments to analytical tasks undertaken by the laboratories. The last trial was processed on 2219195, and the last data set was returned to the trials co-ordinator on 16/11/95.

Reporting tables were provided for the participating laboratories (see appendix 6). This table assumes that the oocysts identified meet the criteria defined in appendix 1, section 2 (Blue book) and therefore fail within the recognised oocyst size range. Thus, due to the already considerable work-load in the analyses and reporting, no documentation of the measurements of oocysts identified were requested. Details of the fluorescence and morphological (shape) characteristics (as detailed in the existing SCA method) were noted in this table. For the subsequent data analysis the results were classed as "good" or "poor" to allow construction of contingency tables. For fluorescence this meant that any oocysts reported as having weak, patchy or uneven fluorescence were scored as "poor" and only strong, even fluorescence was classed as "good". The same was true for rhe morphology (shape) of all oocysts reported, any oocyst that was observed as being broken, "pac-man" shaped or misshapen was classed as "poor".

A potential, additional improvement over the basic reporting characteristics described above was also evaluated in these trials; staining of sporozoite nuclei with the fluorogenic stain, DAPI, has previously been postulated to be of use as an adjunct for the immunofluorescent detection of oocysts (Campbell *et* al.. 1992b) and was subsequently described in a methods paper (Grimason et *al.*, 1993). For these trials, this method was modified into a rapid, user-friendly technique for staining of sporozoite nuclei which could be incorporated into both the SCA and IMS methods (see appendix 1 and 2). To assess the usefulness of DAPI staining as an adjunct for the immunofluorescent detection of oocysts, the inclusion of DAPI into the sporozoite nuclei was compared to the presence of sporulated contents of the oocysts observed by light microscopy.

XIII. Viability assessment of oocysts used in trial

The viability of the oocyst isolates used in these trials was assessed at the SPDL by both the fluorogenic dye method of Campbell *et* al. (1992) and the in *vitro* escystation method of Robertson *et* al. (1993). Full, detailed protocols for the assessment and reporting of oocysts viability was sent to all participants and a detailed viability/IMS procedure for 1 ml sample volumes was supplied for the participants to follow (see appendix 7).

Training and quality control for conducting and enumerating the viability assay are described below in section 6 of this report. training for IMS participants.

In order to compare the effect of rhe IMS technique with the SCA method on oocyst viability at the panicipating laboratories, 2 microcentrifuge tubes containing oocysts in 100µl water were sent to the participating laboratories for analysis and viability assessment. One tube (labelled 'control') was for direct viability analysis. To the other tube, 900µl water was to be added and then processed by the IMS procedure. However, following concentration of the sample by IMS rather than fixing the sample onto slides for enumeration, the viability assay was to be performed, with enumeration of oocysts in suspension to be performed in triplicate and 100 oocysts to be assessed at each viability enumeration.

XIV. Analysis of data

Both the control results accrued by the trials co-ordinator and those reported by the participating laboratories were entered into a Microsoft Excel spread-sheet and analysed using compatible (Microsoft Excel and SPSS for Windows) statistics packages. Statistical tests, including construction of contingency tables for Chi-square analyses, Mann-Whitney U-tests, Kruskall-Wallis, ANOVA, T-tests and calculation of coefficient of variation (cv) values were performed as appropriate. Furthermore, detailed analysis of the results of trials 1-5, 7 and 8 were conducted at PHLS Statistics Unit, Colindale, London by Dr. N. Andrew.

6. TRAINING FOR MS PARTICIPANTS AND TRIAL FEEDBACK

I. Pre-trial quality control on enumeration of oocysts

Slides were prepared for a quality control check by the trials co-ordinator at the SPDL. These were prepared by fixing aliquots (25µl) of a known stock of oocysts onto the 4 wells of a multi-well slide at a concentration in which the calculated distribution of oocysts would result in a statistical probability of some wells being oocyst-negative. These slides were then labelled with FITC-anti-*Cryptosporidium* monoclonal antibody and mounting medium applied. The slide wells were sealed by the addition of cover-slips with the edges sealed with clear lacquer. The slides were examined microscopically by the trials co-ordinator and the oocyst numbers and distribution per well was noted. Counts were confirmed by two senior members of staff at the SPDL, both with >5 years experience of the enumeration of *Cryptosporidium* oocysts. Participating laboratories mere each sent a single slide for examination and it was requested that the number and distribution of oocysts within each slide be reported.

Triplicate 1 ml aliquots of the oocyst stock were also sent to the participants who were requested to enumerate (using standard techniques and immunofluorescence) the number of oocysts in 250µl of each aliquot.

II. Training session

A training session on the IMS technique was held at SPDL on the 25-26 April 1995, organised and conducted by the trials co-ordinator. The training session agenda and report of the training session is detailed in appendix 8.

III. Pre-trial quality control on assessment of viability of oocysts

Although a training session on assessment of oocyst viability using the fluorogenic dye method of Campbell *et al.* (1992) would have been preferable, it was considered that insufficient time was available within the contract period to allow this. Instead, as a quality control for the participating laboratories in the performance of this vital dye assay, a control population of oocysts (-5 $\times 10^5$/ml) was labelled with the dyes at the SPDL and all members of staff at SPDL trained in this method scored the viability of 100 oocysts contained within a 10 µl aliquot using the method in appendix 7. 100 µl aliquots of this labelled population were then sent to the participating laboratories for viability analysis of 100 oocysts in 3 x 10 µl aliquots. Viability assessment was requested to be conducted within a designated 24 h period.

Furthermore two oocyst populations. prepared by mixing the "old" and "new" isolates to give one of relatively "high" viability (approximately 50% viability/excystation) and the other of "low" viability (between IO-15% viability/excystation), were sent at separate times to the participating laboratories. The participants were requested to follow the method provided (see appendix 7) and assess the viability of these oocyst populations. Triplicate assessment of the viability of the oocysts (in 10 µl aliquots) was requested to be reported by each of the participating laboratories within 24 h of a designated date.

IV. Final meeting for IMS participants

A final meeting for all the IMS participants was held at the SPDL on the 15th December 1995. The meeting was organised and chaired by Dr. Andrew Campbell and members of the R & D team from Dynal, Oslo, were also invited to attend as was Mr. Mark Smith of the UK Drinking Water Inspectorate. The meeting agenda included presentation of the results, followed by a discussion of various aspects of the IMS method, including:

1. Any effect of the technique upon the oocysts (viability, morphology, uptake of DAPI etc.) compared to the SCA method.
2. Relative ease of use compared to the SCA method.
3. Possible improvements in the technique which the participants may be able to suggest.

4. Practical considerations of the technique (e.g. time involved, cost etc.)
5. Would the participants use this system for analysing environmental samples now? If not, why not and if so, what advantages would prompt selection of this method?
6. Possible further work including system separation apparatus and use of blocking agents.

7. RESULTS

I. Pre-trial checks on seed counts and control slides

Out of a total 58 oocysts, a mean of 57 (98.3%) were reported. All negative wells were correctly reported. Only one operator missed any oocysts (see table 1) and upon subsequent examination, directed by the trials co-ordiiator, the location of the oocyst was confirmed.

Examination of seed stocks (table 2), revealed percentage means of the mean control results ranging from 55.6% (Lab B) to 101.3% (Lab A).

Table 1. Mean number of oocysts reported on pre-prepared slide

Slide No.	Laboratory	Well			
		1	2	3	4
70187V	Lab A	3	0	3	2
70187V	AC/LR	4	0	3	2
70186M	Lab B	1	3	2	3
70186M	AC/LR	1	3	2	3
70188X	Lab C	4	8	0	2
70188X	AC/ZB	4	8	0	2
70189B	Lab D	6	6	0	0
70189B	AC/ZB	6	6	0	0
		5			
70190x70190x	AC/ZB	5	0	4	5

Table 2. Mean number (n=3) of oocysts reported from examination of seed stock

Laboratory	mean	(range)
Control (n=8)	30.6	(23-36)
Lab A	31.0	(29-36)
Lab B	17.0	(11-24)
Lab C	20.3	(19-21)
Lab D	23.3	(17-28)
Lab E	26.0	(15-39)

II. Training session

1. All participants were supplied with written details, summary and background of the IMS procedure. All panicipants appeared to understand, fully, the scope and limitations of the IMS technique and the aims of the round-robin trials.
2. No problems were encountered during hands-on demonstration. All participants appeared to understand readily the procedures to be followed.
3. In the hands-on trial, two 1 ml samples containing oocysts (theoretical dilution of 33 oocysts/ml) were randomly assigned to each of the four participating laboratories. One sample was to be analysed by the 'modified' SCA method and one by the IMS technique which had been demonstrated and practised the previous day. Results are shown below in table 3 and were considered to be satisfactory.

Table 3. IMS Training session results (target = 33 oocysts)

Participant	No. oocysts detected using modified SCA method	No. oocysts detected using IMS method
Lab B	25	32
Lab C	30	23
Lab D	26	27
Lab E	23	32
Mean ± standard deviation	26 ± 3	28.5 ± 4

III. Trial Data (Recovery)

Described below are summaries of pooled data from all five participating laboratories. Complete data sets for all trails are in appendix 9, including direct, IMS and, when performed, SCA controls. At least five replicates per trial per laboratory were performed. The total numbers of analyses for each technique, when pooling the laboratory data. are for SCA, 25-26, for IMS, 25-27 and for FCM, 20.

1 ml seeded samples (non-turbid water)

In these trials oocysts were seeded into deionised sterile filtered water. (Trials 1 - 4, 6 & 7; tables 4 - 9). Trials 1 to 3 were "old" oocysts (low viability) approximately 8 months old, stored in water at 4°C. Trials 4, 6 & 7 were "new" oocysts (high viability) 2 weeks to 1 month old. Table 4 demonstrates the number of oocysts recovered by each of the techniques, table 5 indicates the degree of dissociation achieved in the IMS technique between the oocyst-bead complex in these trials and tables 6, 7 and 8 are descriptive statistics on the recovery efficiencies of these techniques in these trials. In table 9, the percentage of samples reported as negative by each technique is recorded; as would be expected, when the target seed was high (33 oocysts), none of the laboratories recorded negative results using any of the three techniques, and the greatest number of negative results was recorded when the target seed was low (3.3 oocysts). Tables 6-9 demonstrate that for these trials a consistent pattern of percentage recovery and minimised variability is seen with the 3 techniques. IMS consistently showed highest recoveries (significantly greater than both SCA and FCM in trials 4 and 7), lowest variability and least number of negative results, and FCM consistently showed lowest recoveries, greatest variability and highest number of negative results. The ratio of negative to positive results reported by FCM was calculated to be significantly higher than by either of the other two techniques ($p<0.0001$).

Table 4. Pooled data of number of oocysts recovered by each method

TRIAL (Oocyst seed; NTU of water)	SCA		IMS		FCM	
	Mean	(s.d.)	Mean	(s.d.)	Mean	(s.d.)
1 (33;0)	17.8	(10.1)	22.3	(9.0)	20.2	(9.4)
2 (13;0)	7.8	(5.5)	9.6	(3.5)	7.7	(5.2)
3 (3.3;0)	1.7	(1.2)	2.3	(1.5)	1.5	(1.7)
4 (33;0)	17.0	(8.2)	28.1	(6.5)	17.4	(9.7)
6 (13;0)	12.0	(3.54)	11.8	(3.6)	10.25	(6.7)
7 (3.3;0)	1.2	(1.4)	1.9	(1.4)	0.9	(1.3)

Table 5. Dissociation of oocyst/M450 bead complex

TRIAL (Oocyst seed; NTU of water)	IMS (% Dissociated)
1 (33;0)	89.2
2 (13;0)	86.2
3 (3.3;0)	91.4
4 (33;0)	97.9
6 (13;0)	92.8
7 (3.3;0)	95.8
mean	92.2

Table 6. Mean % Recovery

(% recovery calculated using theoretical seed)

TRIAL (Oocyst seed; NTU of mater)	SCA (Mean % Recovery)	IMS (Mean % Recovery)	FCM (Mean % Recovery)
1 (33;0)	53.9	67.0	61.1
2 (13;0)	60.3	73.5	58.8
3 (3.3;0)	52.1	70.3	45.5
4 (33;0)	51.4	85.2	52.6
6 (13;0)	92.6	90.6	78.8
7 (3.310)	36.4	58.2	27.3
Average mean %	57.8	14.2	54.0

Table 7. Median % Recovery

(% recovery calculated using theoretical seed number)

TRIAL (Oocyst seed; NTU of water)	SCA (Median % Recovery)	IMS (Median % Recovery)	FCM (Median % Recovery)
1 (33;0)	45.5	72.7	54.5
2 (13;0)	53.8	76.9	57.7
3 (3.3;0)	60.6	60.6	30.3
4 (33;0)	50.0	86.4	50.0
6 (13;0)	92.3	92.3	65.4
7 (3.3;0)	30.3	60.6	0.0
Average median %	55.4	74.9	43.0

Table 8. % Coefficient of Variation (cv)

TRIAL (Oocyst seed: NTU of water)	SCA (cv %)	IMS (cv %)	FCM (cv %)
1 (33:0)	56.6	41.2	41.1
2 (13:0)	69.8	37.0	67.4
3 (3.3:0)	70.3	64.3	113.4
4 (33:0)	48.3	23.2	55.6
6 (13:0)	29.4	j0.9	65.6
7 (3.3:0)	115.4	70.4	139.2
Average cv %	65.0	44.5	80.4

Table 9 Percentage of samples reported negative

TRIAL (Oocyst seed; NTU of water)	SCA	IMS (Total)	FCM
1 (33:0)	0	0	0
2 (13:0)	4	0	10
3 (3.3:0)	16	16	40
4 (33:0)	0	0	0
6 (13:0)	0	0	5
7 (3.3:0)	44	12	55

1 ml seeded samples (Turbid water)

In these trials oocysts were seeded into deionised sterile filtered water supplemented with pooled water concentrates to known turbidity. (Trials 5, 8 - 12; tables 10 - 15). In all these trials, oocysts were "new" oocysts (high viability) between 2 weeks to 2 months old, except trial 5 which were "old' oocysts (low viability) approximately 8 months old, stored in water at 4°C.

All trials with defined turbidity water were made using the same pooled water (which included samples from all the participating laboratories), except trial 12, 15 & 16 which used the same pooled concentrate minus the filter back-flush concentrate. Turbidities were set at either 40-60 NTU ("low turbidity" commonly encountered as potable water concentrates) or -600 NTU ("high turbidity" commonly encountered as river water concentrates).

Table 10 demonstrates the number of oocysts recovered by each of the techniques, table 11 indicates the degree of dissociation achieved in the IMS technique between oocyst-bead complex in these trials and tables 12, 13 and 14 are descriptive statistics on the recovery efficiencies of these techniques in these trials. Of particular note in table 12 is that IMS recovered significantly less oocysts than both SCA and FCM in trial 11 ($p<0.001$) and significantly less than FCM only in trial 10 ($p<0.001$).

In table 15, the percentage of samples reported as negative by each technique is recorded; as would be expected, when the target seed was high (33 oocysts) and the turbidity between 40-60 NTU, none of the laboratories recorded negative results using any of the three techniques, although when the target seed was high (33 oocysts) and the turbidity in excess of 600 NTU, negative results were reported using SCA and FCM. For all techniques the greatest number of negative results was recorded when the target seed was low (3.3 oocysts) at turbidities of between 40-60 NTU and at turbidities in excess of 600 NTU. Tables 12-14 demonstrate that for the 'low' turbidity waters (between 40-60 NTU) all three methods apparently performed similarly (in terms of percentage recovery, variability and reporting of negative results). Although in the 'low' turbidity water the

highest number of negative results was again observed with FCM, no statistical difference in ratio of negative to positive results could be demonstrated between the three methods (p=0.07). In the "high" turbidity samples (in excess of 600 NTU) the performance of the IMS technique, and to a lesser extent the SCA method, was reduced. Not only were the recovery efficiencies reduced and variability increased, but the ratio of negative to positive results was significantly lower with FCM than either of the other two techniques (p<0.0001). Also in these "high" turbidity samples the dissociation of beads and oocysts was found to be reduced.

Table 10. Pooled data of number of oocysts recovered by each method

TRIAL (Oocyst seed; NTU of water)	SCA Mean	(s.d.)	IMS Mean	(s.d.)	FCM Mean	(s.d.)
5 (33;40)	19.3	(6.6)	18.2	(5.5)	18.7	(7.5)
8 (3.3;60)	1.2	(1.5)	1.6	(1.8)	1.2	(1.7)
9 (33;60)	15.4	(10.6)	11.6	(9.5)	10.7	(7.7)
10 (33;611)	11.0	(10.7)	4.7	(4.3)	14.1	(10.1)
11 (3.3;611)	0.8	(1.2)	0.2	(0.4)	1.5	(1.6)
12 (33;615)	23.9	(18.8)	22.4	(15.4)	10.0	(13.2)

Table 11. Dissociation of oocyst/M450 bead complex

TRIAL (Oocyst seed; NTU of water)	IMS (% Dissociated)
5 (33;40)	90.1
8 (3.3;60)	90.0
9 (33;60)	91.4
mean	90.5
10 (33.611)	54.2
11 (3.3;611)	50.0
12 (33.615)	63.1
mean	55.8

Table 12. Mean % Recovery

(% recovery calculated using theoretical seed)

TRIAL (Oocyst seed; NTU of water)	SCA (Mean % Recovery)	IMS (Mean % Recovery)	FCM (Mean % Recovery)
5 (25;40)	58.4	55.2	54.5
8 (3.3;60)	37.6	48.5	36.4
9 (33;60)	46.8	35.1	32.4
mean	**47.6**	**46.3**	**41.1**
10 (33;611)	33.3	14.3	42.9
11 (3.3;611)	25.5	4.8	45.5
12 (33;615)	72.5	68.0	90.9
mean	**43.8**	**29.0**	**59.8**

Table 13. Median % Recovery

(% recovery calculated using theoretical seed)

TRIAL (Oocyst seed; NTU of water)	SCA (Median % Recovery)	IMS (Median % Recovery)	FCM (Median % Recovery)
5 (33;40)	57.6	57.6	54.5
8 (3.3;60)	30.3	30.3	0.0
9 (33;60)	42.4	33.3	36.4
mean	**43.4**	**40.4**	**30.3**
10 (33;611)	36.4	15.2	40.9
11 (3.3;611)	0.0	0.0	30.3
12 (33;615)	81.8	75.8	98.5
mean	**39.4**	**30.3**	**56.6**

Table 14. % Coefficient of Variation (cv)

TRIAL (Oocyst seed; NTU of water)	SCA (cv %)	IMS (cv %)	FCM (cv %)
5 (33;40)	34.1	30.4	39.9
8 (3.3;60)	121.6	109.7	139.4
9 (33;60)	68.5	82.4	72.1
10 (33;611)	97.5	91.6	71.5
11 (3.3;611)	140.4	233.9	104.9
12 (33;615)	78.4	68.8	40.4
Average cv %	90.4	102.8	78.0

Table 15. Percentage of samples reported negative

TRIAL (Oocyst seed; NTU of water)	SCA	IMS	FCM
5 (33;40)	0.0	0.0	0.0
8 (3.3;60)	44.0	20.0	55.0
9 (33;60)	12.0	19.2	25.0
10 (33;611)	32.0	28.0	0.0
11 (3.3;611)	56.0	84.0	40.0
12 (33;615)	20.0	16.0	0.0

10 ml seeded samples (non-turbid and turbid water)

In these trials oocysts were seeded into deionised sterile filtered water and supplemented with pooled water concentrates to known turbidity. (Trials 13 - 16; tables 16 - 21). In all these trials oocysts were "new" oocysts (high viability) between 2 weeks to 2 months old.

All trials with defined turbidity water were made using the same pooled water (included samples from all the participating laboratories). except trials 12, 15 & 16 which used the same pooled concentrate minus the filter back-flush concentrate.

Table 16 demonstrates the number of oocysts recovered by each of the techniques, table 17 indicates the degree of dissociation achieved in the IMS technique behveen oocyst-bead complex in these trials and tables 18, 19 and 20 are descriptive statistics on the recovery efficiencies of these techniques in these trials. In table 21, the percentage of samples reported as negative by each technique is recorded; as would be expected, for waters of the same or similar turbidities when the target seed was high (33 oocysts), less negative results were recorded than for when the target seed was low (3.3 oocysts). Whilst for the clean water samples, no significant differences in the negative to positive ratio could be detected behveen the three techniques, there were significant differences detected in the negative to positive ratio in the turbid water samples, with SCA having a significantly higher negative to positive ratio than IMS (p=0.023) and FCM (p=0.003), although no significant difference between these latter two methods was detected, Tables 18-21 demonstrate that for these trials, the same consistent pattern of percentage recovery and miniiised variability seen with the 3 techniques in trials 1-4, 6 & 7 (1 ml seeded samples, non-turbid water) are repeated here, with IMS consistently showing highest recoveries, lowest variability and least number of negative results, although, in these latter trials, FCM did not consistently show the lowest recoveries, greatest variability and highest number of negative results (see trials 15 and 16). These results indicate that by diluting the samples (1 ml 600NTU = 10 ml 60NTU), the problem caused by contaminating debris appears to be reduced for the IMS technique, although not for the SCA method. FCM again appears to be least affected by debris.

Table 16. Pooled data of number of oocysts recovered by each method

TRIAL (Oocyst seed; NTU of water)	SCA		IMS		FCM	
	Mean	(s.d.)	Mean	(s.d.)	Mean	(s.d.)
13 (33:0)	27.1	(13.4)	48.6	(21.7)	27.6	(17.5)
14 (3.3:0)	2.1	(2.2)	4.0	(3.2)	1.9	(1.8)
15 (3.3:60)	1.0	(1.5)	3.6	(3.4)	2.7	(2.1)
16 (33:60)	9.0	(10.4)	18.8	(14.6)	14.0	(12.5)

Table 17. Dissociation of oocyst/M450 bead complex

TRIAL (Oocyst seed; NTU of water)	IMS (% Dissociated)
13 (33:0)	92.4
14 (3.3:0)	89.0
15 (3.3:60)	84.4
16 (33:60)	65.5
mean	82.2

Table 18. Mean % Recovery

(% recovery calculated using theoretical seed)

TRIAL (Oocyst seed; NTU of water)	SCA (Mean % Recovery)	IMS (Mean % Recovery)	FCM (Mean % Recovery)
13 (33;0)	83.2	147.4'	80.9
14 (3.3;0)	64.2	121.2'	57.6
15 (3.3160)	29.1"	109.1	80.3
16 (33;60)	27.2"	56.8	42.4
Average mean %	50.9	108.6	65.3

* IMS significantly greater than both SCA and FCM in trials 13 ($p<0.001$) and 14 ($p<0.04$).
# SCA significantly less than both IMS and FCM in trial 15 ($p<0.002$) and significantly less than IMS only in trial 16 ($p<0.02$).

Table 19. Median % Recovery

(% recovery calculated using theoretical seed)

TRIAL (Oocyst seed; NTU of water)	SCA (Median % Recovery)	IMS (Median % Recovery)	FCM (Median % Recovery)
13 (33;0)	75.8	145.5	98.5
14 (3.3;0)	60.6	121.2	45.5
15 (3.3;60)	0.0	90.9	60.6
16 (33;60)	6.1	48.5	30.3
Average median %	35.6	101.5	58.7

Table 20. % Coefficient of Variation (cv)

TRIAL (Oocyst seed; NTU of water)	SCA (cv %)	IMS (cv %)	FCM (cv %)
13 (33;0)	48.7	44.6	65.7
14 (3.3;0)	101.9	79.7	96.4
15 (3.3;60)	157.6	93.2	79.6
16 (33;60)	116.3	77.9	89.4
Average cv %	106.9	73.9	82.8

Table 21. Percentage of samples reported negative

TRIAL (Oocyst seed; NTU of water)	SC.4	IMS	FCM
13 (33;0)	0	0	10
14 (3.3;0)	32	20	35
15 (3.3;60)	60	24	15
16 (33;60)	28	8	15

1 ml seeded samples including blocking agents (Non-turbid and turbid water)

In these trials oocysts were seeded into deionised sterile filtered water and supplemented with pooled water concentrates to known turbidity. (Trials 17 - 19; tables 22 - 26.) In all these trials oocysts were "new" oocysts (high viability) 2 weeks to 1 month old.

All trials with defined turbidity water were made using the same pooled water (included samples from ail the participating laboratories), except trials 12. 15 & 16 which used the same pooled concentrate minus the filter back-flush concentrate.

Table 22 demonstrates the number of oocysts recovered by each of the techniques, table 23 indicates the degree of dissociation achieved in the IMS technique between oocyst-bead complex in these trials and table 24 provides descriptive statistics on the recovery efficiencies of these techniques in these trials. The experimental blocking agents used showed promise, however at present the available results are not sufficiently consistent to allow rheir recommendation.

The data from trial 20 (in which oocvsts were seeded in to highly turbid water of ~6000NTU) are given in appendix 9. These data are difficult to analyse as, although a higher number of oocysts were recovered by the IMS technique, a larger volume was examined. An absence of background data for this trial means that direct comparisons using rhis data cannot be made.

Table 22. Pooled data of number of oocvsts recovered bv each method

TRIAL (Oocyst seed: NTU of water)	SCA		IMS		FCM	
	Mean	(s.d.)	Mean	(s.d.)	Mean	(s.d.)
17 (33;0)	39.5	(11.5)	4.4	(2.7)	35.0	(16.5)
18 (33;0)	32.8	(11.2)	26.6	(11.0)	28.3	(16.5)
19 (33;617)	103	(11.3)	4.5	(2.6)	13.4	(11.0)

Table 23. Dissociation of oocyst/M450 bead complex

TRIAL (Oocyst seed: NTU of water)	IMS (% Dissociated)
17 (33;0)	83.5
18 (33;0)	62.5
19 (33;617)	76.8

Table 24. Mean % Recovery

(% recovery calculated using theoretical seed)

TRIAL (Oocyst seed; NTU of water)	SCA (Mean % Recovery)	IMS (Mean % Recovery)	FCM (Mean % Recovery)
17 (33;0)	119.8	13.2	105.9
18 (33;0)	99.5	80.7	85.6
19 (33;617)	31.3	13.6	40.5

Controls

Appendix 10 shows the distribution of oocyst numbers detected following direct immunofluorescent labelling in replicate volumes of various stocks. Quality control checks on the anti-*Cryptosporidium* M-450 beads were performed using these stocks throughout the trials.

The individual trial controls (direct, IMS and SCA) are listed along with the data for each trial (see appendix 9).

All negative control samples, for all methods for both clean (trial 6) and turbid (trial 12) samples were reported as negative. All slides recalled (between 5 and 10 slides for each laboratory) have been within 10% of reported numbers.

IV. Trial data (Viability)

The viability of the oocyst isolates used in these trials was assessed at the SPDL by the trials co-ordinator by both the fluorogenic dye method of Campbell *et* al. (1992) and the in *vitro* excystation method of Robertson *et al.* (1993). These results appear in Table 25 below. There was no significant difference between viability scored by in vitro excystation compared to the viability as assessed by vital dyes (DAPI and propidium iodide (PI)). Described in table 26 are summaries of pooled data from all five participating laboratories. Complete data sets for all trails are in appendix 11. No significant difference in the viability of the population of oocysts before and following IMS was detected. The relatively large range of viabilities reported may possibly be due to only 100 oocysts being assessed from a population of $>5\times10^5$ oocysts. although it should be noted that such disparity has never previously been recorded. The range in viabilities reported is larger in the "low" Liability population.

Table 25. Comparison of viability assessed by inclusion/exclusion of DAPI and PI^r and by in vitro excystation of 2 different populations of *Cryptosporidium* oocysts.

	"High" viability population			"Low" viability population		
	DAPI/PI viability	*in vitro* excystation	Sporozoite ratio	DAPI/PI viability	*in vitro* excystation	Sporozoite ratio
	47	47	3.3	14	14	2.5
	52	53	2.7	11	12	3.1
	48	55	2.4	12	14	3.7
	51	54	2.7			
	54					
	55					
mean	51.2	52.3	2.8	12.3	13.3	3.1
s.d.	3.2	3.6	0.5	1.5	1.2	0.6
median	51.5	53.5	2.7	12.0	14.0	3.1

Table 26. Effect of the IMS method on *C.parvum* oocyst viability ("high" viability isolate) using DAPI/PI

	VIABLE (control)	VIABLE (IMS)	POT-VIABLE (control)	POT-VIABLE (IMS)	NON-VIABLE (control)	NON-VIABLE (IMS)
mean	43.6	43.7	9.5	6.4	46.9	49.8
s.d	14.1	10.8	14.0	7.5	15.3	13.3
cv (%)	32.3	24.7	118.0	117.0	32.6	26.7
median	49.0	45.0	2.0	2.0	48.0	46.0
max.	70.0	60.0	43.0	21.0	72.0	74.0
min	20.0	25.0	0.0	0.0	22.0	32.0

V. Comparison of the IMS merhod and the SCA method on the morphology, fluorescence, contents and uptake of DAPI by *C.parvum* oocysts detected following immunofluorescent labelling.

Detailed analysis of the data has been conducted only for trials 1-4 & 6 in which 1315 separate observations for the SCA method and 1917 for the IMS method have been made. These data are described in tables 27-29. These represent 1 ml sample volumes in clean water with both "old" and "new" oocysts only. Further data is provided in appendix 12, and any significant differences identified where appropriate.

Comparison of the morphology (table 27) of observed oocysts detected following the SCA method with the IMS method for "old" oocysts reveals that the SCA method results in better ($p<0.001$) observed shape. However no significant differences for the either the "new " oocysts alone, or when the data for "old" and "new" oocysts are pooled, ($p>0.05$) were detected. Comparison of the "old' oocysts with the "new" oocysts detected following the SCA and IMS methods reveals that the "old" oocysts demonstrated a better ($p<0.001$) observed shape than "new" oocysts using the SCA method. However no significant difference for the IMS merhod ($p>0.05$) was detected. Combination of the data for SCA and IMS methods again reveals that the "old" oocysts apparently demonstrated better ($p<0.001$) observed shape than "new" oocysts using the SCA merhod.

Comparison of the fluorescence (table 28) of obsened oocysts detected following the SCA method with the IMS method for borh "old" and "new" oocysts reveals that the IMS method results in better ($p<0.02$, "old" oocysts; $p<0.00002$, "new" oocysts) obsened fluorescence. Comparison of the "old' oocysts with the "new" oocysts detected following rhe SCA and IMS methods reveals that the "new" oocysts return better ($p<0.0003$) observed fluorescence than "new" oocysts using the IMS method. However no significant difference for the SCA merhod ($p>0.05$) was detected.

Comparison of the DAPI uptake of oocysts (table 29) following the SCA or IMS techniques revealed that whilst no significant difference in the percentage of oocysts with contents was detected for the two techniques, significantly more oocysts incorporated DAPI ($p<0.0001$) and had 4 observable nuclei ($p<0.05$) following the SCA technique than following the IMS technique.

Table 27. Comparison of the IMS method and the SCA method on the morphology of *C.parvum* oocysts detected following immunofluorescent labelling.

% Good Shape	SCA method	IMS method	Combined IMS & SCA method
"Old" oocysts	95.2	90.0	92.0
"New" oocysts	86.4	89.3	88.1
Combined "Old" & "New" oocysts	90.0	89.4	

Table 28. Comparison of the IMS method and the SCA method on the FITC fluorescence characteristics of *C.parvum* oocysts detected following immunofluorescent labelling.

% Good Fluorescence	SCA method	IMS method	Combined IMS & SCA method
"Old" oocysts	80.3	85.1	83.2
"New" oocysts	83.8	90.6	87.7
Combined "Old" & "New" oocysts	82.6	88.0	

Table 29. Comparison of the IMS method and the SCA method on the presence of contents and the uptake of DAPI by sporozoite nuclei in *C.parvum* oocysts detected following immunofluorescent labelling.

	SCX method	IMS method
% with contents	84.5	85.2
% DAPI	92.9	86.8
% 4 nuclei	42.1	38.4

VI. Final meeting for IMS participants

The meeting agenda, minutes of the afternoon session of the meeting and feed-back from the participating laboratories are detailed in appendix 13.

VII. Summary of results

The main points from the results are summarised below in a list format.

1. The IMS technique appeared to be a significantly better method than both SCA and FCM methods at recovering oocysts from 1 and 10 ml clean (very low turbidity) water samples seeded with *C.parvum* oocysts with:
    a) Higher recovery efficiencies
    b) Less variability in the method (% cv)
    c) Less negative results reported
    d) >90% oocysts dissociated from rhe beads in the simple, acid desorption step

2. All three methods showed similar performance characteristics in the recovery of oocysts from low turbidity water (<100 NTU) in 1 ml samples. Interestingly, in clean water samples FCM demonstrated a greater occurrence of negative results than either of the other techniques.

3. IMS and FCM techniques appeared to be consistently better than the SC.4 method in the recovery of oocysts from low turbidity water (<100 NTU) in 10 ml samples.

3. SCA and FCM methods appeared to be consistently better than the IMS technique in the recovery of oocysts from high turbidity water. The recovery efficiency by this technique seems to depend largely upon the water type and/or materials present in sample rather than the turbidity per se. These data indicate that turbidity may not by the correct, or only, parameter for attempting to assess the usefulness of IMS, as high recovery efficiencies were obtained from certain high turbidity waters by IMS.

5. The IMS technique appeared not to affect oocyst viability.

6. Whereas oocyst morphology appeared not to be affected by the IMS method, fluorescence of the FITC anti-*Cryptosporidium* monoclonal antibody appeared to be improved following this technique. However, DAPI staining as an adjunct appeared to be more useful following the SCA method than the IMS method, despite there being no differences in the retention of oocyst contents following either technique. For all three of these parameters (morphology: fluorescence and DAPI staining of sporozoite nuclei). the age of the oocysts also seemed to exert some effect.

8. DISCUSSION

The use of immunomagnetisable separation (IMS) technology for the separation and concentration of target cells is not new, and has. in recent years. been gaining credence and popularity within the bio-medical field, both for routine diagnostic and measurement use and also for application as a research tool. Microbiological applications have included the separation of *Salmonella enteriditis* from artificially contaminated egg yolk (Tuley, 1992), the separation of *Escherichia coli* 0157 from bovine faeces in monitoring of dairy herds (Chapman *et* al.. 1994), the isolation of *E.coli* O157 from food samples (Wright *et* al., 1994) and the concentration of *Giardia lamblia* cysts from water samples (Bifulco and Schaefer: 1993). In the latter case, the authors did not use a direct immunomagnetic concentration technique, but relied instead upon a pre-enrichment flotation step to reduce the excessive particulate debris. prior to immunomagnetic separation.

Whilst, to the authors' knowledge, there has been no full research papers published on the use of IMS technology for the separation and concentration of *Cryptosporidium* oocysts from water, at least two review articles, a PhD thesis and abstracts of m-o presentations (Robertson and Smith, 1992; Smith *et al*, 1993; Parker, 1993; Parker and Smith. 1994; Fricker *et* al., 1995) have provided an indication of the potential for this technique both as a research tool and in routine use in those laboratories which undertake analyses of water samples for *Cryptosporidium* oocysts.

In the work undertaken for rhis report. the use of this technique was tested in five laboratories which undertake routine analyses of water samples for *Cryptosporidium* oocysts, by comparing the recovery efficiency of a carefully designed IMS technique with those techniques in current use (the "Blue Book" Standing Committee of Analysts (SCA) method and flow cytometry (FCM)) from two different volumes of water (1 ml and 10 ml) with different turbidities (clean water, 40-60 nephlometric turbidity units (NTU), >600 NTU), and with different target seeds of oocysts (3.3, 13 and 33 oocysts). Furthermore, as well as allowing comparison of the oocyst recovery efficiency of these three techniques, work was undertaken to identify whether or not the IMS technique affected the viability of oocysts and also to compare the morphology, fluorescence and uptake of 4'6 diamidino-2-phenylindole (DAPI) by the oocysts following this technique.

Whilst inter-laboratory variation occurred (with some laboratories consistently finding higher or loner numbers of oocysts with the different techniques), comparison of the analytical laboratories was not the subject of this study, and the laboratories were anonymised by the use of code letters and in the results section of this report the results from rhe laboratories are combined to allow comparison between methods and other variables without being influenced by the relative efficiencies of the laboratories at the different techniques: results are divided by laboratory in the appendices.

In very low turbidity samples (clean water), the IMS technique appeared to be a significantly better method than both SC.4 and FCM methods at recovering oocysts both from 1 and 10 ml samples. Not only were higher recover) efficiencies reported. but variation in recover) efficiency was reduced and fewer negative results were reported than with the other two techniques. Furthermore, the simple acid desorption step for dissociating the oocysts from rhe beads was considered to be successful, with >90% of the oocysts dissociated from the beads. However, whilst the IMS technique seems to be effective, with reproducible results which are an improvement on the standard techniques, when the water sample is turbid, the efficiency of the ISIS technique is reduced. In one trial with turbid 1 ml samples, significantly less oocysts were recovered using the IMS technique than either of the other methods and in another trial with a 1 ml turbid sample the IMS technique recovered significantly less oocysts than the FCM technique. Assessment of all the results from 1 ml turbid samples indicates that whilst the efficiency of the IMS technique is reduced by turbidity, when the turbidity is relatively low (between 40-60 NTU), all 3 techniques performed with similar efficiency. However, when the turbidity is high (>600 NTU), the efficiency of both the IMS technique and the SCA method are significantly affected. These results suggest that the IMS technique is affected to different extents by different material constituents in water concentrates and that FCM is apparently least affected by interfering debris. Also in these "high" turbidity samples (>600 NTU) the dissociation of beads and

oocysts was found to be reduced. This could either be due to inhibition of the acid desorption of the MAb/epitope or to non-specific matrixing of oocysts by the bead/material complex when the beads arc removed following the dissociation step. Bifulco and Schaefer (1993), also found that in high turbidity samples (>600 NTU) the recovery of *Giardia* cysts was reduced. However, it is not clear whether, in their work ,this represents losses due to the flotation steps used or to the immunomagnetic separation procedure per se. Our research also indicates that the water-type (i.e. the nature of the particulate material which results in the given turbidity measurements) plays a very important role in determining the efficiency of the IMS technique (e.g. 4.8% recovery efficiency was reported in 600 NTU pooled water concentrate, but a 68% recovery efficiency (over 1 log improvement) was reported in the same pooled water concentrate when the filter back-flush water was excluded). Thus, it should be emphasised that the development of any system must be tested in a range of waters.

Whilst FCM appeared to be the technique which provided the most efficient recoveries in these "high" turbidity samples (>600 NTU), it should be noted that in trials with clean water or low turbidity water this technique was the one which consistently reported most negative results (for clean water, this difference was found to be statistically significant), particularly when seed levels were low. As oocysts usually occur in water samples in low numbers, these results may indicate a draw-back to this technique which has not previously been described in the scientific literature. However, when the turbidity increased, the number of negative results reported using FCM was either less than, or similar to, the other two methods.

Attempts were made to address the problems experienced in the IMS technique in samples of high turbidity: by introducing blocking agents into the method protocol. Whilst some of the blocking agents showed promise, insufficient time was available for full development of this improved methodology and subsequent testing by the panicipatirg laboratories. Whilst the efficiency of the IMS technique may be improved by blocking agents, particularly in waters of high turbidity, further extensive testing would be required.

Whilst the IMS technique was found not to have any detectable effect on the viability of oocysts, it did appear to result in significant differences in the oocysts' morphology (if the oocysts were "old"), monoclonal staining characteristics and uptake of DAPI into the sporozoite nuclei as compared to the SCA method. As indicated above, the morphology was only affected in the older population of oocysts, with the reporting of significantly greater numbers of broken, misshapen and 'pat-man' shaped oocysts following this technique. This could be due to mixing of beads with oocysts causing older, physically 'stressed' oocysts to rupture. However, the altered morphology did not apparently hinder rhe operators' identification of the oocysts. Whilst the improved fluorescence with the monoclonal antibody following the IMS technique is intriguing and provides an additional criterion for incorporating this technique into routine use where appropriate. it is not easy to provide explanations which are supported by other research. One suggestion is that rhe acidification of the oocysts increases rhe number of epitopes available for antibody binding. However, work by Vesey *et* al. (1993) has suggested that acidification of oocysts may cause a reduction in fluorescence intensity following monoclonal antibody binding. Nevertheless, in the work by Vesey *et* al. (1993) the observations were made using flow cytometric analysis with 488 nm laser light for fluorescein isothiocyanate (FITC)-excitation and a photo-multiplier tube for measuring FITC-emission, and thus may not be considered to be directly comparable to the work described here in which the FITC-excitation was with high pressure mercury lamps and the distribution and relative brightness of FITC-emission assessed by simple viewing by eye.

The use of DAPI to assist in identification of oocysts has been widely acclaimed, and, again, whilst it is interesting to note that it was considered to be more useful following the SCA method than following IMS, it is not easy to provide suggestions for why this might be observed which are supported by the scientific literature. It is possible, however, that acidification during IMS may cause cross-linking to occur in the oocyst/sporozoite membranes, thus reducing the subsequent ingress of DAPI. However, it should also be noted that these differences were also, in part, due to characteristics of the oocysts themselves and not necessarily due to the techniques *per se*.

Despite the potential difficulties with the IMS in turbid water samples, the results from these trials would indicate that this technique would be a very useful addition to the armoury of methods for the concentration of oocysts from water when undertaking such analyses. Not only did the technique perform significantly better than either FCM and SCA in particular conditions, but it was also considered by the participants to be simple and user-friendly and all the participating laboratories indicated that they would be eager to use it in routine analysis. In practice, this would involve IMS being used for concentration and purification in place of sucrose flotation in the SCA method, following which the sample would either be fired down onto microscope slides and analysed directly by microscopy or would be subsequently analysed following FCM. It should be emphasised that whilst the three techniques compared here were treated separately, there are several opportunities for them to be used in conjunction, so that any problems or short-fall in one, may be compensated for by another. By using these three techniques in this complementary fashion, with selection of appropriate steps from the techniques, depending both upon the questions to be addressed and the type of samples to be analysed, our ability to detect oocysts in water samples should be improved.

The scope for further research on IMS and the use of IMS in the concentration and purification of *Cryptosporidium* oocysts from water samples remains relatively large. The results from this project, have provided data on the usefulness of introducing the technique, as developed, into both routine analytical and research laboratories. Furthermore, they have provided in an indication of the potential that this Technique could have; if further research resources are directed towards its development. In particular, the optimising of the technique for high turbidity (>100 NTU) environmental samples would be of great interest, as would be identifying the nature of inhibitory material(s) and the mechanisms involved in the inhibition of the IMS technique. It would be of pertinence to assess the proportion of environmental waters which contain inhibitory materials and to determine whether they are confined to particular types of samples (e.g. filter back-flush waters, waters from particular geological locations etc.). Whilst the simple acid dissociation step was found to be satisfactory, the requirement for neutralisation and the rate-limiting nature of this step (as identified by the participants) is indicative of the usefulness of developing simple, single-step, specific dissociation methods. Finally, as the participants indicated that they would wish to use IMS in place of sucrose flotation in the SCA method, it would be pertinent to perform simple comparative tests between IMS and the currently-used flotation techniques.

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10. GLOSSARY

***Cryptosporidium*:** A protozoan parasite of the phylum *Apicomplexa*, one species of which (*Cryptosporidium parvum*), is recognised as being pathogenic to humans as well as a range of other mammals.

Cyst: The robust transmission stage of the parasite *Giardia*.

1'6 **diamidino-2-phenylindole (DAPI):** A vital dye which fluoresces when excited by light of wavelength 360-380 nm and the fluorescence intensity of which increases by 20 fold following binding to nucleic acids.

Epitope: The molecular structure which an antibody paratope will recognise and bind to.

Flow cytometry (FCM): The cytometric analysis of fluorescently-labelled cells within a flow cell. In this report fluorescently-labelled organisms (*Cryptosporidium* oocysts) are sorted using pre-defined fluorescent and light-scatter characteristics.

Fluorescein isothiocyanate (FITC): A dye which fluoresces n-hen excited by light of wavelength 470-480 nm and is frequently used in association with antibodies for the recognition of particular organisms or structures.

***Giardia*:** A protozoan parasite of the phylum *Sarcomastigophora*.

IgG: An immunoglobulin (antibody) of the class G

Immunomagnetisable separation (INS): A separation technique based on the use of immunological methods (antibody recognition and binding to particular epitopes) in conjunction with magnetisable separation.

***In* vitro excystation:** The hatching of an organism (in *this* case *Cryptosporidium*) in the laboratory (not within a living host) when exposed to a set of environments and conditions.

Monoclonal antibody: An antibody secreted by cloned myeloma (cancer) cell-lines. The antibody is selected for a specific response to a particular epitope.

Oocyst: The robust transmission stage of the parasite *Cryptosporidium*.

Sporozoite: The infectious stage of the parasite *Cryptosporidium*: each oocyst contains 4 sporozoites.

Streptavidin: A protein which binds with high avidity to biotin.

Turbidity: A measure of the suspended matter content of liquid (measured in nephlometric turbidity units (NTU) and compared to formazan standards)

Viability: A measurement of the ability of an organism to proceed to the next stage in the life-cycle. In this report, usually refers to the ability of *C.parvum* oocysts to excyst with the release of at least one sporozoite from each oocyst.

11. APPENDICES

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APPENDM 1. SECTION I: PROCEDURE FOR THE IMRIUNOMAGNETISABLE SEPARATION OF *CRYPTOSPORIDIUM* OOCYSTS FROM SAMPLES.

1. SCOPE

1.1 This procedure is for the purification of *Cryptosporidium parvum* oocysts from water and other samples using magnetic beads labelled *with* anti-*Cryptosporidium* monoclonal antibody.

1.2 The utility and efficiency of this method is to be compared with the SCA ('Blue book') method for the purification of oocysts from water and other environmental samples.

1.3 The procedures described below do not purport to address all of the safety problems associated with its use. It is the responsibility of the users of these procedures to identify and establish appropriate health and safety practices and determine the applicability of regulatory limitations prior to use. It must be remembered *at* all times *that Cryptosporidium* is a pathogen of man and all samples must therefore be treated as potentially infectious.

2. REFERENCED DOCUMENTS

2.1 Blue book: Isolation and identification of Giardia cysts, Cryptosporidium oocysts and free living pathogenic amoebae in water etc. 1989. Methods for the examination of waters and associated materials. HMSO Publications: London. 1990. ISBN 0 11 752282 1.

3. SUMMARY OF METHOD

3.1 *Cryptosporidium* oocysts will be concentrated from volumes of water or water-based samples by the addition of immunomagnetic beads coated with anti-*Cryptosporidium* monoclonal antibody to the sample. The beads will be thoroughly mixed into the sample to create sufficient opportunity for every oocyst in the sample to be bound to the beads. The magnetic beads with the attached oocysts will then be separated from the remainder of the sample by a magnetic particle concentrator and thus concentrated to a small volume. This volume can then be examined by the modified SCA method (see section II) usually utilised for examination of samples for *Cryptosporidium* oocysts.

4 SIGNIFICANCE AND USE

4 This method is designed to provide an improved method for the concentration of *Cryptosporidium* . s from water and water-based samples.

4.2 The potential advantages of this method as cotnpared to the SCA method are that it provides a final concentrate for examination which contains less contaminating debris and that it enables a higher proportion of the final concentrate to be examined.

4.3 This method will not identify the species of *Cryptosporidium* concentrated nor will it identify host species nor the origin of the oocysts.

4.4 The viability of the separated oocysts may be determined by methods described elsewhere (Section III).

5. INTERFERENCES

5.1 Organisms or objects (particularly ferrous particulate matter) which bind to the magnetic beads by specific or non-specific mechanisms may interfere with this technique.

5.2 Materials/chemicals which may occur in water or water-based samples may inhibit the binding of the oocysts to the monoclonal antibody on the beads. either by altering the antibody or by altering the surface of the oocyst.

5.3 Freezing of oocysts in the samples may inhibit their binding to the magnetic beads and may also interfere with their identification subsequent to separation.

6. APPARATUS

6.1 I.5 ml graduated microfuge tubes made from metal free polypropylene as supplied by Life Sciences International (Cat. No. M109)

6.2 Screw-cau 10 ml test tubes made from glass.

6.3 Gilson (or similar) pipettes and appropriate sterile pipette tips. Flat gel-loading pipette tips will also be required. Gilson pipettes should be P1000, P200 and P20. Pipettes should be calibrated before each set of trials following **the manufacturer's instructions. Records of calibration should be kept.**

6.1 Near vertical rotator e.g. Voss Model 4400. Set up at room temperature.

6.5 Mixer (vortex type) e.g. Fison's Whirlimixer. Set up at room temperature.

6.6 Magnetic particle concentrator for 10 ml test-tubes Dynal MPC-I. Set up at room temperature.

6.7 Magnetic particle concentrator for eppendorf tubes Dynal MPC-M. Set up at room temperature.

6.8 Magnetic panicle concentrator for eppendorf tubes Dynal MPC-E. Set up at room temperature.

6.9 Magnetic stirrer with magnetic followers e.g. Bibby B212. Set up at room temperature.

7. REAGENTS AND MATERIALS

7.1 Purity of reagents Reagent grade chemicals shall be used in all tests unless otherwise indicated.

7.2 Preparation of reagents Prepare all reagents in accordance with the appropriate health and safety guidelines.
7.3 Purity of water Unless otherwise indicated. references to water shall be understood to mean Grade 1 laboratory water.
7.4 Samples Samples will be provided for the analysis. Each sample will be a volume of water or water-based material which has been seeded with *Cryptosporidium parvum* oocysts.
7.5 Magnetic beads Magnetisable beads coated with the anti-*Cryptosporidium* monoclonal antibody shall be provided.
7.6 1 Normal hydrochloric acid solution Caution. Wear gloves and suitable eye protection
7.7 1 Normal sodium hydroxide solution Caution. Wear gloves and suitable eye protection

8. PRECAUTIONS

8.1 The analyst must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using and disposing of sample concentrates. reagents and materials and whilst operating the equipment used.
8.2 Never mouth-pipette

9. PROCEDURE

9.1 Shake the beads for 10 seconds on the miser.

9.2 Shake the sample containing oocysts for 10 seconds on the miser. Ensure that the cap is secure. Wear gloves.

9.3 Immediately after shaking the sample add known volume of 5x PBS and a known volume (see specification sheets supplied with each trial) of the beads to the sample using the appropriate pipette and taking the beads from the centre of the tube/vial containing the beads. Discard pipette tip.

9.4 Affix tube containing sample and beads to near-vertical miser and rotate for 30 min.

9.5 Remove tube from near-vertical miser and place in magnetic particle concentrator (MPC-M).

9.6 Without removing tube from MPC-M gently rock or roll the tube through 180° with the magnet leading and the tube following on the initial rock/roll. Continue for a minute with approximately one 180° roll/rock and return to uptight per second. The beads and oocysts should form a clear 'dot' on the back of the microfuge tube at approximately the 500µl mark.

9.7 Aspirate all the supernatant from the bottom of the tube held in the MPC using a fine pipette tip, preferably using a flat gel-loading type pipette tip. Take care not to disturb the material attached to the wall of the tube adjacent to the MPC-M. Also aspirate any fluid retained within or around the cap of the tube. Do not shake and do not remove tube from MPC-M whilst conducting this step. Ensure that the magnetic strip of the MPC-M is not disturbed.

9.8. Remove tube from MPC-M and resuspend sample in 100µl water. Add 5µl of 1 N hydrochloric acid to microfuge tube and shake on miser for 15 seconds. Allow microfuge to stand in a vertical position (but not in MPC) for 5 min at room temperature then shake on mixer for a further 15 seconds. Again stand microfuge tube at room temperature for 5 min. Add 5 µl of 1 N sodium hydroxide solution and shake on miser for another 15 seconds.

9.9 Flick all sample down to base of tube and immediately place in MPC-E. Allow to stand undisturbed for 20 seconds. Remove 55 µl from centre/base of fluid in tube, but taking care not to disturb beads at back-wall of tube. Place liquid onto well of multi-well slide. Remove the last 55 µl of fluid from the tube again taking care not to disturb beads at back-wall of tube. Place liquid onto well of multi-well slide. Lf any other fluid remains at the base of the tube, cap of tube or sides of tube (not including where the beads are attached to the back wall), distribute this between the two wells of the slide already containing the sample.

9.10 Remove tube from MPC-E and re-suspend sample in 100µl water. Shake on mixer for 15 seconds.

9.11 Flick all sample down to base of tube and immediately place in MPC-E. Allow to stand undisturbed for 30 seconds. Remove 50 µl from centre/base of fluid in tube. Place liquid onto well of multi-well slide. Remove the last 50 µl of fluid from the tube. Place liquid onto well of multi-well slide. If any other fluid remains at the base of the tube, cap of tube or sides of tube distribute this between the two wells of the slide already containing the sample.

9.12 Sample can then be examined for the presence of oocysts of *Cryptosporidium* by the modified SCA method.

APPENDIX 1. SECTION II: PROCEDURE (MODIFIED SCA METHOD) FOR DETECTION AND ENUMERATION OF *CRYPTOSPORIDIUM* OOCYSTS PURIFIED FROM SAMPLES BY THE IMMUNOMAGNETISABLE METHOD OR BY THE SCA METHOD (CENTRIFUGATION).

1. SCOPE

1.1 This procedure describes the detection and enumeration of *Cryptosporidium parvum* oocysts from water and other samples by microscopy using a fluorescence labelled anti-*Cryptosporidium* monoclonal antibody to label the oocysts. The procedure is based upon the "blue book" SCA method with several minor modifications. The SCA method was described over 5 years ago and the modifications have emerged after prolonged use.

1.2 The procedures described below do not purport to address ail of the safety problems associated with its use. It is the responsibility of the users of these procedures to identify and establish appropriate health and safety practices and determine the applicability of regulatory limitations prior to use. It must be remembered at all times that *Cryptosporidium* is a pathogen of man and all samples must therefore be treated as potentially infectious.

2. REFERENCED DOCUMENTS

2.1 Blue book: Isolation and identification of Giardia cysts, Cryptosporidium oocysts and free living pathogenic amoebae in water etc. 1989. Methods for the examination of waters and associated materials. HMSO Publications: London. 1990. ISBN 0 11 752282 1.

3. SUMMARY OF METHOD

3.1 A representative proportion of the concentrated sample is fixed to slides, stained with a fluorescent monoclonal antibody in direct assay and examined with a fluorescence microscope. Oocysts are recognised according to specific criteria (immunofluorescent characteristics. internal morphological characteristics, size, shape and fluorescent dye enhanced morphometrics) and enumerated.

4. SIGNIFICANCE AND USE

4.1 This method is designed for the detection. recognition and enumeration of *Cryptosporidium* oocysts from water and water-based samples.

4.2 The procedure is based upon the SCA method but various modifications have been incorporated since the methodology was first described over 5 years ago.

4.3 This method will not identify the species of *Cryptosporidium* concentrated nor will it identify host species nor the origin of the oocysts. The viability or infectivity of the oocysts will also not be determined.

5. INTERFERENCES

5.1 Inorganic and organic debris which may be naturally occurring or may be added to water in the treatment process (e.g. iron and alum coagulants and polymers) may partially or completely occlude the oocysts.

5.2 Materials/chemicals which may occur in water or water-based samples may inhibit the binding of the oocysts to the monoclonal antibody on the beads. either by altering the antibody or by altering the surface of the oocyst.

5.3 Freezing of oocysts in the samples may deform the oocysts and thus hamper their identification.

5.4 Organisms and debris that autofluoresce or demonstrate non-specific fluorescence when examined by epifluorescence microscopy could interfere with the detection of cysts and oocysts. ***Such*** interference should always be noted.

6. APPARATUS

6.1. Incubator set at 37°C ± 0.5°C

6.2 Epifluorescence and bright field microscope with Nomarski Differential Interference Contrast (DIC) optics and the appropriate filters for FITC and DAPI fluorescence, x20, ×40 dry objectives, ×40 or x50 water or oil immersion objectives. ×100 water or oil immersion objective, eyepiece graticule and calibration slide.

6.3. Multi-spot microscope slides should be of 4 well type, each well capable of containing at least 60µl volume (approximately 10mm diameter) (e.g. PH-068 from C.A. Hendley, Essex).

6.4 Cover slips 50mm x 22 mm (e.g. Shandon 67761315).

6.5 Coplin staining jars at least 2 coplin jars or other suitable container will be required.

6.6 Magnetic stirrer/hot plate with magnetic followers e.g. Bibby B212. Set up at room temperature.

6.7 Gilson (or similar) pipettes and appropriate sterile pipette tips. Gilson pipettes sizes should be P1000, P200 and P20. Pipettes should be calibrated on a daily basis following the manufacturer's instructions. Records of calibration should be kept.

6.8 Positive control slides. Positive control slides are supplied by the manufacturers of the anti-*Cryptosporidium* monoclonal antibody and should be used as recommended by the manufacturers. 'In house' control slides could also be used.

6.9 Negative control slides. Negative control slides are supplied by the manufacturers of the anti-*Cryptosporidium* monoclonal antibody and should be used as recommended by the manufacturers.

6.10 Mixer (vortex type) e.g. Fison's Whirlimiser. Set up at room temperature.

6.11 Humidity chamber Plastic container with lid which can contain slides and tit inside incubator. Absorbent material at the base (e.g. paper towelling) can be soaked in water to ensure a humid atmosphere within the container.

7. REAGENTS AND MATERIALS

7.1 Purity of reagents Reagent grade chemicals shall be used in all tests unless otherwise indicated.

7.2 Preparation of reagents Prepare all reagents in accordance with the appropriate health and safety guidelines.

7.3 Purity of water Unless otherwise indicated, references to water shall be understood to mean Grade 1 laboratory water.

7.4 Sample concentrates Samples will be provided for the analysis. Each sample will be a volume of water or water-based material which has been seeded with *Cryptosporidium parvum* oocysts. These samples will be concentrated either by the immunomagnetic separation procedure or by the SCA method (centrifugation) before detection and enumeration.

7.5 50mM Phosphate buffered saline pH7.2-7.4 (PBS) Prepare a ten times stock solution by dissolving 10.7g disodium hydrogen onhophosphate (anhydrous) (Na_2HPO_4), 3.9g sodium dihydrogen onhophosphate ($NaH_2PO_4.2H_2O$) and 85g sodium chloride (NaCl) in a sufficient quantity of water to produce a final volume of 1L (1000 ml). Dilute one volume of the solution with 9 volumes of water before use. The stock phosphate buffered saline (10 times) can be made up in advance and stored at room temperature in the dark for a maximum of 4 weeks. the diluted phosphate buffered saline can be stored for up to 1 week at room temperature.

7.6 Fluorescein isothiocyanate (FITC)-conjugated-anti-*Cryptosporidium* monoclonal antibody. Anti-*Cryptosporidium* monoclonal antibody directly conjugated FITC (e.g. as supplied by Cell Labs, Shield Diagnostics, Waterborne Inc. or Meridian Diagnostics etc.). The anti-*Cryptosporidium* monoclonal antibody should be made up, stored, and used according to the manufacturers' instructions.

7.7 4'6 diamidino-2-phenyl indole (DAPI) in phosphate buffered saline (PBS). Prepare a stock solution of DAPI by dissolving I mg DAPI (Sigma D 9542) in 0.5 ml methanol. To 50 ml of diluted PBS add 10 µl of stock DAF'I. The stock DAPI can be made up in advance and stored at 4°C in the dark for a maximum of 4 weeks. The DAF'I in PBS should be made up fresh for each day's use.

7.8 Mounting medium: 1.4 diazabinclo 12.2.21 octane (DABCO)/glycerol in PBS Pre-warm 30 ml glycerol to approximately 35°C with a heated stir-plate and a magnetic follower. Add 20 ml of diluted PBS and lg DABCO (Sigma D 2522) to the warm glycerol with continuous stirring (CAUTION hygroscopic, causes burns. avoid-inhalation. skin and eye contact: wear gloves). Store at 1°C in the dark. Can be stored for a maximum of 8 weeks.

7.9 Methanol (ANALAR)

8. PRECAUTIONS

8.1 The analyst must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using and disposing of sample concentrates, reagents and materials and whilst operating the equipment used.

8.2 Never mouth-pipette

9. PROCEDURE: Preparation of slides for microscopic examination

9.1 Mark each 4 welled slide with sample number. Use a marker which will not be removed by immersion in methanol e.g. wax pencil or on frosted slides ordinary lead pencil.

9.2 Shake the sample concentrate for 15 seconds on the miser. Ensure that the cap is secure. Wear gloves.

9.3 Immediately after shaking the sample use the P200 pipette to dispense 25 µl onto each well of the labelled 4 well slide Ensure even coverage of each well. Take the 25µl aliquots from the centre of the tube/vial containing the sample concentrate. Discard pipette tip between each sample concentrate.

9.4 Air-dry slides in an incubator or fan oven.

9.5 Methanol-fix the slides by total immersion of the slide in methanol in a Coplin staining jar or other suitable container for 5 min at room temperature. This step is contrary to that described in the original "blue book" SCA method. Remove slides from jar and allow methanol to evaporate from slide. Methanol should be

discarded in accordance with COSHH regulations. Alternatively, 25 µl methanol can be added to each well of the slide and allowed to evaporate to dryness.

9.6 Apply 25 µl of anti-*Cryptosporidium* monoclonal antibody at working strength to each well of the slide. Ensure complete coverage of each well of the slide. Place the slides in the humidity chamber with the slides elevated above the absorbent material (ensure that the absorbent material is wet). Place in incubator for 30 "Ii".

9.7 Rinse each slide individually with a gentle stream of PBS to remove residual monoclonal antibody. Immerse slides in a staining jar or other suitable container containing PBS for 5 minutes. Positive control slides must be washed in a separate jar and slides from different samples must also be washed in separate jars. Remove slides from jar, discard PBS and replace with new PBS. Immerse slides in replacement PBS for 5 minutes.

9.8 Remove slides from jar. discard PBS and refill jar with DAPI in PBS solution. Immerse slides in DAPI in PBS for 5 minutes. Remove slides from jar and discard DAPI in PBS solution.

9.9 Fill Coplin jar nith water and dip each slide in jar for between 1-3 seconds to remove residual PBS and DAPI.

9.10 Air dry slides in incubator or fan oven.

9.11 Apply one drop mounting medium with P200 pipette to each well of the slide. allowing the drop to fall freely (i.e. avoid contact between slide and pipette tip) and apply cover-slip to slide. Do not press cover-slip. Slides should be examined as soon as possible.

10. PROCEDURE: Microscopic examination of slides

10.1 The microscope should be set up in accordance with the manufacturer/suppliers' instructions. Ensure that the fluorescence field is evenly illuminated and covers the total area covered by the objective. The eye-piece graticule should be calibrated using a calibration slide supplied by the manufacturer/supplier of the microscope. The microscope should be sited in a darkened room.

10.2 Scan the positive control slide with rhe FITC ("blue") block inserted and at a total magnification of at least X200. Oocysts should appear as apple green. spherical objects of between 4-6 µm in diameter. Background fluorescence should be either very dim or non-existent. If no oocysts are detected then a) the staining procedure has not worked, b) the positive control is faulty or c) the microscope has been incorrectly set up. do not examine the sample concentrates. Re-check reagents and procedures to determine the problem.

10.3 When an oocyst has been detected transfer to the DAPI (ultra-violet) block to visualise the sporozoite nuclei. Within each oocyst with contents there should be up to 4 sporozoite nuclei which appear as 4 points of shy-blue fluorescence within the oocyst. Not all oocysts will necessarily have contents. The presence of oocyst contents can be confirmed by examining the oocyst by light microscopy with DIC optics.

10.4 Scan the negative control slide with the FITC ("blue") block inserted and at a total magnification of at least X200. Oocysts should not be observed. If oocysts are observed then reagents and/or equipment must be contaminated. discard all reagents and disposable equipment and then repeat all procedures.

10.5 Using epifluorescence with the FITC block (blue) in place, scan the sample concentrate slides at no less than X200 total magnification. Cover the whole area of each well with vertical or horizontal sweeps. Ensure that the whole area of each well is scanned.

10.6 When a presumptive oocyst is detected (apple green fluorescence. spherical object of between 4-6 µm diameter). score fluorescence from weak to strong and note whether fluorescence is even or patchy, measure using eye-piece micrometer at a minimum of x1000 total magnification. examine by light microscopy, preferably using DIC optics. and note whether contents are present. and examine under ultra-violet block for DAPI staining of nuclei. The characteristics of the oocysts detected should be recorded in tables as described. The number of oocysts detected in each well must be recorded.

APPENDIX 2. SECTION I: IMS PROCEDURE: DETAILED PROTOCOL FOR 1 ML SAMPLES

1. Wear gloves.
2. Shake the sample containing oocysts for 10 seconds on the mixer. Ensure that the cap is secure.
3. Shake the beads for 10 seconds on the miser.
4. Immediately add 200 µl of 5 x PBS/Tween 20 and 10 µl of the M-450 beads (10 mg/ml) to the sample using the appropriate pipette and taking the beads from the centre of the tube/vial containing the beads. Discard the pipette tip.
5. Affix tube containing sample and beads to near-vertical miser and rotate for 30 min.
6. Remove tube from near-vertical miser and place in magnetic particle concentrator (MPC-M).
7. Without removing tube from MPC-M gently rock or roll the tube through 180° with the magnet leading and the tube following on the initial rock/roll. Continue for 1 minute with approximately one 180° roll/rock and return to upright per second. The beads and oocysts should form a clear 'dot' on the back of the tube at approximately the 500µl mark.
8. Aspirate all the supernatant from the bottom of the tube held in the MPC using a tine pipette tip, preferably using a flat gel-loading type pipene tip. Take care not to disturb the material attached to the wall of the tube adjacent to the MPC-M. Also aspirate any fluid retained within or around the cap of the tube. Do not shake the tube and do not remove tube from MPC-M whilst conducting this step. Ensure that the magnetic strip of the MPC-M is not disturbed.
9. Remove tube from MPC-M and re-suspend sample in 100µl water. Add 5µl of 1 N hydrochloric acid to the tube and shake on miser (vortex) for 15 seconds. Allow tube to stand in a vertical position (but not in MPC) for 5 min at room temperature then shake on mixer for a further 15 seconds. Stand tube at room temperature for a further 5 min. Add 5 µl of 1 N sodium hydroxide solution and shake on miser for another 15 seconds.
10. Flick all of the sample down to the base of the tube and immediately place the tube in MPC-E. Allow the tube to stand undisturbed for 20 seconds. Remove 55 µl from centre/base of fluid in tube. but taking care not to disturb beads at back-wall of tube (i.e. wall of tube nearest to the magnet). Place liquid onto a well of a labelled multi-well slide. Remove the last 55 µl of fluid from the tube again taking care not to disturb beads at back-wail of tube. Place liquid onto another well of the same multi-well slide. If any other fluid remains at the base of the tube. cap of tube or sides of tube (not including where the beads are attached to the back wall), distribute this between the two wells of the slide already containing the sample.
11. Remove tube from MPC-E and re-suspend sample in 100µl water. Shake on mixer for 15 seconds.
12. Flick all sample down to base of tube. Remove 50 µl from centre/base of fluid in tube. Place liquid onto the 3rd well of the same multi-well slide. Remove the last 50 µl of fluid from the tube. Place liquid onto the final well of the same multi-well slide. If any other fluid remains at the base of the tube, cap of tube or sides of tube distribute this between the two wells of the slide already containing the sample.
13. Air-dry slides in an incubator or fan oven.
14. 50 µl (one drop) methanol is then added to each well of the slide and allowed to evaporate to dryness at room temperature.
15. Apply 25 µl of anti-*Cryptosporidium* monoclonal antibody at working dilution to each well of the slide. Ensure complete coverage of each well of the slide. Place the slides in the humidity chamber with the slides elevated above the absorbent material (ensure that the absorbent material is moist). Place in incubator at 37°C for 30 min.
16. Gently aspirate the monoclonal antibody from the wells.

17. Apply 50µl PBS to each well and allow to stand for 2 min.

18. Gently aspirate the PBS from each well. Again apply 50µl PBS to each well and allow to stand for a further 2 min. before gently aspirating the PBS.

19. Apply 50 µl (one drop) DAPI in PBS solution to each well and allow to stand for 2 min.

20. Gently aspirate the DAPI in PBS solution from each well.

21. Appiy 50 µl (one drop) of water to each well and leave for 1-3 seconds to remove residual PBS and DAPI.

22. Gently aspirate the waler from each well.

23. Apply one drop mounting medium with P20 pipette to each well of the slide, allowing the drop to fall freely (i.e. avoid contact between slide and piperte tip) and apply cover-slip to slide. Do not press cover-slip. Slides should be examined as soon as possible.

APPENDIX 2. SECTION II: IMS PROCEDURE; DETAILED PROTOCOL FOR 10 ML SAMPLES

1. Wear gloves.

2. Shake the M-450 beads (10 mg/ml) for 10 seconds on the miser.

3. Shake the sample containing oocysts for 10 seconds on the mixer. Ensure that the cap is secure.

4. Immediately after shaking the sample add 2 ml of 5× PBS/Tween 20 and 50 µl of the M-450 beads to the sample using the appropriate pipette and taking the beads from the centre of the tube/vial containing the beads. Discard pipette tip.

5. Affix tube containing sample and beads to near-vertical miser and rotate for 30 min.

6. Remove tube from near-vertical miser and place in magnetic particle concentrator (MPC-1).

7. Without removing tube from MPC-1 place the magnet side of the MPC-1 downwards (tube is horizontal). Gently rock the tube end to end. tilting cap-end and base-end of the tube up and down in turn. Do not remove the tube from the MPC-1. Continue the tilting action for 1 minute with approximately one tilt per second.

8. Return the MPC-1 to the upright position. tube vertical, with cap at top. Aspirate all the supernatant from the bottom of the tube held in the MPC-1 using a fine pipette tip. Take care not to disturb the material attached to the wall of the tube adjacent to the MPC-1. Also aspirate any fluid retained within or around the cap of the Nbe. Do not shake the tube and do not remove tube from MPC-1 whilst conducting this step.

9. Remove tube from MPC-1 and re-suspend sample in 500µl 1x PBS/Tween 20. Mix gently to re-suspend all material in the tube.

10. Remove all the liquid from the tube and place in 1.5 ml microfuge tube.

11. Add a further 500µl 1x PBS/Tween 20 to the 10 ml tube and rinse as before.

12. Affix microfuge tube containing sample and beads to near-vertical miser and rotate for 15 min.

13. Remove tube from near-vertical miser and place in magnetic particle concentrator (MPC-M).

14. Without removing tube from MPC-M gently rock or roll the tube through 180° with the magnet leading and the tube following on the initial rock/roll. Continue for 1 minute with approximately one 180° roll/rock and return to upright per second. The beads and oocysts should form a clear 'dot' on the back of the microfuge tube at approximately the 500µl mark.

15. Aspirate all the supernatant from the bottom of the tube held in the MPC using a fine pipette tip, preferably using a flat gel-loading type pipette tip. Take care not to disturb the material attached to the wall of the tube adjacent to the MPC-M. Also aspirate any fluid retained within or around the cap of the tube. Do not shake the tube and do not remove tube from MPC-M whilst conducting this step. Ensure that the magnetic strip of the MPC-M is not disturbed.

16. Remove tube from MPC-M and re-suspend sample in 100µl water. Add 5µl of 1 N hydrochloric acid to microfuge tube and shake on miser (vortex) for 15 seconds. Allow microfuge to stand in a vertical position (but not in MPC) for 5 min at room temperature then shake on mixer for a further 15 seconds. Stand microfuge tube at room temperature for a further 5 min. Add 5 µl of 1 N sodium hydroxide solution and shake on mixer for another 15 seconds.

17. Flick all of the sample down to the base of the tube and immediately place the tube in MPC-E. Allow the tube to stand undisturbed for 20 seconds. Remove 55 µl from centre/base of fluid in tube, but taking care not to disturb beads at back-wall of tube (i.e. wall of tube nearest to the magnet). Place liquid onto a well Of a labelled multi-well slide, Remove the last 55 µl of fluid from the tube again taking care not to disturb beads at back-wall of Nbe. Place liquid onto another well of the same multi-well slide. If any other fluid remains at the base of the tube, cap of tube or sides of tube (not including where the beads are attached to the back wall), distribute this between the two wells of the slide already containing the sample.

18. Remove tube from MPC-E and re-suspend sample in 200µl water. Shake on mixer for 15 seconds.

APPEKDM 3. MICROSCOPIC EXAMINATION OF SLIDES

1. The microscope should be set up in accordance with the manufacturer/suppliers' instructions. Ensure that the fluorescence field is evenly illuminated and covers the total area covered by the objective. The eye-piece graticule should be calibrated using a calibration slide supplied by the manufacturer/supplier of the microscope. The microscope should be sited in a darkened room.

2. Scan the positive control slide with the FITC ("blue") block inserted and at a total magnification of at least X200. Oocysts should appear as apple green, spherical objects of between 4-6 µm in diameter. Background fluorescence should be either very dim or non-existent. If no oocysts are detected then a) the staining procedure has not worked, b) the positive control is faulty or c) the microscope has been incorrectly set up. Do not examine the sample concentrates. Recheck reagents and procedures to determine the problem.

3. When an oocyst has been detected transfer to the DAPI (ultra-violet) block to visualise the sporozoite nuclei. Within each oocyst with contents there should be up to 4 sporozoite nuclei which appear as 4 points of sky-blue fluorescence within the oocyst. Not all oocysts will necessarily have contents. The presence of oocyst contents can be confirmed by examining the oocyst by light microscopy with DIC optics.

4.Scan the negative control slide with the FITC ("blue") block inserted and at a total magnification of at least X200. Oocysts should not be observed. If oocysts are observed then reagents and/or equipment must be contaminated. Discard all reagents and disposable equipment and then repeat all procedures.

5. Using epifluorescence with the FITC block (blue) in place. scan the sample concentrate slides at no less than X200 total magnification. Cover the whole area of each well nith vertical or horizontal sweeps. Ensure that the whole area of each well is scanned.

6. When a presumptive oocyst is detected (apple green fluorescence, spherical object of between 4-6 µm diameter), score fluorescence from weak to strong and note whether fluorescence is even or patchy, measure using eye-piece micrometer at a minimum of x1000 total magnification, examine by light microscopy, preferably using DIC optics, and note whether contents are present, and examine under ultra-violet block for DAPI staining of nuclei. The characteristics of the oocysts detected should be recorded in tables as described. The number of oocysts detected in each well must be recorded.

19. Flick all sample down lo base of tube. Remove 50 µl from centre/base of fluid in tube. Place liquid onto the 1st well of a second multi-well slide. Repeat, removing 50 µl of fluid from the tube and placing the liquid on to the 2nd, 3rd and 4th wells of the slide. If any other fluid remains at the base of the tube, cap of tube or sides of tube distribute this between the wells of the slide already containing the sample with beads.

20. Air-dry slides in an incubator or fan oven.

21. 50 µl (one drop) methanol is then added to each well of the slide and allowed to evaporate to dryness at room temperature.

22. Apply 25 µl of anti-*Cryptosporidium* monoclonal antibody at working dilution to each well of the slide. Ensue complete coverage of each well of the slide. Place the slides in the humidity chamber with the slides elevated above the absorbent material (ensure that the absorbent material is moist). Place in incubator at 37°C for 30 min.

23. Gently aspirate the monoclonal antibody from the wells.

24. Apply 50µl PBS to each well and allow to stand for 2 min.

25. Gently aspirate the PBS from each well. Again apply 50µl PBS to each well and allow to stand for a further 2 min, before gently aspirating the PBS.

26. Apply 50 µl (one drop) DAPI in PBS solution to each well and allow to stand for 2 min.

27. Gently aspirate the DAPI in PBS solution from each well.

28. Apply 50 µl (one drop) of water to each well and leave for 1-3 seconds to remove residual PBS and DAPI.

29. Gently aspirate the water from each well

30. Apply one drop mounting medium with P20 pipette to each well of the slide, allowing the drop to fall freely (i.e. avoid contact between slide and pipette tip) and apply cover-slip to slide. Do not press cover-slip. Slides should be examined as soon as possible.

APPENDIX 4. FLOW CYTOMETRIC METHODS

LAB D PROTOCOL

Samples supplied for flow cytometry were centrifuged and the supernatant aspirated off to leave a pellet of approximately 100 µl volume. This was resuspended and transferred to a flow cytometry sample tube along with an aliquot (equivalent to 10% of the sample volume) of bovine serum albumin (BSA). The final concentration of BSA was 1%. The required volume (equal to pellet volume) of monoclonal antibody (Cell-Labs FITC, as supplied by Bradsure Biologic&) was added to the microcentrfuge tube and mixed before adding to the pellet/BSA mixture. This step was performed as a wash step, to ensure minimal oocyst loss of on tube transfer. The pellet/BSA/monoclonal antibody mixture was then incubated at 37°C for 30 min.

Before running the samples through the flow cytometer (a Coulter EPICS Elite), an aliquot of Coulter fluorescent alignment beads was added to each as an internal standard. These beads are a continual visual alignment check and sort monitor (being sorted along with any oocysts).

The samples were then run on the flow cytometer, the machine sorting on the basis of side scatter and fluorescence onto target slides. These slides were then air-dried and mounted with Citifluor mountant before being read by fluorescent/DIC microscopy.

LAB B PROTOCOL

CRYPTOSPORIDIUM - IMS TRIAL

PROTOCOL FOR FLOW CYTOMETRY.

PREPARATION OF SAMPLE

1. Samples received from the distributing laboratory were concentrated by centrifugation at 13000 rpm for 1 minute.
2. The pellet was washed with 2ml Hank's Balanced Salt Solution (HBSS) and centrifuged at 13000 rpm for 1 min.
3. The supernatent was removed and and the pellet resuspended in 100ul HBSS
4. 100ul FITC CRYPTOSPORIDIUM MONOCLONAL ANTIBODY (BRADSURE) was added, mixed and Incubated for 30-45 mins. At 370C
5. The sample was washed 2x in HBSS by centrifugation, removal of supernatent and reconstitution in 2ml HBSS.
6. After the final wash the pellet was reconstituted to 500ul.

FLOW CYTOMETRY BY FACSort.

This instrument has an aerosol free sorting system with two light scatter detectors and three fluorescence detectors. A catcher tube captures the sorted particles and deposits them on a polycarbonate filter.

1. The detected signals used for the samples were Forward Scatter (FSC) and Fluorescein Isothiocyanate (FITC) which were recorded on a logarithmic scale
2. CallBRITE FITC Flow Cytometer Beads were used as an instrument check.
3. *Cryptosporidium sp.* oocysts were used to set the instrument Controls
4. The Instrument Controls were sot so that the stained oocysts appeared in the top of an FSC-FITC Dot Plot. These control settings were stored and used In subsequent tests.
5. A Sort Region was defined in the FSC-FITC Dot Plot to include all the oocysts.
6. The FACSort was cleaned with FACSafe before running the samples,

TRIAL SAMPLE

1. The FACSort was checked with the Calibrite beads and the positive sample of oocysts to ensure that the Instrument Controls and Sort Regions were correctly set
2. The trial samples were sorted and collected onto 1.2um membrane filters
3. The membranes were mounted on glass slides and covered with a coverslip using glycerol mountlng medium. The slides were sealed with colourless nail varnish.
4. Slides were kept in the dark until examined on a fluorescence microscope

APPENDIX 1

DETECTION AND ENUMERATION OF *CRYPTOSPORIDIUM* OOCYSTS USING FLOW CYTOMETRY

SECTION 1 SCOPE AND FIELD OF APPLICATION

1.1 The method described is suitable for the detection of *Cryprosporidium* oocysts in water, sewage and related materials by flow cytometry.

1.2 The normal volumes for the test and the concentration methods used are described in 50.4.0.

1.3 The method implements conventional microscopy for the detection of oocysts by 'sorting' stained samples onto a microscope slide for confirmation.

SECTION 2 REFERENCES

2.1 Isolation and Identification of *Giardia* Cysts, *Cryprosporidium* Oocysts and Free Living Pathogenic Amoebae in Water etc, 1989.
HMSO. ISBN 0 11 752282. 1

2.2 'In-house' method based upon the development of a new technology.

SECTION 3 PRINCIPLE OF THE METHOD

3.1 Definition and Description of the Organism

See 50.4.0

3.2 Pathogenicity

See 50.4.0

3.3 General Principle

The concentration techniques used in 50.4.0 are applicable. The staining and examination techniques differ in that the flow cytometer is used to 'sort' the sample onto a microscope slide using sort parameters designed to select *Cryprosporidium* oocysts and eliminate the majority of unwanted material.

SECTION 4 HAZARDS

See 50.4.0

The use of a free flowing liquid to interrogate the sample does not present a health risk to the operator through the generation of aerosols.

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Direct eye contact with laser light may cause blindness. Under no circumstances should pieces of the equipment be removed allowing direct laser light to be radiated into the laboratory.

High pressure mercury vapour lamps and DABCO used in the mounting medium are dangerous. Reference should be made to NAM 50.4.0 for advice on safe handling. The Dacos detergent is also dangerous and safety glasses should be worn when the detergent solution is being made up.

SECTION 5 PERFORMANCE DATA

See 50.4.0

SECTION 6 REAGENTS

The only reagent used in the procedure is sheath fluid, the carrier fluid used for the sample during interrogation by the laser beam. This is available commercially 2s 2 particle free fluid 2nd should be used according to the manufacturers instructions.

SECTION 7 APPARATUS

Flow cytometer
Sample tubes
Microscope siides
Vortex mixer
Incubator $37^{0}C \pm 1^{o}C$
Calibration beads 2, 6 or 10µm diameter.

SECTION a CALIBRATION

8.1 Calibration of the Flow Cytometer

To achieve optimum recovery of oocysts from samples, the machine must be accurately aligned. Alignment consists of

The correct positioning of the nozzle with respect to the laser beam

The correct focusing of the laser beam

Alignment of collecting lenses and filters to give optimum signals with calibration beads together with minimum instrument settings.

It is important to keep the nozzle clean and therefore once 2 week before switching on the machine remove the nozzle and clean by sonicating in detergent in a water bath for 5 ± 1 min. Replace the nozzle making sure that it is secure and that the

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stream of sheath fliud falls centrally into the waste when the fliudics are switched 0".

8.1.1 Flow cytometer settings

The alignment parameters are forward scatter height (FSC - H), side scatter height (SSC - H), green detector height (FL1 - H) and red detector height (FL2 - H). These parameters are viewed as dot histograms on the display. The threshold level for forward scatter FSC -H should be between 60 - 70 and the voltage setting for the photomultiplier tubes (PMT) should be SSC - H 250, FL1 - H - 300, FL2 - H - 300. The amp gains should be 2, 4, 4 and 4 respectively.

Using 10μm beads aiign the machine to achieve coefficient of variance for FCC - H, FL1 - H and FL2 - H of less than 3.5 at detection channels as close to 200 as possible. Once the machine is aligned, the dot histograms together with the instrument settings should be printed to provide a record of correct alignment and this must be signed by the operator. If there is difficulty in alignment, a senior microbiologist should be consulted.

Note:

The machine may be aligned using 2μm or 6μm beads and the instrument setting may vary slightly from those described above.

8.1.2 Optimisation of fluidics

With the drop drive engaged. the machine should be adjusted to give a drop delay of between 10.5 and 15.0 (with correctly focused side streams) using the autosort. The drop drive frequency should be between 23,000 and 26,000 cycles per second. If these cannot be achieved the nozzle should be adjusted and flushed until these settings are achieved.

8.1.3 Instrument settings for sorting

Once alignment and fluidics are optimised, the machine can be set for sorting *Cryptosporidium.* Turn pulse processing on and change SSC - H to forward scatter width FSC - W. The PMT are now adjusted to threshold FSC - H - 60-70, SSC - 300, FL1 600 and FL2 400. The amp gains are reset to 2, Log, Log and Log respectively. Draw the appropriate sort region on a dot plot of FSC - W against FL1 - H and load this into the computer.

Once all these checks are complete, the machine is ready for a sort test.

8.1.4 Instrument optimisation check

In order to be sure that the machine is properly aligned a sample containing a known number of *Cryptosporidium* oocysts should be sorted and counted. In practical terms, the risk of contaminating routine samples makes this impossible. However, green latex beads of similar size to oocysts provide a suitable check for alignment. Prepare a sample containing a known

number of beads in 10µl aliquots and sort 2 separate aliquots diluted to approximately 1ml with sheath fluid. Count the beads and record the result in NAM Y. If the instrument is correctly aligned, the count for each sample should fall between ± 20% of the actual value. If the count is lower than 20% of the actual value. the procedures described in 8.1.1 to 8.1.3 should be repeated to check that alignment is satisfactory.

One sample of calibration beads should be tested each morning following alignment and a further aliquot tested after each 5 samples. Where 5 samples are not analysed during the morning, a calibration sample should also be run at the beginning of the afternoon and after each 5 samples. These results should be recorded in NAM Y.

8.2 Positive and Negative Controls

In conjunction with the analysis detailed in this method, at the beginning of each day a positive control consisting of a suspension of oocysts will be prepared and stained in accordance with the method described in 50.4.0. Similarly a negative control consisting of an environmental sample known not to contain the organisms will also be stained. The results of all positive and negative controls are recorded in NAM Y. In addition, a blank sample consisting of sheath fluid is run between each test sample. The blank samples are not examined if a test sample is negative but are incorporated to ensure that there is no carry-over of oocysts from a positive sample to the following sample. Where an environmental sample is positive, the negative controls before and after that sample are examined microscopically and the results recorded in NAM Y. If a negative control is positive, the machine should be flushed thoroughly with detergent and a further negative control examined before any further samples are analysed.

8.3 Calibration of the Microscope Eyepiece Graticule

Although the flow cytometer will detect and 'sort' *Cryptosporidium* oocysts, enumeration against a background of environmental material is difficult. Visual confirmation of 'sorted' samples is essential and the eyepiece graticule should be calibrated according to 50.4.0.

SECTION 9 SAMPLE COLLECTION AND PRESERVATION

See 50.4.0

SECTION 10 ANALYTICAL PROCEDURE

Samples are processed according to 50.4.0.

10.1 Staining

Analysis of samples by flow cytometry requires that they must be stained in suspension. Cell Lab monoclonal antibodies are preferable for this purpose because they contain only a minimum of Evans blue counterstain and can therefore be

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analysed without the need to wash the sample.

Pipette the required volume to be tested into an eppendorf tube. Add an equal volume of monoclonal antibody and incubate at 37°C ± 1°C for 30 min. After staining, clean samples should be diluted to lml with sheath fluid and analysed. Dirty samples may be diluted in up to 6ml of sheath fluid and dispensed into separate tubes in lmi aliquots for analysis. Where stained samples cannot be anaiysed immediateiy they may be stored at 2-8°C for 24h before analysis. Once each sample has been sorted the machine is rinsed with sheath fluid for 5 min and a negative control sorted before the next sample is tested.

Material sorted onto microscope slides is dried, mounted and examined as described in 50.4.0.
The Labman number of each sample analysed should be recorded in NAM Y.

SECTION 11 CALCULATIONS

See 50.4.0

SECTION 12 NOTES

See 50.4.0

The Examination of Sample Concentrates for Cryptosporidium Oocysts using Flow Cytometry / Microscopy

LAB E PROTOCOL

Introduction

A sample concentrate prepared in the normal way, following the Standing Committee of Analysts method, is stained in suspension using a commercially availabie monocional antibody conjugated with F.I.T.C.

The stained sample is then sorted on a flow cytometer (Coulter Epics Elite) using side scatter and F.I.T.C fluorescence as parameters. The sort zone is amorphous and extends approximately an. order of magnitude around the cloud produced by oocysts obtained from Moredum Animal Health Ltd. Three drops are sorted when an event occurs within the sort zone.

The sorted material is examined by fluorescence microscopy for oocysts as defined in the S.C.A. method with the addition of using D.A.P.I. to stain nuclear DNA as an aid to identification.

A low number of fluorescent microspheres are added to the samples. Their presence on the slide of sorted material is taken as demonstration that the sorting procedure has worked, especially when the sample is very low in naturally fluorescent particles.

PROCEDURE

1) The flow cytometer is on switched following the instrument start up procedure, and the protocol for cryptosporidium anaiysis selected.

2) After a 30 minute warm up time. the instrument alignment and sort conditions are set up and checked using Couiter Immunockeck fluorescent microspheres.

3) The sample concentrate is thoroughly mixed and (normally) 100μl measured into a micro test tube containing 100μl of fluorescent antibody and 10μl of a fluorosphere suspension containing approximately 20 microspheres.

4) With each batch of samples, a positive control containing approximately 100 oocysts and a blank of de-ionised water are also stained.

5) The sample/stain mixture are allowed to react for 30 minutes at 37°C.

6) The positive control, blank and samples are processed in turn on the flow cytometer, collecting sorted 'material onto microscope slides. When the contents of a tube have passed through the flow cell, the sample lime and microtube are back flushed **with 6** drops of sheath fluid which is then also passed through the instrument.

7) Between samples the sample line is back flushed then washed for a minimum of 30 seconds with 10% bleach followed by a minimum of' 30 seconds with Coulterclenz.

8) The slide is dried and then stained for 10 minutes with 2μg/ml DAPI at room temperature.

9) The DAPI is aspirated off the slide. a drop of mountant is placed on the slide and a cover slip put in place.

10) The slide is then examined under a fluorescent microscope equipped with appropriate filtration for F.I.T.C. AND D.A.P.I. Scanning is carried out using a X25 objective with closer examination and measurement using X40 and X100 objectives.

APPENDIX 5. IMS SPECIFICATION SHEET

IMS ROUND ROBIN TESTING

SPECIFICATION SHEET FOR TRIAL 12 (615 NTU) - To be processed Friday 21/7/95.

ITEMS ENCLOSED

3 X 6 MICROFUGE TUBES OF OOCYSTS FOR ANALYSIS. AT LEAST 5 TUBES TO BE ANALYSED BY EACH METHOD (SCA, IMS, FLOW CYTOMETRY) AND ONE TUBE SPARE FOR EACH METHOD. ALL THE SAMPLE TO BE ANALYSED.
ONE NEGATIVE CONTROL SAMPLE (615 NTU WATER- SO OOCYSTS SEEDED). TO BE PROCESSED FOR EACH METHOD

IMS BEADS: 100 µL OF 10 MG/ML MS BEADS. FOR EACH 1 ML IMS SAMPLE USE 10µL OF THESE BEADS.

APPENDIX 6. TABLE FOR REPORTING *CRYPTOSPORIDIUM* OOCYSTS

SAMPLE NUMBER:

Oocyst number	Fluorescence score (+ to +++) (even or patchy)	Shape	Contents by light microscopy	Nuclei stained with DAPI (1, 2 3 or 4)
1	+++	OVAL	YES	3

Date analysed:
Analysed by:
FITC-MAb used:

APPENDIX 7. SECTION I: VIABILITY OF *CRYPTOSPORIDIUM PARVUM* OOCYSTS USING FLUOROGENIC VITAL DYES (DAPI AND PI)

Pre-acidification of oocysts.

Incubate 100µl of oocysts suspension and 1 ml of acidified HBSS (pH to 2.75 with HCl) at 37°C for 1 h. Following this pre-treatment oocysts are washed twice before being subjected to the DAPI/PI incubation step.

Incubation of oocysts with DAPI and PI.

Working solutions of DAPI (2 mg/ml in absolute methanol) and PI (1 mg/ml in 0.1 M PBS, pH 7.2) are prepared and stored at 4°C in the dark.
Oocysts are suspended in Hanks Balanced Salt Solution (HBSS) and 100µl of suspension is incubated simultaneously with 10µl of DAPI working solution and 10µl of PI working solution at 37°C for two hours. If FITC Mab is required incubate with this for the last 30 min.
Oocysts are then washed twice in HBSS before being viewed by epifluorescence microscopy.

Microscopy.
Ten microlitre aliquots of oocysts suspension are viewed under both DIC (Nomarski) optics and epifluorescence with a" Olympus BH2 microscope equipped with a UV filter block (350-nm excitation.>450- nm emission) for DAPI and a, green filter block (535-nm excitation, >590-nm emission) for PI. Proportions of ruptured (ghost). PI positive (PI+), DAPI positive/PI negative (DAPI+/PI-), DAPI negative/PI negative (DAPI-/PI-) oocysts (Table 1) are quantified by enumerating 100 oocysts in each sample (see Table 2 for example). Ghost oocysts are easily identified under Nomarski optics, being non-refractile apart from the residual body.

PI+ oocysts fluoresce bright red under the green filter block, and this red fluorescence varies from distinct points of intense fluorescence corresponding to the locations of sporozoite nuclei to a more diffuse fluorescence within the oocyst. Oocysts are considered DAPI+/PI- only if they do not include PI and if the nuclei of the sporozoites fluoresce a distinctive sky blue under the W filter block. Those oocysts which are "either PI+ nor **ghosts** and which show either a rim fluorescence or a" absence of fluorescence under the *W* filter block are considered DAPI-/PI- (Table 1).

Table 1. Correlation of oocyst viability, visualisation of oocyst contents by Nomarski (DIC) microscopy, and exclusion or inclusion of DAPI and PI.

Type of oocyst[a]	Sporozoites seen by Nomarski microscopy	Inclusion of: PI	Inclusion of: DAPI	Viability
Ghost	No	No	No	Dead
PI+	Yes	Yes	Yes	Dead
DAPI+/PI-	Yes	No	Yes	Viable at assay
DAPI- /PI-	Yes	No	No	Viable after further trigger
c(DAPI+)/PI-	Yes	No	Yes[b]	Dead

[a] DAPI- PI- oocysts can be converted to DAPI+ PI- oocysts and vice versa
[b] = Cytoplasmic DAPI staining - not solely nuclear staining

Table 2: Quantifying oocyst viability with DAPI/PI

Ghosts	PI+	c(DAPI+)/PI-	DAPI-/PI-	DAPI+/PI-

Count 100 oocysts.

% Oocyst viable is calculated using the following formulae:

$$\%\text{Viable} = \frac{\text{DAPI+/PI-}}{100} = \frac{\qquad}{100} = \%$$

% Oocyst potentially viable is calculated using the following formulae:

$$\%\text{ Potentially Viable} = \frac{\text{(DAPI+/PI-)+(DAPI-/PI-)}}{100} = \frac{\qquad}{100} = \%$$

APPENDIX 7. SECTION II: METHOD FOR ASSESSING VIABILITY OF *CRYPTOSPORIDIUM PARWM* OOCYSTS USING FLUOROGENIC VITAL DYES (DAPI AND PI)

1. Incubate 100µl of oocyst suspension with 1 ml of acidified HBSS at 37°C for 1 h.

   (pH HBSS to 2.75 with HCI before use)

2. Wash oocysts twice with HBSS (1 1,000-13,000*g*, 30 Sec) and resuspend in 100µl HBSS.

3. To this 100µl oocysts in HBSS add 10µl of DAPI working solution (2 mg/ml in MeOH) and 10µl of PI working solution (1 mg/ml in PBS). Incubate at 37°C for 1h 30 min.

4. Add FITC Mab to the DAPI/PI oocyst suspension and incubate at 37°C for a further 30 min.

5. Wash Oocysts twice in HBSS and resuspend in 100µl HBSS, before viewing 10µL aliquots by epifluorescence microscopy.

APPENDIX 7. SECTION III: METHOD FOR ASSESSING VIABILITY OF *CRYPTOSPORIDIUM PARVUM OOCYSTS* USING FLUOROGENIC VITAL DYES (DAPI AND PI) FOLLOWING LMS OF OOCYSTS.

1. Shake the beads for 10 seconds on the mixer.
2. Shake the sample containing oocysts for 10 seconds on the miser. Ensure that the cap is secure
3. Immediately after shaking the sample add 200 µl of 5× PBS and 10 µl of the beads to the sample using the appropriate pipette and taking the beads from the centre of the tube/vial containing the beads. Discard pipette tip.
4. Affix tube containing sample and beads to near-vertical mixer and rotate for 30 min.
5. Remove tube from near-vertical miser and place in magnetic particle concentrator (MPC-M)
6. Without removing tube from MPC-M gently rock or roll the tube through 180° with the magnet leading and the tube following on the initial rock/roll. Continue for 1 minute with approximately one 180° roll/rock and return to upright per second. The beads and oocysts should form a clear 'dot' on the back of the microfuge tube at approximately the 500µl mark.
7. Aspirate all the supernatant from the bottom of the tube held in the MPC using a fine pipette tip, preferably using a flat gel-loading type pipette tip. Take care not to disturb the material attached to the wall of the tube adjacent to the MPC-M. Also aspirate any fluid retained within or around the cap of the tube. Do not shake the tube and do not remove tube from MPC-M whilst conducting this step, Ensure that the magnetic strip of the MPC-M is not disturbed.
8. Remove tube from MPC-M and resuspend sample in 1 ml of acidified HBSS at 37°C for 1 h. (pH HBSS to 2.75 with HCl before use). Vortex sample for 10 sec after 30 min. of this period.
9. Place in magnetic particle concentrator (MPC-M) to separate M-450 beads. Remove all the acidified HBSS solution containing the oocysts and place in new labelled microfuge tube.
10. Wash oocysts twice with HBSS (1 1,000-13,000*g*, 30 Sec) and re-suspend in 100µl HBSS.
11. To this 100µl oocysts in HBSS add 10µl of DAPI solution (2 mg/ml in MeOH) and 10µl of PI solution (1 mg/ml in PBS). Incubate at 37°C for lh 30 min.
12. Add FITC Mab to the DAPI/PI oocyst suspension and incubate at 37°C for a further 30 min
13. Wash Oocysts twice in HBSS and resuspend in 100µl HBSS, before viewing 10µL aliquots by epifluorescence microscopy.

APPENDIX 8. REPORT OF TRAINING SESSION FOR IMS PARTICIPANTS

Training session was held at Scottish Parasite Diagnostic Laboratory, Stobhill NHS Trust. Springbum, Glasgow G21 3UW, 25-26 April 1995. The training session was organised and conducted by Dr. Andrew Campbell.

Training session attendees:
N. Sykes, Thames Water Utilities
J. Watkins & P.Kemp, Yorkshire Environmental
I. Mapletden. Southern Science
(J. Simonnette. Strathclyde Water Services)

Training session agenda:
1. Discussion and overview of IMS technique.
2. Hands-on practical demonstration with all participants following instructions on the procedure.
3. Hands-on trial on procedure with all participants comparing IMS with the 'modified' SCA method (details of modified SCA method have been circulated lo all participants).
4. Discussion on time-scale of trials and reporting of results.
5. Discussion on feed-back required by participants from SPDL on progress through the trials.
6. Discussion on time-scale for small/large volumes and turbidity trials.
7. Discussion on proponion of each sample to be analysed by each method.
8. Discussion on inclusion of flow cytometry into trials.

Report on points on agenda
1. All participants were supplied with written details, summary and background of IMS procedure. All participants appeared to understand fully the scope and limitations of the IMS technique and the aims of the round-robin trials.
2. No problems were encountered during hands-on demonstration. All participants appeared to understand readily the procedures to be followed.
3. In the hands-on trial, two 1 ml samples containing oocysts (theoretical dilution of 33 oocysts/ml) were randomly assigned to each of the four participating laboratories. One sample was to be analysed by the 'modified' SCA method and one by the IMS technique which had been demonstrated and practised the previous day. Results are shown in the table below.

Participant	No. oocysts detected using modified SCA technique	No. oocysts detected using IMS technique
(Strathclyde Water Services	25	32)
Thames Water Utilities	30	23
Yorkshire Environmental	26	27
Southern Science	23	32
Mean +/- standard deviation	26 +/- 3	28.5 +/- 4

4. The numbers of samples which will be sent out at any one time was discussed, analysis of samples on the selected day was discussed and it was agreed that due to other laboratory commitments etc. samples would be analysed either on the day specified. on the day before or on the subsequent day. Advanced notification (4 weeks) for samples was agreed. The filling in of tables for reporting of the results was discussed. It was agreed that cumulative totals would be included. When flow cytometry is used, only the total numbers of oocysts detected will be reported. Reporting of results to the SPDL was agreed to be within a 2 week period (dates specified for each trial); if the dead-line cannot be met, participants will give advance notification. If results are not received by the dead-line SPDL will contact the participant.
Throughout the trials. all slides shall be retained by the participating laboratories and may be requested to be returned for confirmation at SPDL. Requests for return of slides for confirmation will take place if (a) a

particular problem is considered to be occurring and (b) will also be carried out at random throughout the period of the trials.

5. Participants requested feed-back. It was agreed that tables of recoveries for all techniques will be distributed to the participants. The target number (theoretical dilution) will be provided as will the results obtained by each laboratory. The tables will be anonymous. Tables will be sent out approximately 1 week after SPDL receives a complete data set for each trial.

6. It was agreed that initial trials will be of 1 ml samples in laboratory grade 1 water samples. The next set of trials will be 1 ml samples but in water of a range of defined turbidities. Larger volumes will be analysed later on in the trial. Participants will provide SPDL with information on the range of turbidities commonly encountered in environmental concentrates and will also be supplying SPDL with waters of high turbidities which have been screened as negative for oocysts. Such a pool of waters was considered to be preferable to making high turbidity samples in the laboratory for the trials.

7. It was agreed that for 1 ml samples the total sample will be analysed. This will require concentration by centrifugation for the 'modified' SCA method and for flow cytometry. This was agreed as this is the procedure used in all participating laboratories for environmental samples. For the larger volumes, the total volume will be analysed using the IMS technique, but only a proportion for the other methods. The proportion to be analysed shall be agreed before this stage of the trials commences, but is likely to be between 10-50%.

8. All participants were keen to incorporate the flow cytometric method into the trials.

Conclusions

The training session was considered to be successful nith all participating laboratories apparently assimilating the techniques readily. Panicipants took back IMS beads to their own laboratories for their own practice sessions before the trials begin and for any 'in house' training that they consider necessary. It is hoped that the participants felt similarly positive after the training session and it was emphasised that the trials should not be considered as inter-laboratory competitions but a co-operative venture.

Appendix 9. **Trial 1** (1ml; **33** oocysts; **old)**

	SCA	IMS	IMS	FCS		Control	Control	Control
Lab A		(Total)	(Dissociated)			Direct	SCA	IMS
	42	28	25			39	36	31
	28	31	22			35	29	33
	28	29	22			29	28	30
	40	34	32			34	25	27
	30	21	19				17	31
mean	33.6	28.6	24			34.3	27.0	30.4
s.d.	6.8	4.8	4.9			4.1	6.9	2.2
median	30	29	22			34.5	28.0	31.0
Lab B								
	15	22	19	11				
	16	27	25	14				
	13	21	19	12				
	12	12	11	21				
	13	25	22	13				
mean	13.8	21.4	19.2	14.2				
s.d.	1.6	5.8	5.2	4.0				
median	13	22	19	13				
Lab C								
	10	11	9	12				
	3	6	6	16				
	8	8	3	16				
	4	7	7	16				
	11	6	5	14				
mean	7.2	7.6	6	14.8				
s.d.	3.6	2.1	2.2	1.8				
median	8	7	6	16				
Lab D								
	17	26	26	18				
	22	24	23	18				
	27	21	20	21				
	15	22	22	24				
	11	27	27	20				
mean	18.4	24	23.6	20.2				
s.d.	6.2	2.6	2.9	2.5				
median	17	24	23	20				
Lab E								
	23	37	35	19				
	17	25	22	38				
	10	36	30	33				
	11	26	22	29				
	19	21	20	38				
		26	25					
mean :	**16**	28.5	**25.7**	**31.4**				
s.d.	**5.5**	**6.51**	5.8	**7.9**				
median	**17**	26	**23.5**	**33**				
no zeros/								

Trial 2 (1 ml; 13 oocysts; old)

	SCA	IMS (Total)	IMS (Dissociated)	FCS	Control Direct/	Control / SCA	Control / IMS
Lab A							
	10	16	11		141	9	14
	161	16	11		19	12	11
	14	12	11		11	12	15
	221	4	4i		10	4	14
	18	6	6		12		9
mean	16	10.81	8.6		13.2	9.3	12.6
s.d.	4.5	5.6	3.41		3.6	3.81	2.5
median	16	12	111		12.0	10.51	14.0
Lab B							
	11	9	9	5			
	10	10	9	6			
	7	8	8	0			
	5	7	6	10			
	11	17	13	0			
mean	8.8	10.2	9	4.2			
s.d.	2.7	4.0	2.5	4.3			
median	10	9	9	5			
Lab C							
	1	5	5	4			
	3	6	5	4			
	0	10	8	1			
	7	12	10	6			
	2	5	4	10			
mean	2.6	7.6	6.4	5			
s.d.	2.7	3.2	2.5	3.3			
median	2	6	5	4			
lab D							
	7	11	11	11			
	3	11	11	4			
	4	11	11	6			
	12	10	10	10			
	4	4	4	11			
mean	6	9.4	9.4	8.4			
s.d.	3.7	3.0	3.0	3.2			
median	4	11	11	10			
Lab E							
	5	10	8	12			
	8	10	9	11			
	3	10	9	9			
	7	11	8	11			
	6	8	5	22			
mean	5.8	9.8	7.8	13			
s.d.	1.9	1.1	1.6	5.2			
median	6	10	8	11			
% zero	4	0		10			

Trial 3 (1 ml: 3.3 oocysts; old)

	SCA	IMS (Total)	IMS (Dissociated)	FCS	Control/ Direct	Control SCA	Control IMS
Lab A							
	0	2	2		2	0	3
	4	2	2		0	1	3
	2	2	2		4	0	1
	2	1	1		1	0	2
	2	0	0				
mean	2	1.4	1.41		1.8	0.31	2.3
s.d.	1.4	0.9	0.91		1.7	0.5	1.0
median	2	2	2		1.5	0.01	2.5
Lab B							
	1	4	4	0			
	0	3	2	0			
	2	4	4	0			
	3	5	5	0			
	3	2	2	0			
mean	1.8	3.6	3.4	0			
s.d.	1.3	1.1	1.3	0			
median	2	4	4	0			
Lab C							
	2	0	0	1			
	1	2	2	1			
	0	4	3	0			
	2	0	0	0			
	0	1	1	0			
mean	1	1.4	1.2	0.4			
s.d.	1	1.7	1.3	0.6			
median	1	1	1	0			
Lab D							
	1	2	2	2			
	2	3	3	2			
	1	1	1	4			
	1	0	0	1			
	2	3	2	4			
mean	1.4	1.8	1.6	2.6			
s.d.	0.6	1.3	1.1	1.3			
median	1	2	2	2			
Lab E							
	1	4	3	2			
	4	4	2	3			
	4	3	3	6			
	1	3	3	2			
	2	4	4	2			
mean	2.4	3.6	3.0	3			
s.d.	1.5	0.5	0.7	1.7			
median	2	4	3	2			
% zero	16	16		40			

Trial 4 (1ml; 33 oocysts; new)

	SCA	IMS	IMS	FCM		Control	Control	Control
Lab A		(Total)	(Dissociated)			Direct	SCA	IMS
	6	21	21			28	13	29
	5	24	24			32	17	24
	10	39	36			35	21	26
	16	29	26			34	11	35
	12	27	25					30
	15	34	33					
mean	10.7	29.0	27.5			32.3	15.5	28.8
s.d.	4.5	6.6	5.8			3.1	4.4	4.2
median	11.0	28.0	25.5			33.0	15.0	29.0
Lab B								
	35	19	19	13				
	14	23	23	4				
	22	31	31	13				
	36	38	38	4				
	24	30	30	13				
mean	26.2	28.2	28.2	9.4				
s.d.	9.3	7.4	7.4	4.9				
median	24	30	30	13				
Lab C								
	11	20	20	21				
	5	26	23	13				
	17	32	30	14				
	18	28	28	18				
	17	30	30	19				
mean	13.6	27.2	26.2	17				
s.d.	5.5	4.6	4.5	3.4				
median	17	28	28	18				
Lab D								
	23	37	37	16				
	27	33	33	17				
	13	14	14	6				
	4	36	35	22				
	17	25	25	9				
mean	16.8	29	28.8	14				
s.d.	9.0	9.6	9.4	6.4				
median	17	33	33	16				
Lab E								
	22	20	20	45				
	23	35	35	33				
	16	23	23	21				
	18	32	32	24				
	15	25	25	22				
mean	18.8	27	27	29				
s.d.	3.6	6.3	6.3	10.1				
median	18	25	25	24				
no zeros								

Trial 5 (lml; 50NTU; 33 oocyst)

	SCA	IMS	IMS	FCM		Control	Control	Control
Lab A		(Total)	(Dissociated)			Direct	IMS	SCA
	21	20	17			13	22	28
	18	19	17			12	20	16
	14	9	7			25	17	16
	19	17	16			18	24	25
	30	13	11			15		
		14	13			27		
						15		
mean	20.4	15.3	13.5			24		
s.d.	5.9	4.1	4.0					
median	19	15.5	14.5		mean	18.6	20.8	21.3
					s.d.	5.9	3.0	6.2
Lab B					median	16.5	21	20.5
	26	19	18	20				
	34	20	19	25				
	20	8	6	39				
	17	33	19	11				
	34	20	20	13				
mean	26.2	20	16.4	21.6				
s.d.	7.8	8.9	5.9	11.2				
median	26	20	19	20				
Lab C								
	16	15	14	22				
	18	13	12	20				
	8	9	8	12				
	14	22	17	23				
	16	18	13	10				
mean	14.4	15.4	12.8	17.4				
s.d.	3.8	4.9	3.3	6.0				
median	16	15	13	20				
Lab D								
	7	16	16	15				
	16	19	19	13				
	20	20	20	14				
	21	26	26	9				
	22	24	24	17				
mean	17.2	21	21	13.6				
s.d.	6.1	4.0	4.0	3.0				
median	20	20	20	14				
Lab E								
	21	15	15	19				
	20	19	19	24				
	14	24	21	29				
	21	20	18	14				
	15	22	22	25				
mean	18.2	20	19	22.2				
s.d.	3.4	3.4	2.7	5.8				
median	20	20	19	24				
no zeros								

Trial 6 (1 ml; 13 oocysts; new)

	SCA	IMS	IMS	FCS		Control	Control
Lab A		(Total)	(Dissociated)			Direct	IMS
	13	6	5			16	16
	8	2	2			12	14
	14	11	11			14	14
	9	11	11			13	11
	17	15	15			11	12
		16	16			15	18
		14	13				9
							14
mean	12.2	10.7	10.4				
s.d.	3.7	5.1	5.2		mean	13.5	13.5
median	13.0	11.0	11.0		s.d.	1.9	2.8
					median	13.5	14
Lab B							
	17	6	6	3			
	10	9	7	0			
	17	10	9	5			
	9	9	8	4			
	13	10	10	1			
mean	13.2	8.8	8	2.6			
s.d.	3.8	1.6	1.6	2.1			
median	13	9	8	3			
Lab C							
	15	14	13	9			
	15	11	7	7			
	6	19	17	7			
	7	14	13	7			
	16	9	9	4			
mean	11.8	13.4	11.8	6.8			
s.d.	4.9	3.8	3.9	1.8			
median	15	14	13	7			
Lab D							
	12	15	15	14			
	13	13	13	19			
	9	14	14	23			
	12	9	9	13			
	10	12	12	21			
mean	11.2	12.6	12.6	18			
s.d.	1.6	2.3	2.3	4.4			
median	12	13	13	19			
Lab E							
	8	15	13	14			
	10	13	12	14			
	19	12	10	16			
	10	16	14	8			
	12	13	11	16			
mean	11.8	13.8	12	13.6			
s.d.	4.3	1.6	1.6	3.3			
median	10	13	12	14			
% zero	0	0		5			

Trial 7 (1ml; **3.3 oocysts; new)**

	SCA	IMS (Total)	IMS (Dissociated)	FCM		Control Direct	Control SCA	Control IMS
Lab A								
	0	1	1			4	0	4
	2	3	3			4	0	1
	1	1	1			2	3	3
	0	4	4			3	1	4
	0	2	2					
mean	0.6	2.2	2.2			3.3	1.0	3.0
s.d.	0.9	1.3	1.3			1.0	1.4	1.4
median	0	2	2			3.5	0.5	3.5
Lab B								
	3	1	1	0	??	Mechanical fault in		
	2	1	1	0	??	proportioning valve of		
	3	3	3	0	??	flow cytometer reported		
	5	0	0	3	??			
	3	2	2	0	??			
mean	3.2	1.4	1.4	0.6				
s.d.	1.1	1.1	1.1	1.3				
median	3	1	1	0				
Lab C								
	0	2	2	0				
	0	2	2	1				
	1	1	1	1				
	1	1	1	0				
	0	0	0	1				
mean	0.4	1.2	1.2	0.6				
s.d.	0.5	0.8	0.8	0.5				
median	0	1	1	1				
Lab D								
	0	2	2	0				
	0	0	0	0				
	0	1	1	0				
	0	6	6	1				
	0	2	2	0				
mean	0	2.2	2.2	0.2				
s.d.	0	2.3	2.3	0.4				
median	0	2	2	0				
Lab E								
	1	3	3	3				
	2	3	3	4				
	1	2	1	2				
	3	3	2	2				
	2	3	2	0				
mean	1.8	2.8	2.2	2.2				
s.d.	0.8	0.4	0.8	1.5				
median	2	3	2	2				
% zero	44	12		55				

Trial 8(1ml; 60NTU; 3.3 oocyst)

	SCA	IMS	IMS	FCM		Control	Control
ab A		(Total)	(Dissociated)			Direct	IMS
	1	3	3			3	0
	1	8	8			1	4
	3	2	2			2	1
	0	4	4			1	1
	1	3	3				2
ıean	1.2	4	4			1.8	1.6
d.	1.1	2.3	2.3			1.0	1.5
ıedian	1	3	3			1.5	1
ab B							
	2	1	1	0			
	0	1	0	1			
	3	1	1	0			
	6	1	0	0			
	3	1	1	0			
ıean	2.8	1	0.6	0.2			
.d.	2.2	0.0	0.5	0.4			
ıedian	3	1	1	0			
ab C							
	3	0	0	0			
	0	1	1	5			
	0	1	1	0			
	2	1	1	2			
	2	1	1	1			
ıean	1.4	0.8	0.8	1.6			
.d.	1.3	0.4	0.4	2.1			
ıedian	2	1	1	1			
ab D							
	0	1	1	0			
	0	0	0	0			
	0	0	0	0			
	0	0	0	0			
	0	0	0	1			
nean	0	0.2	0.2	0.2			
.d.	0	0.4	0.4	0.4			
nedian	0	0	0	0			
ab E							
	0	1	1	3			
	2	2	2	3			
	1	4	2	4			
	1	1	1	4			
	0	2	2	0			
nean	0.8	2	1.6	2.8			
.d.	0.8	1.2	0.5	1.6			
nedian	1	2	2	3			
% zero	44	20		55			

Trial 9 (1ml; 60 Nru: 33)

	SCA	IMS (Total)	IMS (Dissociated)	FCM	Control! Direct	Control IMS
Lab A						
	12	15	15 /new beads'		33	35
	13	0	0		371	29
	14	0	0		29	32
	25	0	0		301	32
	17	0	0			25
		1	0			
mean	16.21	2.7	2.5:		32.3	30.6
s.d.	5.3	6.1	6.1		3.6	3.8
median	14	0	0		31.5	32
Lab B						
	25	361	36	0		
	251	6	3	0		
	19	21	18	0		
	28	24	21	0		
	22	12	7	0		
mean	23.8	19.8	17	0		
s.d.	3.4	11.5	13.0	0		
median	25	21	18	0		
Lab C						
	30	20	20	17		
	25	26	25	15		
	23	13	11	17		
	26	20	19	9		
	34	23	22	21		
mean	27.6	20.4	19.4	15.8		
s.d.	4.4	4.8	5.2	4.4		
median	26	20	20	17		
Lab D						
	1	9	9	15		
	2	4	4	17		
	0	0	0	7		
	0	11	11	18		
	0	10	10	15		
mean	0.6	6.8	6.8	14.4		
s.d.	0.9	4.7	4.7	4.3		
median	0	9	9	15		
Lab E						
	10	11	7	11		
	4	4	3	9		
	7	11	11	6		
	13	13	13	13		
	11	11	10	24		
mean	9	10	8.8	12.6		
s.d.	3.5	3.5	3.9	6.9		
median	10	11	10	11		
% zero	12	19.2		25		

Trial 10 (1 ml; 611 NTU; 33)

	SCA	IMS (Total)	IMS (Dissociated)	FCS		Control Direct	Control IMS
ab A							
	37	13	11			18	17
	26	6	5			35	18
	30	9	7			13	16
	14	13	8			33	21
	20	5	4			31	
						22	
						31	
nean	25.4	9.2	7			27	
.d.	8.9	3.8	2.7				
nedian	26	9	7		mean	26.3	18.0
					s.d.	7.8	2.2
.ab B					median	29	17.5
	9	7	1	2			
	13	12	3	4			
	15	7	0	6			
	12	10	1	6			
	15	4	1	4			
nean	12.8	8	1.2	4.4			
.d.	2.5	3.1	1.1	1.7			
nedian	13	7	1	4			
.ab C							
	0	3	3	5			
	2	7	3	5			
	0	7	2	7			
	4	0	0	3			
	0	5	5	3			
nean	1.2	4.4	2.6	4.6			
.d.	1.8	3.0	1.8	1.7			
nedian	0	5	3	5			
.ab D							
	0	0	0	20			
	0	1	1	27			
	0	0	0	24			
	0	0	0	24			
	0	0	0	25			
nean	0	0.2	0.2	24			
.d.	0	0.4	0.4	2.5			
nedian	0	0	0	24			
.ab E							
	13	3	3	28			
	8	0	0	25			
	13	5	5	22			
	24	0	0	21			
	20	1	1	22			
mean	15.6	1.8	1.8	23.6			
s.d.	6.3	2.2	2.2	2.9			
median	13	1	1	22			
% zero	32	28		0			

Trial 1 1(**1 ml; 611** NTU;3.3)

	SCA	IMS	IMS	FCS	Control	Control
Lab A		(Total)	(Dissociated)		Direct	IMS
	0	0	0		2	1
	1	1	1		2	2
	0	0	0		3	0
	2	0	0		0	3
	2	0	0			1
mean	1	0.2	0.2		1.8	1.4
s.d.	1	0.4	0.4		1.3	1.1
median	1	0	0		2	1
Lab B						
	2	0	0	1		
	3	0	0	0		
	0	1	1	1		
	1	1	0	2		
	1	1	0	1		
mean	1.4	0.6	0.2	1		
s.d.	1.1	0.5	0.4	0.7		
median	1	1	0	1		
Lab C						
	0	0	0	0		
	0	0	0	0		
	0	0	0	0		
	0	0	0	0		
	0	0	0	0		
mean	0	0	0	0		
s.d.	0	0	0	0		
median	0	0	0	0		
Lab D						
	0	0	0	5		
	0	0	0	0		
	0	0	0	3		
	0	0	0	0		
	0	0	0	3		
mean	0	0	0	2.2		
s.d.	0	0	0	2.2		
median	0	0	0	3		
Lab E						
	1	0	0	3		
	3	0	0	4		
	0	0	0	2		
	4	0	0	3		
	1	0	0	2		
mean	1.8	0	0	2.8		
s.d.	1.6	0	0	0.8		
median	1	0	0	3		
% zero	56	84		40		

Trial 12 (1ml; 615 NTU: 33)

	SCA	IMS	IMS	FCS	Control	Control
Lab A		(Total)	(Dissociated)		Direct	IMS
	38	23	9	I	36	37
	39	36	271		29	40
	35	25	23		37	36
	44	2	0		42	33
	29	23	15			
mean	37	21.8	14.8		36.0	36.5
s.d.	5.5	12.3	10.8		5.4	2.9
median	38	23	15		36.5	36.5
Lab B						
	21	26	14	17		
	29	9	4	10		
	28	34	17	19		
	23	29	7	6		
	27	35	11	12		
mean	25.6	26.6	10.6	12.8		
s.d.	3.4	10.5	5.2	5.3		
median	27	29	11	12		
Lab C						
	18	12	6	34		
	6	26	5	25		
	2	13	9	18		
	12	18	7	32		
	5	25	12	44		
mean	8.6	18.8	7.8	30.6		
s.d.	6.4	6.5	2.8	9.8		
median	6	18	7	32		
Lab D						
	0	12	12	38		
	0	0	0	33		
	0	0	0	47		
	0	0	0	28		
	0	0	0	26		
mean	0	2.4	2.4	34.4		
s.d.	0	5.4	5.4	8.4		
median	0	0	0	33		
Lab E						
	56	36	34	36		
	48	42	39	46		
	37	53	34	40		
	49	37	30	36		
	52	45	39	53		
mean	48.4	42.6	35.2	42.2		
s.d.	7.1	6.9	3.8	7.3		
median	49	42	34	40		
% zero	20	16		0		

Trial 13 (10ml; 33 oocysts)

	SCA	IMS (Total)	IMS (Dissociated)	FCS	Control Direct	Control IMS
Lab A						
	16	41	41		42	44
	16	14	11		39	39
	14	45	44		31	51
	14	51	50		36	32
	24	48	48			
mean	16.8	39.8	38.0		37	41.5
s.d.	4.1	14.9	15.9		4.7	8.0
median	16	45	44		37.5	41.5
Lab B						
	48	45	41	5		
	201	471	47	1		
	121	431	351	1		
	351	641	61	0		
	221	62	591	0		
mean	27.4~	52.21	48.6	1.4		
s.d.	14.2	10.01	11.31	2.1'		
median	22	47	47i	1		
Lab C						
	15	51	44	25		
	3	71	0	38		
	41	7	1	45		
	351	531	48	45		
	33	61	51	48		
mean	41.4	35.8	28.8'	40.2:		
s.d.	8.6	26.6'	26.0	9.3;		
median	41	51	44	45		
Lab D						
	10	68	68:	36		
	251	541	52;	42		
	25	781	61	35		
	8	28	28:	19		
	27	361	361	29		
mean	19	52.0	49i	32.2'		
s.d.	9.21	21.0	16.8'	8.7		
median	251	54	52	351		
Lab E						
	31	40	37	30		
	21	64	60	45i		
	38	641	631	40		
	53	107	104	11		
	20	381	34	39:		
mean	32.6;	62.6	59.6'	33'		
s.d.	13.61	27.8,	28.1	13.41		
median	31	64	60	391		
% zero	0	0		10		

Trial 14 (10ml; 3.3 oocysts)

	SCA	IMS (Total)	IMS (Dissociated)	FCS		Control Direct!	Control IMS
Lab A							
	2	4	3			3	5
	3	1	1			6	7
	4	10	10			6i	2
	1	5	4			4	6
	2	3	3				
mean	2.41	4.6	4.21			4.8	5.0
s.d.	1.1	3.4	3.4			1.51	2.2
median	2	4	3			5	5.5
Lab B							
	7	3	3	1			
	3	4	2	0			
	5	5	5	1			
	0	10	9	4			
	0	6	6	0			
mean	3	5.6	5	1.2			
s.d.	3.1	2.7	2.7	1.6			
median	3	5	5	1			
Lab C							
	0	0	0	3			
	2	0	0	2			
	0	0	0	0			
	2	0	0	3			
	1	2	0	1			
mean	1	0.4	0	1.8			
s.d.	1	0.9	0	1.3			
median	1	0	0	2			
Lab D							
	0	4	4	0			
	2	0	0	0			
	0	3	3	0			
	0	3	3	4			
	0	2	2	0			
mean	0.4	2.4	2.4	0.8			
s.d.	0.9	1.5	1.5	1.8			
median	0	3	3	0			
Lab E							
	8	4	4	5			
	4	6	6	3			
	1	7	5	5			
	2	10	9	4			
	4	8	7	2			
mean	3.8	7	6.2	3.8			
s.d.	2.7	2.2	1.9	1.3			
median	4	7	6	4			
% zero	32	20		35			

Trial 15 (10ml; 63 NTU; 3.3)

	SCA	IMS (Total)	IMS (Dissociated)	FCS	Control Direct	Control IMS
Lab A						
	3	3	2		5i	5
	1	1	1		1	0
	2	7	7		3	4
	31	5	5		1	7
	1	12	12		I	8
mean	2	5.6	5.41		2.5	4.8
s.d.	1	4.21	4.41		1.9	3.1
median	2	5	5		2	5
Lab B						
	0	5	3	2		
	0	3	3	1		
	0	4	4	0		
	0	1	0	1		
	0	4	3	1		
mean	0	3.4	2.6	1		
s.d.	0	1.5	1.5	0.7		
median	0	4	3	1		
Lab C						
	0	1	1	4		
	0	3	0	4		
	0	2	0	4		
	0	2	1	7		
	0	0	0	5		
mean	0	1.6	0.4	4.8		
s.d.	0	1.1	0.5	1.3		
median	0	2	0	4		
Lab D						
	0	0	0	3		
	0	0	0	0		
	0	0	0	1		
	0	0	0	1		
	0	0	0	0		
mean	0	0	0	1		
s.d.	0	0	0	1.2		
median	0	0	0	1		
Lab E						
	6	8	8	2		
	21	8	a	4		
	3	a!	6	6		
	1	8	a	5i		
	2	5	4	2		
mean	2.8	7.4	6.8	3.8		
s.d.	1.91	1.3	1.8	1.8		
median	2	a	8	4		
% zero	60	24		15		

Trial 16 (10ml; 63 NTU;33)

	SCA	IMS	IMS	FCS		Control	Control
Lab A		(Total)	(Dissociated)			Direct	IMS
	20	32	15			30	24
	28	16	10			28	28
	14	26	5			16	36
	5	21	2			31	23
	23	25	5				
	18	24	7.4			26.3	27.8
	8.9	6.0	5.1			6.9	5.9
	20	25	5			29	26
Lab B							
	0	17	17	0			
	1	10	10	6			
	2	13	12	1			
	5	16	16	5			
	0	14	14	0			
	1.6	14	13.8	2.4			
	2.1	2.7	2.9	2.9			
	1	14	14	1			
Lab C							
	2	23	10	24			
	1	11	9	36			
	2	11	6	30			
	9	16	11	25			
	2	16	15	14			
	3.2	15.4	10.2	25.8			
	3.3	4.9	3.3	8.1			
	2	16	10	25			
Lab D							
	0	2	2	3			
	0	0	0	6			
	0	2	2	3			
	0	1	1	0			
	0	0	0	1			
	0	1	1	2.6			
	0	1	1	2.3			
	0	1	1	3			
Lab E							
	25	18	6	26			
	29	32	20	27			
	16	54	49	23			
	24	44	38	25			
	16	49	32	25			
	22	39.4	29	25.2			
	5.8	14.5	16.6	1.5			
	24	44	32	25			
% zero	28	8		15			

Trial 17 (1ml;33)

	SCA	IMS (Total)	IMS (Decoupled)	FCS		Control Direct	Control IMS	Control IMS (PBS)
Lab A								
	44	0	0			381	7	42
	30,	5	5			45	5	40
	50	3	3			31	2	53
	441	2	1			331	1	48
	47	4	4				13	
mean	43	2.8	2.61			36.81	5.6	45.8
s.d.	7.71	1.91	2.1			6.2	4.8	5.9
	44	3	3			35.5	5	45
Lab B ,								
	35	3	3	141				
	39	3	3	241				
	41	4	2	11				
	31	9	71	14				
	43	9	6	39				
mean	37.81	5.6	4.21	20.4.				
s.d.	4.8	3.11	2.2:	11.5				
median	39	41	3	14				
Lab C								
	36	1	1	29				
	48	3	3	38				
	46	8	5	39				
	26	3	2	261				
	40	4	4	30				
mean	39.2	3.81	3i	32.4				
s.d.	8.8	2.6	1.6	5.8				
median	40	3	3	30				
Lab D								
	251	8	7i	26				
	241	11	11	24				
	24	4	3i	28				
	22	1'	1	51				
	25	3	31	30				
mean	24	5.4	5	31.8				
s.d.	1.2	4.0	4.0	11.0				
median	24	4	3	28				
Lab E								
	39	4	3	63				
	59	6	5	56				
	59	3	3	74				
	54	3	2	37				
	57	5	4	46				
mean	53.6	4.2	3.4	55.2				
s.d.	8.4	1.3	1.1	14.4				
median	57	4	3	56				

Inhibition of magnetic particle collection

Trial 18 (1ml; 33)

	SCA	IMS (Total)	IMS (Decoupled)!	FCS		Control Direct	Control! IMS	Control IMS (PBS)
Lab A								
	30	37	331			39	34	32
	51	34	22			25	28	27
	32	40	31			61	36	4.5
	32	25	16			341	41	26
	29	32	28					
mean	34.8	33.6	26			39.81	34.6,	32.5
s.d.	9.1	5.7	7.0			15.3	5.4	6.7
median	32	34	28			36.5	35	29.5
Lab B								
	31	271	201	16				
	361	41	23	20				
	34	22	14	14				
	37	23;	14	301				
	33	291	18	15				
mean	34.2	28.4	17.81	19				
s.d.	2.4	7.61	3.9	6.6’				
median	34	27	18	16				
Lab C								
	24	19	18	32				
	20	24	22	13				
	26	30	26	30				
	19	26	12	18				
	30	27	11	16				
mean	23.8	25.2	17.8	21.8				
s.d.	4.5	4.1	6.4	8.6				
median	24	26	18	18				
Lab D								
	19	17	15	21				
	25	13	13	28				
	22	13	11	11				
	27	15	15	17				
	18	14	11	15				
mean	22.2	14.4	13	18.4				
s.d.	3.8	1.7	2.0	6.5				
median	22	14	13	17				
Lab E								
	47	56	25	49				
	60	23	4	61				
	45	38	4	48				
	501	34	8	531				
	441	7	2	58				
mean	49.21	31.6	8.6	53.8:				
s.d.	6.51	18.2	9.4	5.6				
median	471	341	4	53				

Trial 19 (1ml; 617NTU;33)

	SCA	IMS	IMS	FCS		Control	Control
Lab A		(Total)	(Decoupled)			Direct	IMS
	3	6	4			35	16
	0	0	0			28	13
	0	2	1			21	27
	2	11	8			29	21
	3	8	7				
mean	1.6	5.4	4			28.3	19.3
s.d.	1.5	4.4	3.5			5.7	6.1
median	2	6	4			28.5	18.5
Lab B							
	16	4	2	0			
	2	4	4	2			
	2	4	3	0			
	6	5	4	0			
	9	5	3	0			
mean	7	4.4	3.2	0.4			
s.d.	5.8	0.5	0.8	0.9			
median	6	4	3	0			
Lab C							
	18	7	5	28			
	11	1	1	21			
	15	0	0	16			
	11	5	5	17			
	15	5	0	17			
mean	14	3.6	2.2	19.8			
s.d.	3	3.0	2.6	5.0			
median	15	5	1	17			
Lab D							
	0	5	5	14			
	0	5	5	10			
	0	6	6	3			
	0	2	2	2			
	0	6	5	11			
mean	0	4.8	4.6	8			
s.d.	0	1.6	1.5	5.2			
median	0	5	5	10			
Lab E							
	32	4	3	36			
	27	8	5	22			
	25	5	4	19			
	37	3	3	20			
	24	1	1	29			
mean	29	4.2	3.2	25.2			
s.d.	5.4	2.6	1.5	7.2			
median	27	4	3	22			

Trial 20 (1ml; 6000 NTU; 33)

	SCA		IMS	IMS	FCS		Control
Lab A			(Total)	(Decoupled)			Direct
	2	*	8	6			28
	2	*	9	7			25
	2	*	9	6			37
	0	*	7	5			22
	2	*	9	6			
mean	1.6		8.4	6			28.0
s.d.	0.9		0.9	0.7			6.5
median	2		9	6			26.5
Lab B							
	2	*	4	2	7		
	3	*	11	2	2		
	1	*	11	5	1		
	0	*	14	8	4		
	1	*	11	8	3		
mean	1.4		10.2	5	3.4		
s.d.	1.1		3.7	3.0	2.3		
median	1		11	5	3		
Lab C							
	1	*	0	0	*4*	*	
	0	*	2	0	*4*	*	
	2	*	4	1	*1*	*	
	1	*	1	1	*4*	*	
	2	*	0	0	*3*	*	
mean	1.2		1.4	0.4	3.2		
s.d.	0.8		1.7	0.5	1.3		
median	1		1	0	4		
Lab D							
	0	*	0	0	*1*	*	
	0	*	0	0	*1*	*	
	0	*	0	0	*2*	*	
	0	*	0	0	*0*	*	
	0	*	0	0	*0*	*	
mean	0		0	0	0.8		
s.d.	0		0	0	0.8		
median	0		0	0	1		
Lab E							
	2	*	17	5	*3*	*	
	4	*	26	4	*5*	*	
	4	*	23	2	*4*	*	
	3	*	15	2	*2*	*	
	2	*	18	4	*6*	*	
mean	3		19.8	3.4	4		
s.d.	1		4.5	1.3	1.6		
median	3		18	4	4		
	*	10 % analysed					

Appendix 10. Direct replicate stock counts

	Old oocysts stock used in trials 1,2, 3 & 5.	New oocysts stock used in trials 4,7 - 20.
	Direct counts	Direct counts
	15	8
	11	14
	13	15
	11	18
	11	9
	10	18
	13	15
	11	18
	16	25
	21	20
	17	24
	21	27
	19	27
	14	32
	18	32
	18	36
		29
		32
		32
		29
TOTAL		
mean	14.9	23.0
s.d.	3.7	8.3
median	14.5	24.5
cv %	25.0	36.2

Appendix 11 Prestained viability test

	VIABLE (DAPI+/PI-)	POT-VIABLE (DAPI-/PI-)	NON-VIABLE (PI+; Ghosts)
Lab A			
	48	0	52
	48	0	52
	48	0	52
	58	2	40
mean	50.5	0.5	49.0
s.d.	5.0	0.9	5.9
median	48.0	0.0	52.0
Lab B			
	69	4	27
	57	3	40
	68	3	29
mean	64.7	3.3	32.0
s.d.	6.7	0.6	7.0
median	68.0	3.0	29.0
Lab C			
	80	2	18
	751	1	24
	74	1	25
mean	76.3	1.3	22.3
s.d.	3.21	0.6	3.8
median	75.01	1.01	24.0
Lab D			
	52	3	45
	46	7	47
	451	5	50
mean	47.7	5.0	47.3
s.d.	3.81	2.0	2.5
median	46.0	5.0	47.0
Lab E			
	58	0	42
	491	0	51
	57	2	41
mean	54.7	0.71	44.7
s.d.	4.91	1.2	5.5
median	57.0	0.01	42.0
TOTAL			
mean	58.2	2.1	39.7
s.d.	11.5	2.01	11.5
median	57.01	1.91	41.5

Viability test 1

	VIABLE (DAPI+/PI-)	POT-VIABLE (DAPI-/PI-)	NON-VIABLE (PI+; Ghosts)
Lab A			
	55	7	38
	47	1	52
	47	0	53
	51	1	48
	48	1	51
mean	49.6	2.0	48.4
s.d.	3.4	2.8	6.1
median	48.0	1.0	51.0
Lab B			
	46	11	43
	56	1	43
	52	6	42
mean	51.3	6.0	42.7
s.d.	5.0	5.0	0.6
median	52.0	6.0	43.0
Lab C			
	54	3	43
	53	2	45
	51	1	48
mean	52.7	2.0	45.3
s.d.	1.5	1.0	2.5
median	53.0	2.0	45.0
Lab D			
	34	7	59
	38	10	52
	45	5	50
mean	39.0	7.3	53.7
s.d.	5.6	2.5	4.7
median	38.0	7.0	52.0
Lab E			
	59	3	38
	64	1	35
	53	3	44
mean	58.7	2.3	39.0
s.d.	5.5	1.2	4.6
median	59.0	3.0	38.0
TOTAL			
mean	50.2	3.7	46.1
s.d.	7.2	3.4	6.3
median	51.0	3.0	45.0

Viability test 2

	VIABLE (DAPI+/PI-)	POT-VIABLE (DAPI-/PI-)	NON-VIABLE (PI+; Ghosts)
Lab A			
	14	0	86
	11	0	89
	12	0	88
mean	12.3	0.0	87.7
s.d.	1.5	0.0	1.5
median	12.0	0.0	88.0
Lab B			
	27	7	66
	28	14	58
	30	0	70
mean	28.3	7.0	64.7
s.d.	1.5	7.0	6.1
median	28.0	7.0	66.0
Lab C			
	8	0	92
	12	0	88
	12	0	88
mean	10.7	0.0	89.3
s.d.	2.3	0.0	2.3
median	12.0	0.0	88.0
Lab D			
	49	5	46
	42	9	49
	47	6	47
mean	46.0	6.7	47.3
s.d.	3.6	2.1	1.5
median	47.0	6.0	47.0
cv %	7.8	31.2	3.2
Lab E			
	15	0	85
	21	0	79
	13	0	87
mean	16.3	0.0	83.7
s.d.	4.2	0.0	4.2
median	15.0	0.0	85.0
TOTAL			
mean	22.7	2.7	74.5
s.d.	13.8	4.4	17.0
median	15.0	0.0	85.0

Viability following IMS

	VIABLE / VIABLE		POT-VIABLE	POT-VIABLE	NON-VIABLE	NON-VIABLE
	Control	IMS	Control	IMS	Control	IMS
Lab A						
	44	45	1	2	55	53
	50	47	0	1	50	52
	49	44	1	0	50	56
mean	47.7	45.3	0.7	1.0	51.7	53.7
s.d.	3.2	1.5	0.6	1.0	2.9	2.1
median	49.0	45.0	1.0	1.0	50.0	53.0
cv %	6.7	3.4			5.6	3.9
Lab B						
	30	46	33	13	37	40
	38	47	29	21	33	32
	20	57	43	11	37	32
mean	29.31	50.0;	35.01	15.0	35.7	34.7
s.d.	9.0	6.1	7.21	5.3	2.31	4.6
median	30.0	47.01	33.0	13.0	37.01	32.0
cv %	30.7	12.2'	20.6	35.3	6.5	13.3
Lab C						
	30	25	3	1	67	74
	28	32	0	0	721	68
	28	27	0	0	72	73
mean	28.7	28.0	1.0	0.3	70.31	71.7
s.d.	1.2	3.6	1.7	0.61	2.91	3.2
median	28.01	27.01	0.01	0.0	72.0	73.0
cv %	4.0	12.9			4.1	4.5
Lab D						
	70	38	8	171	221	45
	60	421	13	121	271	46
	56	351	8	15	361	50
mean	62.01	38.3'	9.71	14.7	28.3	47.0
s.d.	7.21	3.5	2.9	2.51	7.1	2.6
median	60.01	38.0,	8.01	15.0	27.0	46.0
cv %	11.6	9.21	29.9:	17.2,	25.01	5.6
Lab E						
	501	58	2	0	48	42
	51	60	1	1	48	39
	501	531	0	2	50	45
mean	50.3	57.0;	1.01	1.0	48.7	42.0
s.d.	0.6	3.6	1.0	1.0	1.2	3.0
median	50.01	58.0	1.01	1.01	48.01	42.0
cv %	1.11	6.3			2.41	7.1
TOTAL						
mean	43.6	AR.7	9 5	6.41	46.91	49.8
s.d.	14.1	IO.81	14.01	7.5	15.3	13.3
cv %	32.3	24.71	148.01	117.0'	32.6	26.7
median	49.0	45.0	2.01	2.0	48.0	46.0
Range: maxi	70.0	60.01	43.0	21 .0	72.0	74.0
min	20.0	25.01	0.0	0.0	22.0	32.0

Appendix 12. oocyst fluorescence

LAB	TRIAL	SCA Y	SCA N	IMS Y	IMS N
Lab A	1	84	0	1431	0
	2	40	0	48	6
	3	5	0	7	0
	4	631	1	171	3
	6	611	0	751	0
		70			
Lab E	II	28	10	153	18
	2		1	49;	0
	3	121	0	15	3
	4	1001	12	1321	3
	6	59	0	69	0
Lab B	1	68	1	101	6
	2	421	2	48	3
	3	9	0	16	2
	41	131	0	138	3
	61	64	21	431	1
Lab C	1	14	221	8	301
	2	121	1	33	5
	3	5	0	7	0
	4	631	5	128.	8
	6	571	2	58	2
Lab D	1;	44;	48	81	39;
	2	13	17	321	15
	3	3	4	3	5
	4	101	741	86	59
	6	29	27	43,	19
sum		1086	229	1687	230
% good fluorescence =		82.6		88.0	

OLD OOCYSTS

sum	415	1021	6961	122 Sig at p= 0.00001
% good fluorescence =	80.3		85.1	

NEW OOCYSTS

sum	637	123	943i	98
% good fluorescence =	83.8		90.6	Sig at p= 0.02183

Not Sig p>0.1 Sig at p= 0.00027

OLD VS NEW COMBINED IMS AND SCA

old	1111	224	% good FITC=	83.2 Sig at p= 0.00002
new	1580	2211	% good FITC=	87.71

Oocyst shape

LAB	TRIAL	SCA Y	SCA N	IMS Y	IMS N
Lab A	1	82	2	133:	10
	2	40	0	54'	0
	3	5	0	7	0
	4	571	7	164	10
	6	47	14	66	9
Lab E	1	75	3'	138	331
	2	26	3	46	3
	3	121	0	121	6
	4	105	7	132	3i
	6	55	4	66	3
Lab B	1	67	2	93	14
	2	36	8	44	7
	3	6	3	14	4
	4	114	17	120	21
	6	51	15	33	11
Lab D	1	91	1	119	1
	2	30	0	47	0
	3i	7	0	a	0
	41	841	0	145	0
	6	47	9	57	5
Lab C	1	32	6	33	5
	2	13	0	29:	9
	3	5	0	6	1
	6	4463	15	59	41
		1184	131	1713:	204

% good shape =	90.01		89.4	
OLD OOCYSTS				
sum	492	25	736	82 Not Sig p>0.5
% good shape =	95.2		90.0	
NEW OOCYSTS				
sum	657	103	930	111
% good shape =	86.4		89.3	Sig at p= 0.00067
	Sig at p= 0.0000		Not Sig at p= 0.654	
O L D V S N E W				
COMBINED IMS AND SCA				
/old	1228	107	% good shape=	92.0 Not Sig at p= 0.06123
new	1587	214	% good shape=i	88.1

Oocyst contents and DAPI

LAB	TRIAL	SCA Contents	SCA DAPI+	SCA 4 nuclei	SCA Empty	IMS Contents	IMS DAPI+	IMS 4 nuclei	IMS /Empty
Lab A	1	71	59	33	13	123	741	35	20
	2	361	36	22	4	48	28	9	6
	3	3	4	3	1	7	3	2	0
	4	551	531	12	9	155	1371	14	19
	6	58	49	8	3	65	62	12	10
Lab E	1	701	51	341	10	161	130	48	10
	2	271	25	18	2	431	37	20	6
	3	121	111	5	0	151	13	101	3
	4	106	104	34	6	131	117	34	4
	6	54	50	22	5	65	64	281	4
Lab B	1	531	461	221	16	94	88	40	13
	2	40	38	231	4	43	41	26	8
	3	7	5	2	2i	15	13	6	3
	4	114	111	43	151	137	1331	43	4
	6	65	65	20	1	35	34	10	9
Lab D	1	79	77	431	13	91	80	63	29
	2	25	21	15	5	41	301	21	6
	3	6	6	3	1	6	61	4	2
	4	76	73	321	8	130	111	65	15
	6	471	47	23	9	58	56	33	4
Lab C	1	17	151	7	191	241	17i	10	13
	2	7	6	3	6	28	281	17	10
	31	2	2	2	3	6	61	3	1
	4	37	37	231	31	56	54	35	80
	6	42	39	151	17	55	54	381	5
sum		11091	1030	467	203'	1632	1416'	626i	284

% with contents =	84.5	Not Sig at p=0.612	85.2
OF THOSE WITH CONTENTS			
% dapi =	92.9	Sig at p=0.0000	86.8
OF THOSE WITH CONTENTS			
% 4 nuclei	42.1	Sig at p=0.0489	38.4

APPENDIX 13. REPORT OF FINAL MEETING FOR MS PARTICIPANTS

This meeting was held at the Scottish Parasite Diagnostic Laboratory, Stobhill NHS Trust, Springburn, Glasgow G21 3UW on the 15th December 1995. The meeting was organised and chaired by Dr. Andrew Campbell.

Meeting attendees:

A. Campbell (AC), L. Robertson (LR) & C. Paton (CP), Scottish Parasite Diagnostic Laboratory.
M. Smith (MS), Drinking Water Inspectorate.
N. Sykes (NS), Thames Water Utilities
J. Watkins (JW), Yorkshire Environmental
R. Down (RD), Southern Science
J. Gibson (JG) & J. Coyle (JC), Strathclyde Water Services
B. Grön (BG), Dynal R&D

Apologies:

H. Smith, SPDL

Meeting agenda:

1. Practical demonstration of trial preparation, including seed calculation, distribution and random allocation of samples sent to participants.
2. Presentation of pooled results for the Round Robin trial of IMS and active discussion of the results. Specific points raised addressed by AC.
    A. Discussion of results obtained for seed distribution.
    B. Discussion of results obtained for 1 ml clean water samples
    C. Discussion of results obtained for 1 ml turbid samples
    D. Discussion of results obtained for 10 ml samples.
    E. Brief discussion of results obtained using blocking agents and very high turbidity (600-6000 NTU) samples
3. Summary of the results on the effect of the IMS technique on oocyst viability.
4. Summary of the results on the effect of the IMS technique and the age of the oocysts on the immunofluorescence, morphology, numbers sporulated and the uptake of DAPI by oocysts.
5. Summary of the pooled results.
6. Feedback on Round Robin testing from participants.
    A. Methods
        1. Feasibility of currently written method.
        2. Relative ease, convenience and time of applying the methods
        3. Adoption of method into routine sample analysis
        4. Any useful changes and additions
        5. User-friendliness of form of words
        6. Inconsistencies etc.
    B. Trial system
        1. Methods of reporting
        2. Time scale of reporting
        3. Feedback to participants from supervising laboratory

Report on meeting:

1. All participants understood the practical limitations of the seeding/distribution methods and agreed that the trial arrangement could be considered as "double blind".

2. Presentation of the results

A. Discussion of results obtained for seed distribution. All participants agreed that the seed distribution varied extensively and had encountered comparable ranges when conducting similar experiments themselves. The samples distributed to the participants would have contained unknown numbers of oocysts and although a theoretical seed of either 3 3, 13 or 3.3 oocysts were the targets (estimated from previous seed counts) the relative performance of each method will be analysed

B. Discussion of results obtained for 1 ml clean water samples. All participants agreed that the method that consistently produced the highest recoveries in this data set was the IMS method. The method with the largest variability in results was the FCM method. RD suggested that this might be a reflection of differences in methodology between the laboratories for using FCM, as both IMS and SCA methods were detailed by AC. AC thought these results were very encouraging especially the recovery in the samples of 3.3 oocysts.

C. Discussion of results obtained for 1 ml turbid samples. These data sets were split into low turbidity (40-60 NTU) or high turbidity (- 600 NTU). All participants agreed that the low turbidity samples were representative of the potable water concentrates routinely examined for *Cryptosporidium* sp. oocysts in their laboratories. All methods produced similar results within this data set.
All participants agreed that the high turbidity samples were representative of the raw water concentrates (possibly obtained from filtering 100-500 L raw water) that they routinely examined for *Cryptosporidium* sp. oocysts in their laboratories and that the majority of samples that they routinely examined for *Cryptosporidium* sp. oocysts were of high turbidity (>90%). The performance of the IMS method deteriorated in some of these high turbidity samples. When this occurred this was identified to be due, in part, to non-specific binding of material to the bead/oocysts complex and the decrease in the dissociation efficiency. It was also agreed that water type was probably more important than turbidity of the samples in affecting the efficiency of the IMS method, as the efficiency of the MS method improved on removing the filter back-flush concentrates which contain both ferric and alum flocculating agents.

D. Discussion of results obtained for 10 ml samples. The same pattern of results was obtained with 10 ml samples as 1 ml samples: IMS performed significantly better in clean samples. In low turbidity samples (- 60 NTU), IMS consistently performed better than the other two techniques. It was suggested by AC that this could be a reflection of a decrease in the efficiency of the other two techniques (FCM and SCA) as the greater volume required further centrifugation steps. By extrapolation, a 10 ml 60 NTU sample is equivalent to a 1 ml high turbidity (- 600 NTU) sample, as discussed above (point C); this suggests one potential area for improving the current FCM and SCA techniques and all participants stated that they would use IMS for clarification of samples at this stage (10 ml or greater volumes) in preference to sucrose flotation.

E. Brief discussion of results obtained using blocking agents and very high turbidity (6006000 NTU) samples. Highly variable results were obtained with the IMS technique, although the inclusion of blocking agents led to an overall improvement in the recovery of oocysts in high turbidity samples containing back-flush water. The data set in which the same representative volume was not analysed (trial 20) was agreed by all participants to be difficult to interpret. The other data sets (17-19) would be not be analysed in detail due to the use of experimental blocking agents; the use of these reagents in IMS is at a very early stage of development.

3. A summary of the results on the effect of the IMS technique on oocyst viability was presented. All participants agreed that there was no significant difference between viability of the oocysts that had been concentrated by centrifugation and by IMS separation. The IMS method does not, therefore, appear to select for a particular viability-subset of the oocyst population. It was agreed that a training session on the viability assay may have been of assistance, however all participants were confident in the assay procedure as detailed. At the time of the viability analyses, other monitoring commitments reduced the time available being directed to the viability analyses that the participants would have wished.

4. A summary of the results on the effect of the IMS technique and the age of the oocysts on the immunofluorescence, morphology, numbers spomlated and uptake of DAPI by oocysts was presented. All participants agreed that whilst there were differences between the data sets of oocysts that had been concentrated by the SCA method and following the IMS method, these differences were relatively minor. All participants were interested in the observations that the age of oocysts(> 1 month & < 8 months) and viability of the oocyst population(>85% & < 5%) had only a limited effect upon the immunofluorescent detection and subsequent confirmation of presumptive oocysts.

5. A summary of the pooled results was presented. All participants were agreed that this was a good representation of the trial outcome. Trial controls and quality controls showed accuracy in enumeration of oocysts by participants.

6. Discussion on feedback of the Round Robin testing. Points listed in point 6 of the agenda were introduced by AC and discussion of the individual points made is covered in the afternoon meeting. Minutes of the afternoon session are enclosed.

Minutes of afternoon session

Attendees:

A. Campbell (AC) & C. Paton (CP), Scottish Parasite Diagnostic Laboratory.
M. Smith (MS), Drinking Water Inspectorate.
N. Sykes (NS), Thames Water Utilities
J. Watkins (JW), Yorkshire Environmental
R Down (RD), Southern Science
J. Gibson (JG) & J. Coyle (JC), Strathclyde Water Services

AC. Reintroduced questions on feedback.

JW: Would use the technique to replace sucrose flotation step of the SCA method. Found it more user friendly and could be applicable to multiple sample processing. However, was concerned about the application of IMS to dirty samples as most of the water samples examined at Yorkshire Environmental are of these type. Would hope to be able to examine back samples and see if IMS produced similar or better results to those obtained when sucrose flotation was used. As well as looking at back samples would also attempt seeding experiments to compare sucrose flotation and IMS.

CP: Indicated the routine work that she was most involved with was sewage and that IMS had not been identified nor extensively tested for sewage purification.

RD: Agreed with the comments of JW, but would be more interested in the application of IMS at the 10 ml stage.

NS: Suggested that even larger volumes than 10 ml might be usefully purified using IMS.

JG: Suggested use of IMS at 1L stage.

AC: Pointed out that at such a large volume the number of beads required may make the process economically impractical.

RD: Asked about the cost of the beads.

AC: Does not know the cost of the beads but has been informed by Dynal that they would be of comparable price to other reagents marketed for similar techniques (e.g. *E.coli* O157).

JG: Pointed out that the major problem with samples at Strathclyde Water Service is that even at the 50 ml stage they are very thick and sludge like, so would need to be diluted for IMS. However, agreed that the place for IMS at present in the routine analysis is to replace sucrose flotation.

NS: Pointed out that Thames Water Utilities and other laboratories that use the calcium carbonate flocculation method would have to check whether aspects of this (e.g. pH) may affect IMS.

AC: Asked whether the participants would use flow cytometry with IMS.

NS/RD/JW: Would use flow cytometry.

JW May use either. For example, if the sample is very clean, may do manually (e.g. without flow cytometry) to save time. Flow cytometry is added to the SCA method as an additional option, rather than as a replacement similar to the IMS technique.

AC: Asked if the participants felt that there was a further requirement for blocking agent research. or if they felt that the differences between environmental samples may be so great that it would not be feasible for a single blocking agent to be ideal.

JW: Felt it was feasible, and indicated that if a blocking agent was required then it should be provided by Dynal.

AC: Asked if participants would be interested in putting IMS samples both with and without blocking agent through flow cytometer, instead of fixing directly to slides.

JW: Stated that he would be interested in trying this.

NS: Stated that in their laboratory, oocysts stained with Cell-labs monoclonal antibody following IMS were less bright than those stained in the FCM process and therefore he wondered whether the flow cytometer would be able to detect the oocysts following the IMS procedure.

AC: Suggested this disparity in fluorescence intensity was probably due to the fixed mount (with IMS) as compared to the liquid mount (with flow cytometry) and not an effect of the IMS technique per se.

JG: Suggested that even if the oocysts were less fluorescent, the gatings on the flow cytometer could be altered accordingly.

JW: Indicated that he felt that the difference in fluorescence intensity was due, as AC suggested, to the liquid mount as compared to the fixed mount and that any difference in fluorescence intensity due to the IMS technique would be so slight that a human eye would be unable to visualise it.

AC: Asked if the participants felt that the de-coupling device (acid) should be improved.

JW: Stated that he felt that looking at the beads, rather than just at the material de-coupled from the beads was a nuisance.

NS/RD: Agreed with JW.

AC: Stated that it seemed that everyone agreed that they did not wish to have to examine the beads and suggested that, other than the extra time and effort involved, there were problems associated with screening the beads including ensuring that the beads fixed to the microscope slides and autofluorescence from the beads. He stated that he felt that there might be the possibility of devising another method for de-coupling the beads, 'a specific chemical scissors', and wondered whether the participants would have found such a mechanism useful.

JW: Asked whether such a mechanism make the procedure more simple; would it involve one step, rather than two.

AC: Stated that he thought that it would.

NS: Stated that he felt that the acid/alkali steps in the present system were the most fiddly part of the technique and that if it could be replaced by another system then it would be an improvement.

AC: Asked whether the participants had any opinions about wash steps during the techniques: should they be included?

JW: Suggested that the importance of wash steps was the same through out this, and other, techniques, e.g. filter washing. For each wash there was a diminishing amount of return and he felt that as in clean samples a 90% recovery occurred with the present system there was no need for additional wash steps.

AC: Asked if it was satisfactory to JW to know that he was missing approximately 10% of the potential oocysts in the de-coupling step.

JW: Stated that he would find this satisfactory.

JC: Returned the discussion to the introduction of a method for de-coupling other than the acid/alkali system in the present technique and suggested that it would involve a lot of extra work to test out new de-coupling methods.

AC: Suggested that in the first instance testing out such a mechanism, if one could be identified, would be up to Dynal R & D.

JG: Returned again to the topic of wash steps and suggested that it would be an improvement if 10% of the potential oocysts were not lost. Asked JW if he would be happy at losing 10% in a final water.

JW: Stated that it would be satisfactory and pointed out that at present a sucrose step has approximately 25% efficiency, therefore a 90% recovery using IMS without a wash step must be better.

JG: Agreed, but also stated that if one extra step increased the possibility of finding that 10% then perhaps it should be included.

AC: Stated that the reason that wash steps were not included in the technique initially was because he thought that more manipulations may result in more losses and that a direct, simple capture step, without washes, might be preferable.

JW: Stated that he felt that laboratories undertaking these analyses should be divided into 2 groups: those with flow cytometers and those without. He felt that extra wash steps may assist those without flow cytometers, but he felt that for those with flow cytometers, such a step would give negligible advantage.

AC: Asked if the participants could suggest any other improvements to the technique.

RD: Stated that a 10 ml 'batch magnet' would be useful.

NS: Stated that he found the 10 ml magnet relatively fiddly to manipulate initially.

AC: Asked what were the specific points which made IMS same a more preferable option to sucrose.

JG: Stated that it was much quicker than sucrose, and that the sucrose flotation method also involved up to 4 wash steps.

JW: Added that the SDS also made the sucrose flotation step more lengthy.

JG: Stated that not all laboratories used SDS.

JW: Reiterated that he felt that speed was very important in these analyses.

JG: Pointed out that speed meant less technician time, which in turn meant less cost.

JW: Stated that in 1 ml volumes there was the option of using a microfuge.

AC: Asked if the sample was clean and of 1 ml volume, would IMS be preferable to microfuge.

JW: Replied that in such an instance he would use the microfuge but that 99% of samples are not like that.

AC: Stated that whilst the participants had now discussed where they would use IMS in the method quite fully, and all seemed agreed that it would be useful to replace sucrose, he would like the discussion to move onto the next point, changes and additions to the method.

NS: Stated that as previously discussed an improvement in the acid de-coupling step.

AC: Asked if this was the only place for improvement.

JG: Asked if the mixing time of 30 min could be reduced.

AC: Replied that whilst longer mixing times seem to have no effect on the technique, he found variability in results when the time was reduced to 15 min. However, further replicates would need to be undertaken to test this fully. Asked if the 30 min mixing time was a problem within the technique.

NS: Replied that as other work could be continued during this period it was not a problem in their laboratory, however he wondered if a reduction in mixing time might reduce non-specific binding.

AC: Replied that he found that non-specific binding occurred within 5 min, so felt that reduction of mixing time would not improve this.

NS: Reiterated that he felt that 30 min was satisfactory as other work could be continued with and the machine did not have to be watched during this period.

MS: Indicated that he had assumed that 30 min was the minimum mixing time, so that one was not tied to returning to the machine in exactly 30 min.

AC: Agreed that this was so.

JG: Said that the mixing could be done over night.

AC: Asked if the form of words in his written instructions was satisfactory.

JC: Replied that as he was not at the training session, the written instructions were his main source of instruction; he found them to be satisfactory although some of the changes were slightly confusing.

AC: Replied that the changes had been made after the start of the project when samples were sent out and he understood how some confusion might arise.

RD: Suggested that it would be difficult to explain the 'rock and roll' motion clearly in words without a demonstration as well.

NS: Added that the 'tap' on the end of the magnet which made a difference could also only clearly be explained by an actual demonstration and would be difficult to explain with written words only.

JW: Stated that he found that some individuals in his laboratory seemed to find it very difficult to obtain a neat 'dot' at the back of the tube and always got a smear and, as it seemed to vary from person to person, it seemed to be an individual thing.

MS: Suggested that an indication of the diameter of the dot which should be obtained might assist.

AC: Moved the discussion on to point 6, concerned with inconsistencies.

JW: Stated that he felt that the multi-well slide being used was not ideal; he would prefer a smaller size of well.

AC: Moved the discussion onto the next point, concerned with reporting.

JW: Stated that in their laboratory they do not normally use DAPI, but he found it useful and thought that the technique should be added to the new SCA manual.

JG: Suggested that a column in the reporting table for oocyst size would have been useful.

AC: Replied that he had assumed for the purposes of this trial, only oocysts would be being reported and hence the size could be assumed to be within the usual range.

JW: Added that measuring the oocysts would have required extra time.

MS: Added that extra time and effort would also have to have been directed at calibrating the graticule etc.etc.

RD: Suggested that a coding system to use on the report table throughout the trial might have helped.

AC: Agreed.

JG: Suggested that a coding system for fluorescence would have been useful and suggested that for fluorescence it could have ranged from + (good) to +++ (brilliant).

AC: Agreed.

JG: Repeated that such a coding system should have been in place for the trial.

AC: Agreed. Moved the discussion on to the time scale for reporting results. Stated that he would have preferred much more rapid reporting of results to enable him to decide how to structure the next part of the trial and also to allow him to start analysing the data, however he appreciated that the participants would have had routine commitments. All the same, he felt that the tendering laboratories had been made aware of the extent of the trials and the commitment in terms of time which would be required, but again he appreciated that the people actually carrying out the work, may have no input into making decisions on this.

MS: Stated that the trials had been pared down as much as possible.

AC: Asked if there had been room for further trials in the time allocated.

MS: Stated that he felt no more trials could have been conducted in the time.

AC: Asked if the participants felt that the feedback from SPDL had been satisfactory.

JG: Replied that it had been good.

NS: Agreed that it had been good and that if any problems arose, AC dealt with them rapidly.

AC: Suggested that MS may wish to make further points on the DWI position.

MS: Thanked everyone for their participation. Stated that more analysis of the data was required before the final report could be written, but by the end of February 1996, the report should be ready to go into the public domain and that all participating laboratories would be sent a copy. He stated that he felt that it was particularly useful as it was the first trial in which 3 techniques, SCA, IMS and flow cytometry, were compared.

AC: Asked if anyone had any other business to report or discuss.

Any other business

JG: Asked if 10 L grab sampling had been validated compared to filtering for sampling.

JW: Replied that both techniques would be put in the new SCA hand book. He suggested that either technique may be appropriate depending upon the questions being asked and that either technique may miss oocysts. Stated that in his experience 10 L grab samples were adequate for nearly all situations.

RD: Suggested that during an incident or suspected incident a 24 h sampler might be useful.

JW: Replied that he felt that during an incident or suspected incident he would recommend filtration as well, but time is also important and that grab samples give a more rapid response time.

RD: Stated that during incidents they do filters and grab samples.

JG: Asked what had happened to the oocysts in Loch Lomond.

AC handed out copies of the summary of the results to participants and forms to fill in giving comments on the IMS trial and methods.

AC: Asked if anyone would be interested in looking at the validity of extrapolating data on dilution, for example from 5% to 50%.

JW: Asked what he wanted to be done.

AC: Suggested seeding 6 samples and in 3 samples looking at 50% of the sample and in 3 samples looking at 5% of the samples and seeing if the extrapolated data is significantly different.

JW: Asked by what technique he would want such samples analysed.

AC: Replied that any technique would provide useful data on this question.

JW: Said that he would be interested.

Close of meeting.

Minutes of meeting taken and reported by L Robertson:

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METHOD WRITE UP FOR BLUE BOOK

NAME: .....

LABORATORY.....

Please comment on the following:

1. FEASIBILITY OF CURRENTLY WRITTEN METHOD
2. RELATIVE EASE, CONVENIENCE AND TIME OF APPLYING THE METHODS
3. USEFUL CHANGES AND ADDITIONS
4. USER-FRIENDLINESS OF FORM OF WORDS .
5. INCONSISTENCIES ETC.
6. METHODS OF REPORTING
7. USE OF DAPI AS AN ADJUNCT TO IMMUNOFLUORESCENCE FOR IDENTIFYING/CONFIRMING OOCYSTS
8. HOW WOULD YOU ENVISAGE THE IMS TECHNIQUE BEING UTILISED IN YOUR LABORATORY.

METHOD WRITE-UP FOR BLUE BOOK

Nelson Sykes
Thames Water Laboratory, Reading

1.FEASABILITY OF CURRENTLY WRITTEN METHOD:

At the present time the method would only be applicable to clean water samples, where oocyst recoveries using IMS were significantly better than with the Blue Book and flow-cytometry methods. However, the inconsistency and variability of IMS results
when applied to turbid samples would indicate its unsuitability for raw waters.

Further work may be required to develop broad-spectrum dispersant and wash buffers. This could aleviate problems with oocyst dissociation and debris interference.

Recovery trials comparing IMS with sucrose concentration are recommended- any significant increase in oocyst recovery would be encouraging.

2.RELATIVE EASE, CONVENIENCE AND TIME OF APPLYING THE METHODS:

The methods are user-friendly, with straight-forward techniques and simple apparatus.

3.USEFUL CHANGES AND ADDITIONS:

An improvement in the oocyst/beads dissociation method would be useful (acidification/neutralization sample by sample plus the minute quantities used is tedious).

Using larger sample volumes might be an option, especially where turbid samples are tested (? 50 ml). Are there magnets large enough to cope? What would be the cost implication?

4.USER-FRIENDLINESS OF FORM OF WORDS:

No problems.

5.INCONSISTENCIES:

None as far as methods are concerned, only some results.

6.METHODS OF REPORTING:

Possibly some slight changes, i.e. set scores (+,++,+++) for fluorescence, and codes for shape (R=round, O=oval, B=burst)

7.USE OF DAPI AS AN ADJUNCT TO IMMUNOFLUORESCENCE FOR IDENTIFYING/CONFIRMING OOCYSTS:

This would be recommended, along with DIC, especially with raw waters.

8.HOW WOULD YOU ENVISAGE THE IMS TECHNIQUE BEING UTILISED IN YOUR LABORATORY:

As a possible replacement for sucrose concentration in grossly turbid samples, and a general concentration aid in samples with lower turbidities, given the improvements necessary as mentioned in the answer to Q1.

METHOD WRITE UP FOR BLUE BOOK

COMMENTS FROM STRATHCLYDE WATER LABORATORIES

1. FEASABILITY- this is quite suitable.

2. RELATIVE EASE - the method was fairly simple and would be time saving if it replaced the sucrose stage and should reduce the loss of oocysts at this stage.

3. USEFUL CHANGES- include recipes for IMSdispersant and wash buffers

   ADDITIONS- see separate sheets

4. USER FRIENDLINESS OF THE WORDS are quite accceptable to us but perhaps not to "STANDING COMMITTEE OF ANALYSTS".

5. INCONSISTENCES- no apparent inconsistences except for the use of "mixer" and "rotator".

6. METHODS OF REPORTING- we presume you mean recording and the recording tables should include a column for size and flourescence should have a scoring system with guide lines.

7. USE OF DAPI- this is a good adjunct to microscopy of routine slides.

8. IMS TECHNIQUE- this would appear to us as an improved substitute for the sucrose stage especially if larger, dirtier volumes could be processed.

We understand the trial was the study of cryptosporidium but almost always we examine for giardia and cryptosporidium in the one process.

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METHOD WRITE UP FOR BLUE BOOK

NAME: IAN MAPLESDEN

LABORATORY: SOUTHERN SCIENCE.

Please comment on the following:

1. FEASABILITY OF CURRENTLY WRITTEN METHOD
2. RELATIVE EASE, CONVENIENCE AND TIME OF APPLYING THE METHODS
3. USEFUL CHANGES AND ADDITIONS
4. USER-FRIENDLINESS OF FORM OF WORDS
5. INCONSISTENCIES ETC.
6. METHODS OF REPORTING
7. USE OF DAPI AS AN ADJUNCT TO IMMUNOFLUORESCENCE FOR IDENTIFYING/CONFIRMING OOCYSTS
8. HOW WOULD YOU ENVISAGE THE IMS TECHNIQUE BEING UTILISED IN YOUR LABORATORY.

(1) FEASABLE FOR CLEANER SAMPLES, NEEDS IMPROVEMENT FOR DIRTIER SAMPLES.

(2) FAIRLY EASY TO USE APPART FROM FIDDLY DECOUPLING PROCEDURE. EXTRA SLIDE SCANNING TIME INVOLVED COMPARED TO FLOW CYTOMETRY

(3) IMPROVED DECOUPLING PROCEDURE, + NOT HAVING TO SCAN BEAD WELLS ON SLIDES.

(4) EASY TO UNDERSTAND.

(5) NONE REMEMBERED.

(6) REPORTING FORMS TIME CONSUMING TO FILL IN.

(7) DAPI ALREADY USED IN THIS LABORATORY AND IS FOUND USEFUL.

(8) IN PLACE OF THE SUCROSE FLOTATION CLEAN UP PROCEDURE PRIOR TO FLOW CYTOMETRY.

METHOD WRITE UP FOR BLUE BOOK

NAME: ROGER DOWN

LABORATORY SOUTHERN SCIENCE

Please comment on the following:

1. FEASABILITY OF CURRENTLY WRITTEN METHOD
2. RELATIVE EASE, CONVENIENCE AND TIME OF APPLYING THE METHODS
3. USEFUL CHANGES AND ADDITIONS
4. USER-FRIENDLINESS OF FORM OF WORDS
5. INCONSISTENCIES ETC.
6. METHODS OF REPORTING
7. USE OF DAPI AS AN ADJUNCT TO IMMUNOFLUORESCENCE FOR IDENTIFYING/CONFIRMING OOCYSTS
8. HOW WOULD YOU ENVISAGE THE IMS TECHNIQUE BEING UTILISED IN YOUR LABORATORY.

(1) FEASABLE FOR RELATIVELY CLEAN SAMPLES eg BOREHOLES, MOST TREATED WATER. PROBABLY UNSUITABLE FOR MOST OF OUR SURFACE WATERS.

(2) QUITE EASY TO USE AND REASONABLE WITH DEMANDS ON TIME IF BATCH PROCESSED. DECOUPLING FIDDLY.

(3) MAKE DECOUPLING SIMPLER (+ PERHAPS MORE EFFICIENT). DROP COUNTING OF OOCYSTS STILL ATTACHED TO BEADS

(4) NO PROBLEMS

(5) NONE NOTED.

(6) THE FORMS WERE TIME CONSUMING TO FILL IN - WE HAD TO DEVISE OUR OWN CODING SYSTEM TO HELP SPEED UP THE PROCEDURE

(7) DAPI HAS BEEN USED IN THIS LABORATORY FOR MORE THAN TWO YEARS AND IS VERY USEFUL AS AN AID TO IDENTIFICATION.

(8) TO REPLACE SUCROSE FLOTATION BEFORE FLOW CYTOMETRY. POSSIBLE SUPPLEMENTARY STAGE TO SUCROSE FOR VERY DIRTY SAMPLES WHERE THE SAMPLE IS STILL TOO TURBID AFTER FLOTATION.

DRAFT

METHOD WRITE UP FOR BLUE BOOK

NAME: John Watkins

LABORATORY Yorkshire

Please comment on the following:

1. FEASABILITY OF CURRENTLY-WRITTEN METHOD
2. RELATIVE EASE, CONVENIENCE AND TIME OF APPLYING THE METHODS
3. USEFUL CHANGES AND ADDITIONS
4. USER-FRIENDLINESS OF FORM OF WORDS
5. INCONSISTENCIES ETC.
6. METHODS OF REPORTING
7. USE OF DAPI AS AN ADJUNCT TO IMMUNOFLUORESCENCE FOR IDENTIFYING/CONFIRMING OOCYSTS
8. HOW WOULD YOU ENVISAGE THE IMS TECHNIQUE BEING UTILISED IN YOUR LABORATORY.

1. Current written method feasable.

2. Although it looks a realatively detailed method, once it has been used several times it becomes reasonably easy. It's not time consuming and a number of samples can be processed simultaneously. This fact should be made clear!

3. Pictures and or diagrams might help to clarify the separation technique and the equipment. Some details of times say for IMS 30 - 45 minutes and which stages can be done for a number of samples and which need to be done individually and which are time critical.

5 No problems.

6. No problems. Assessing DAPI staining and internal contents was obviously time consuming for samples with high numbers of oocysts.

7. Excellent. We have used this to good effect as an additional confirmation, even removing coverslips staining and re-mounting. It's quick, simple and effective.

8. Replacement for culture on a routine basis and for 'one-off' samples where flow cytometry was impractical. Would be prepared to conduct further limited trials