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Detection of *Cryptosporidium* oocysts in water: techniques for generating precise recovery data

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D.T. REYNOLDS, R.B. SLADE, N.J. SYKES, A. JONAS AND C.R. FRICKER. 1999. When determining the recovery efficiency of a procedure for the detection of *Cryptosporidium* or the removal efficiency of a treatment process, it is necessary to accurately enumerate a 'seed dose'. Conventional techniques for this are highly variable and consequently, can result in misleading data. In this study, a flow cytometric method was developed for the production of suspensions of *Cryptosporidium* oocysts in which the number of organisms could be precisely determined. A Becton Dickinson FACScalibur flow cytometer was employed to produce oocyst suspensions containing 100 oocysts. Analysis of these suspensions resulted in a mean dose of 99·5 oocysts (S.D. = 1·1, %cv = 1.1). These results indicate that the use of such suspensions to seed test systems generates far more accurate data than is presently possible using conventional techniques. In addition, the use of immunomagnetic separation (IMS) for the isolation of oocysts from three different water matrices, after seeding with oocysts counted using flow cytometry, was investigated. The recovery efficiency of the IMS procedure was found to be high, with the percentage recovery of oocysts ranging from 82·3 to 86·3%, and the use of precise numbers of oocysts allowed accurate recovery efficiency data to be generated. A laser scanning instrument (Chem*Scan* RDI) was employed for the rapid detection and enumeration of oocysts after capture using membrane filtration. This technique was found to be faster and easier to perform than conventional epifluorescence microscopy. These findings demonstrate that the Chem*Scan* RDI system may be used as alternative procedure for the routine examination of IMS supernatant fluids for the presence of *Cryptosporidium*.

INTRODUCTION

Methods for the detection of oocysts of the protozoan parasite *Cryptosporidium parvum* have received much attention over recent years, largely due to an increase in the number of reported water-borne outbreaks of cryptosporidiosis. Routine procedures for the detection of oocysts involve the concentration of large volumes of water by filtration or flocculation, followed by further concentration using centrifugation. A variety of techniques is being developed to detect oocysts from such water concentrates, including the polymerase chain reaction (PCR) (Johnson *et al*. 1995), reverse transcription PCR (Stinear *et al*. 1996), fluorescent *in situ* hybridization (FISH) (Lindquist 1997) and tissue culture infectivity (Slifco *et al*. 1997). Nevertheless, most laboratories continue to rely on immunofluorescence techniques. These procedures involve the incubation of samples with a fluorescein–isothiocyanate conjugated *Cryptosporidium* sp. specific monoclonal antibody (FITC-mAb) prior to examination for the presence of labelled organisms using epifluorescence microscopy.

Although water concentrates may be analysed directly using immunofluorescence techniques, the presence of particulate material, which accumulates during the concentration procedure, often interferes with the microscopic analysis. Conventional methods used to reduce levels of such debris involve the use of flotation procedures (Arrowood and Sterling 1987) or flow cytometry (Vesey *et al*. 1991, 1993a;

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Veal *et al.* 1997). Of these two techniques, flow cytometry has proved most useful, although it does require highly skilled operators and is a relatively slow procedure. Recently, however, immunomagnetic separation procedures (IMS) have become commercially available which have been reported to provide a simple and rapid alternative to presently used separation techniques (Campbell *et al.* 1997; Bukhari *et al.* 1998). Using this technology, magnetic beads coated with monoclonal antibody specific for *Cryptosporidium* sp. are used to selectively isolate oocysts from background debris.

Following IMS, oocyst detection relies on the examination of sample preparations using epifluorescence microscopy. Although this procedure is widely employed, it is time consuming and can cause operator fatigue. Consequently, a reliable automated procedure for the detection of labelled organisms would be of considerable benefit. The Chem*Scan* RDI instrument (Chemunex, Maisons Alfort, France) has previously been used for the enumeration of fluorescently labelled bacteria from potable water (Reynolds and Fricker 1999). Using this technique, organisms are captured by membrane filtration, labelled, and the filter subsequently scanned with a laser inside the instrument. As the laser passes over the membrane, fluorescent events (including labelled organisms) are detected by a series of optical sensors. Finally, the signals generated undergo a sequence of computer analyses in order to identify events that are labelled organisms. This permits an accurate enumeration to be made down to one organism on a membrane. A visual validation of all the Chem*Scan* results can then be made by transferring the membrane to an epifluorescence microscope that is fitted with a motorized stage. This stage, which is controlled by the Chem*Scan* RDI, can be driven to the location of each fluorescent event for visual confirmation of all results (Fig. 1).

Numerous scientific publications report the efficiency of new procedures for the isolation of oocysts, and many of these indicate percentage recoveries that are far in excess of the methods currently used within the water industry. However, such high recoveries may be due to an underestimation of the number of oocysts added to the test system (the seed dose). The enumeration of oocysts within seed doses is extremely difficult due to inherent inefficiencies of counting techniques (Klonicki *et al.* 1997) and the fact that oocysts commonly have an uneven distribution within stock suspensions (Bukhari and Smith 1995; Drozd and Schwartzbrod 1996). In our experience, when counting the number of oocysts within replicates of a stock suspension, coefficients of variation in excess of 10% are often obtained. Consequently, when using subsequent aliquots of the stock suspension to seed a test system, it is not possible to determine the exact number of oocysts added and therefore it is difficult to interpret recovery data.

Recent studies in this laboratory indicate that flow cytometry coupled with cell sorting may be used to overcome this problem, producing seed doses in which the number of oocysts can be precisely determined. This technique relies on the ability of the instrument to analyse particles in a fluid stream. As each particle passes through the flow cytometer's laser beam, several light scatter properties are collected. These measurements correlating to the size and internal complexity of the particle. Using this information, flow cytometer–cell sorters have the ability to count and separate particles of interest from the main body of liquid (Fig. 2).

In this study, the use of flow cytometry for the rapid production of *Cryptosporidium* oocyst 'seed doses' within

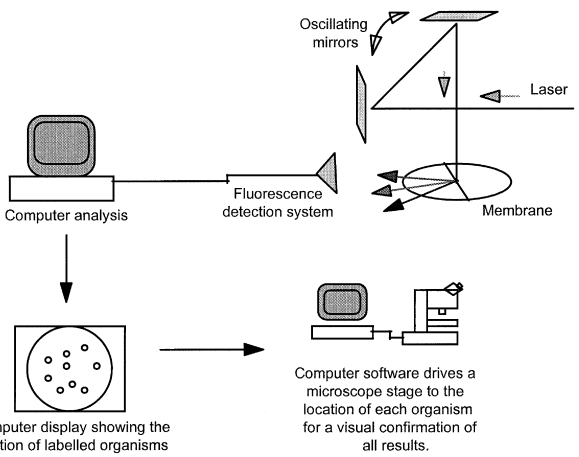

Fig. 1 Detection of labelled organisms using the Chem*Scan* RDI instrument. After analysis, the system permits all fluorescent events detected on the membrane to be visually validated

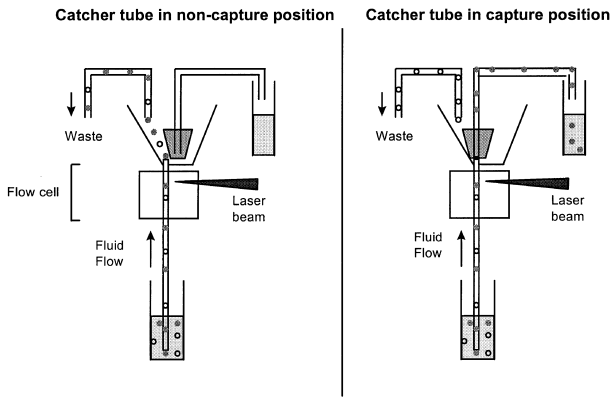

Fig. 2 Sorting and enumeration of particles of interest using Flow cytometry. The sort mechanism uses a catcher tube positioned above the flow cell that rapidly moves in and out of the sample stream to capture designated cells after they pass through the laser. The catcher tube can move up to a rate of 300 times s^{-1}. The captured cells are then directed to the collection tube for further analysis

which the number of oocysts can be accurately determined is reported. Furthermore, preliminary data are presented which demonstrate the potential of laser scanning for the rapid detection of oocysts from water concentrates after their isolation using IMS.

MATERIALS AND METHODS

Organisms

Cryptosporidium parvum oocysts (Harley Moon strain, NADC, Ames, IA, USA), produced in neonatal Holstein calves and purified from faeces using discontinuous sucrose and caesium chloride centrifugation gradients, were obtained from the Sterling Parisitology Department, University of Arizona (USA) at a concentration of $1{\cdot}0 \times 10^8$ ml^{-1}. The oocysts were stored at 2–8 °C in 0·01 mol l^{-1} phosphate-buffered saline (PBS) containing Tween-20 (0·01%) and antibiotics (gentamicin, 100 μg ml^{-1}, penicillin 100 μ ml^{-1} and streptomycin 100 μg ml^{-1}). At the time of this investigation, the oocysts were approximately 3 months old. Prior to the study, oocysts within an aliquot of this suspension were captured by membrane filtration and labelled with FITC-mAb. Examination of this preparation using epifluorescence microscopy demonstrated that $> 95\%$ of organisms exhibited excellent immunofluorescent staining.

Enumeration and sorting of unlabelled oocysts using flow cytometry

Flow cytometry was performed using a modified Becton Dickinson FACScalibur flow cytometer (Becton Dickinson, NJ, USA). Filtered, de-ionized water was employed as sheath fluid and the sample flow rate was set on the 'Lo' speed. The wavelength of the laser was 488 nm, the power setting was 15 mW. PMT voltages were set at EOO (linear gain) for forward angle light scatter detection and at 400 (logarithmic gain) for side angle light scatter detection. The data acquisition dot plot parameters used during the analysis were forward angle light scatter *vs* side angle light scatter.

Oocysts were introduced into the flow cytometer at a concentration of approximately $1{\cdot}0 \times 10^5$ ml^{-1} (suspended in de-ionized water). Events on the resultant dot plot were allowed to accumulate until a series of oocyst populations were discernible. These populations represented signals obtained from single oocysts and from those which were aggregated. A sort region was then drawn which encompassed the region known to be characteristic of only single oocysts. The sort total on the instrument was set at 100, the sort facility being 'single cell'. On each acquisition, oocysts emerging from the sort line were captured by membrane filtration (Cycloblack polyester membranes, 25 mm diameter, 2 μm pore size, Whatman, Basingstoke, UK) or directly into Leighton tubes for IMS recovery studies. After the completion of each acquisition, sheath fluid was allowed to run through the sort line onto the membrane or into the Leighton tube for approximately 10 s, to ensure the collection of all oocysts from the sort line.

To assess the ability of the instrument to accurately sort a defined number of oocysts, 100 replicate membranes were prepared, incubated with FITC-mAb and analysed using the Chem*Scan* RDI instrument. After this analysis, the number of oocysts on 10% of the membranes was also determined using epifluorescence microscopy.

Recovery of *Cryptosporidium* oocysts from water concentrates using immunomagnetic separation (IMS)

Concentration procedure. Samples of river water (30 litre) were taken from various sites within the Thames Water Utilities catchment area. Water from each site was processed in 10 litre volumes using calcium carbonate flocculation/centrifugation (Vesey *et al.* 1993b). Concentrates from each site were mixed to create a single water concentrate (matrix) from each location.

Prior to concentration, the turbidity of each sample was determined using a Hach 2100N Turbidmeter (Hach, CO, USA). The pH of each was measured using a Corning 140 pH meter (Corning, Essex, UK). The level of total dissolved solids (TDS) was determined using a Merck DIST WPI TDS meter (BDH/Merck, Leicestershire, UK) and the amount of particulate organic carbon (POC) and Chlorophyll A in each sample was determined using standard methods (Anon 19791980).

Oocyst seeding. Aliquots (500 μl packed pellet volume, 1050 *g*, 10 min) of each water concentrate were added to Leighton tubes containing a 'seed dose' of 100 oocysts produced using flow cytometry. Three replicates of each water concentrate were prepared. In addition, a further aliquot of each water concentrate was added to a clean Leighton tube in order to determine the presence of any indigenous oocysts.

Prior to the seeding of Leighton tubes with oocysts, instrument performance was assessed by attempting to sort 100 oocysts onto each of 10 membranes. The number of oocysts on each was then determined using the Chem*Scan* RDI system. The mean number of oocysts detected was subsequently used to determine the percentage recovery of oocysts from the seeded samples.

Immunomagnetic separation. This procedure was based on the Dynal IMS procedure (Dynabeads anti-*Cryptosporidium*; product no. 730.0. Dynal AS, Oslo, Norway). The volume in each Leighton tube was adjusted to 10 ml with de-ionized

water. Following this dilution, 1 ml 10 × SL Buffer A (Dynal), 1 ml 10 × SL Buffer B (Dynal) and 100 μl anti-*Cryptosporidium* paramagnetic Dynabeads (Dynal) were added. Each sample was incubated on a rotating mixer at room temperature for 1 h. Immediately after incubation, the paramagnetic beads were captured using a magnetic concentrator (MPC-1, Dynal) and the supernatant fluid carefully poured to waste. The beads were re-suspended in 1 ml 1 × SL buffer A and transferred to a 1·5 ml Eppendorf tube where they were recaptured using a second magnetic particle concentrator (MPC-M, Dynal). After the resultant supernatant fluid had been discarded, the beads were re-suspended in 100 μl 0·1 mol l^{-1} hydrochloric acid and incubated at room temperature for 5 min to release oocysts. After incubation, the beads were recaptured and the acid supernatant fluid containing oocysts was transferred to a clean Eppendorf tube. This acid incubation was repeated.

Oocyst permeabilization. This procedure was performed to permit subsequently effective oocyst labelling with 4,6-diamidino-2-phenylindole (DAPI). The acidified supernatant fluid (200 μl) obtained from the IMS procedure was neutralized by the addition of 200 μl MOPS buffer (1 mol l^{-1}, pH 7, Sigma). Each sample was then diluted with 400 μl ethanol (99·9%) and incubated in a water bath (80 °C, 10 min). Oocysts within this final supernatant fluid were then captured by membrane filtration (Cycloblack polyester membranes) prior to incubation with FITC-mAb/DAPI.

Enumeration of *Cryptosporidium* oocysts using the Chem*Scan* RDI system

This was performed following instructions given by the manufacturers of the 'Chem*Scan* Detection of *Cryptosporidium* oocysts on a Membrane filter Kit' (Chemunex, Maison Alfort, France). Briefly, after oocyst capture, each membrane was carefully transferred onto a sintered glass vacuum support (Millipore, Basingstoke, UK) and rinsed with 500 μl of a 1 : 5 dilution of ChemSol B12, which is a buffered detergent solution (Chemunex). Subsequently, each membrane was carefully loaded on a droplet (100 μl) of labelling solution and incubated in a humid chamber at 37 °C for 1 h.

For labelling of oocysts which had been sorted onto membranes using flow cytometry, the labelling solution was prepared by mixing ChemID (Chemunex), which contains a *Cryptosporidium* specific FITC-mAb, and CSH (Chemunex) which contains an antibody blocking reagent as instructed. For the labelling of oocysts captured from IMS supernatant fluids, DAPI was included in the labelling solution (20 μg ml^{-1}, final concentration).

After incubation, each membrane was transferred onto a sintered glass vacuum support (Millipore), which allowed any labelling solution to be removed from the surface of the membrane. Each membrane was then placed onto a membrane holder (Chemunex) and analysed with the Chem*Scan* RDI instrument following the instructions given by the manufacturer. After analysis, all fluorescent events on the membrane, which were identified by the Chem*Scan* RDI as potentially being an oocyst, were microscopically validated. During this procedure, each membrane was transferred to an epifluorescence microscope (Leica DMRB, Leica), the stage of which was driven to the location of each possible oocyst by the Chem*Scan* RDI. This allowed a visual confirmation as to whether the fluorescent event detected was a labelled oocyst by assessing FITC-mAb/DAPI staining characteristics.

Enumeration of *Cryptosporidium* oocysts using manual microscopy

Membranes were air dried and mounted on a microscope slide using 20 μl mounting medium (2·5% DABCO, 10% glycerol, 5% sodium chloride (5 mol l^{-1}), 5% formalin–80% PBS (0·1 mol l^{-1})). Immediately after mounting, the number of oocysts on each membrane was determined manually using an epifluorescence microscope (Leica DMLB, Leica).

Epifluorescence microscopy

In this study, a Leica DMRB (Leica, Milton Keynes, UK) was used. This microscope was equipped with a blue filter block for the detection of FITC-mAb labelled oocysts (490 nm excitation wavelength, 510 nm emission wavelength) and a u.v. filter block for the detection of DAPI labelling (400 nm excitation wavelength, 420 nm emission wavelength). Oocysts were viewed at a magnification of × 620 during the validation of the Chem*Scan* results. When enumerating the number of oocysts using manual epifluorescence microscopy, a magnification of × 400 was used.

RESULTS

Production *Cryptosporidium* oocyst 'spike doses' using flow cytometry

The ability of a Becton Dickinson FACScalibur flow cytometer to accurately enumerate and sort unlabelled oocysts was assessed. Suspensions of 100 oocysts were produced.

The results generated by the Chem*Scan* were in the form of two maps (Fig. 3). The first, the 'All Data Map', showed the number and location of all fluorescent events on the membrane which possessed fluorescence characteristics similar to that of fluorescein isothiocyanate (FITC). The second map, the 'Results Map', showed only those events whose

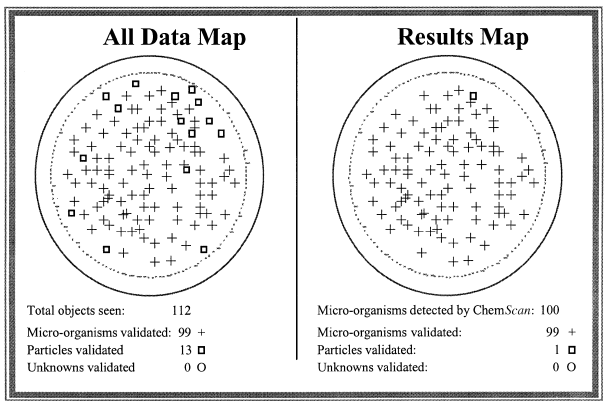

Fig. 3 Typical Chem*Scan* RDI results with complete visual microscopic validation. The All Data Map depicts the location of all fluorescent objects on the membrane which possessed fluorescence characteristics similar to that of fluorescein ($n = 112$). The Results Map indicates which of these objects the Chem*Scan* RDI identified as labelled oocysts ($n = 99$). The manual microscopic validation results are shown below each scan map. In this example, 112 fluorescent objects were detected by the Chem*Scan*, 85 of which were visually identified as oocysts and 13 of which were found to be interfering fluorescent particles. All oocysts were identified by the Chem*Scan* resulting in no false-negatives. However, one particle was wrongly identified by the Chem*Scan* as being a labelled oocyst, therefore resulting in one false-positive. It should be noted that during this study, only the Results Map was routinely validated

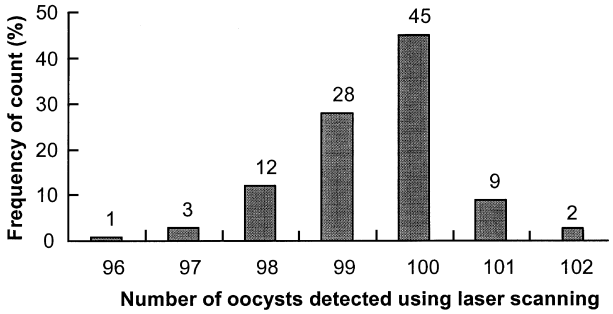

Fig. 4 Frequency of counts generated, using laser scanning, from the analysis of filter membranes onto which 100 *Cryptosporidium* oocysts had been sorted using flow cytometry. Number of counts = 100. The mean number of oocysts detected per membrane was 99·5 (S.D. ± 1·1, %cv = 1·1). Each count represents the number of oocysts, confirmed as such, following the microscopic validation of each membrane

size and fluorescence intensity were characteristic of labelled oocysts. The discrimination parameters used by the instrument were optimized prior to this study to ensure the recognition of all labelled organisms (data not shown). As a result, only fluorescent events within the 'Results Map' were routinely viewed during microscopic validation.

The mean number of oocysts recovered from each of the doses produced was 99·5 (S.D. = 1·1, %cv = 1·1, $n = 100$). Exactly 100 organisms were detected in 45% of the samples analysed, with counts ranging from 96 to 102 organisms. More than 100 organisms were recovered from 11% of the doses analysed. In all of these samples, small oocyst aggregates of two organisms were present. This indicated that the instrument had sorted these in error, identifying them as being a single organism (Fig. 4).

Ten (10%) of the samples analysed were also examined using manual epifluorescence microscopy and the results compared with those using the Chem*Scan* RDI system (Table 1). Identical oocyst counts were obtained from analysis of eight of these membranes. However, Chem*Scan* analysis resulted in counts slightly higher than manual microscopy in two instances (i.e. membrane number 40: 100 oocysts (Chem*Scan*), 99 oocysts (manual microscopy); membrane number 80: 100 oocysts (Chem*Scan*), 98 oocysts (manual microscopy)). As all of the oocysts detected by the Chem*Scan* system were visually confirmed, the results suggest that manual microscopy failed to give accurate counts for these two samples.

Table 1 Comparison between the number of *Cryptosporidium* oocysts enumerated using laser scanning (validated count) and the manual microscopic examination of filter membranes

Replicate number	Number of oocysts observed using laser scanning	Number of oocysts observed using manual epifluorescence microscopy
10	101	101
20	100	100
30	100	100
40	100	99
50	100	100
60	99	99
70	100	100
80	100	98
90	100	100
100	102	102

A difference between the two enumeration methods was observed in two instances (membranes 40 and 80). In both cases, analysis by laser scanning resulted in a slightly higher count than manual microscopic examination (1 and 2 oocysts, respectively).

Table 2 Characteristics of the three river waters evaluated

River Site	Turbidity (NTU)	pH	TDS (mg l^{-1})	POC (mg l^{-1})	Chlorophyll A (μg l^{-1})	Total packed pellet volume (30 l)	Raw water equivalent volume analysed (500 μl packed pellet)
1	5·8	8·0	260	0·88	2·22	3·8 ml	4·0 l
2	4·6	7·8	290	0·99	3·31	5·3 ml	2·8 l
3	6·1	7·7	280	0·88	2·42	4·8 ml	3·1 l

Prior to the concentration of each water type (30 l) measurements of turbidity and pH were taken in addition to determining the concentration of total dissolved solids (TDS), particulate organic carbon (POC) and Chlorophyll A in each. After concentration, the packed pellet volume of each was recorded and subsequently the volume of raw water equivalent to 500 μl of these matrices calculated.

Recovery of *Cryptosporidium* oocysts from river water concentrates using immuno-magnetic separation (IMS)

The characteristics of each of the three water types examined in this study are summarized in Table 2. After concentration, aliquots of each matrix ($n = 3$) were seeded with approximately 100 oocysts using flow cytometry (mean seed dose 99·3 $\pm$ 1·2, range 98–101, $n = 10$). The Chem*Scan* RDI system was then employed to determine the number of oocysts recovered from each after isolation using IMS.

The recovery of oocysts from each of these matrices using IMS was found to be high (Table 3), with a mean percentage recovery of 84·3% $\pm$ 2·0 (i.e. Matrix 1, 86·3% $\pm$ 5·2; Matrix 2, 84·3% $\pm$ 10·5; Matrix 3, 82·3% $\pm$ 4·2). Subsequent analysis of these recovery data demonstrated no significant difference between recoveries obtained from these different matrices (*t*-test, $P > 0{\cdot}05$).

During the analysis of these IMS supernatant fluids using the Chem*Scan* system, a number of fluorescent events were detected on each membrane which were not confirmed as being labelled oocysts during the microscopic validation procedure (i.e. Matrix 1, 34·3 events ($\pm$ 19·4); Matrix 2, 18·3 ($\pm$ 10·1); Matrix 3 13·0 events ($\pm$ 4·0). These appeared to be either particulate debris or cellular material to which the FITC-mAb had non-specifically bound. Although these events interfered with the analysis, the microscopic validation system allowed rapid validation of the results (greater than 100 events in 3–4 min). As the instrument has the capability of scanning a membrane while a previous membrane is being validated, it was possible to validate all membranes prior to the Chem*Scan* completing its analysis of the 'next membrane'. Consequently, it was possible to analyse approximately 10 membranes per hour.

DISCUSSION

When assessing the ability of a process to recover or remove *Cryptosporidium* oocysts from water, it is essential that the number of oocysts initially added to the test system is accurately determined. In general, procedures for the enumeration of oocysts within such seed doses involve counting the number of organisms within aliquots of a suspension (using a

Table 3 Recovery of oocysts from water concentrates using immunomagnetic separation. The mean number of oocysts recovered from three different water matrices using IMS was 84·3 $\pm$ 2·0%. The mean seed dose was 99·3 ($\pm$1·2) oocysts

Sample	Number of oocysts recovered from unseeded controls ($n = 1$)	Mean number of oocysts recovered from seeded matrices ($n = 3$)	Mean percentage recovery ($n = 3$)	Mean number of events detected by Chem*Scan* RDI which were not visually confirmed as oocysts
Matrix 1	0	85·7 $\pm$ 5·1 (range 80–90)	86·3 $\pm$ 5·2	34·3 $\pm$ 19·4
Matrix 2	0	83·7 $\pm$ 10·4 (range 72–92)	84·3 $\pm$ 10·5	18·3 $\pm$ 10·0
Matrix 3	0	81·7 $\pm$ 4·2 (range 77–85)	82·3 $\pm$ 4·2	13·0 $\pm$ 4·0

The mean number of oocysts recovered from three different water matrices using IMS was 84·1% $\pm$ 1·9. The mean seed dose was 99·3 (%1·2) oocysts.

haemocytometer or by epifluorescence microscopy) prior to the addition of a further aliquot to the test system. This technique has proved highly inefficient for a number of reasons. Firstly, studies indicate that all commonly used enumeration techniques can result in an underestimation of the number of oocysts present, either because of the loss of oocysts during sample preparation or, in the case of immunofluorescence techniques, the 'non-staining' of some organisms (Frederickson 1995; Anon 1996; Klonicki *et al.* 1997). Secondly, oocysts in suspension often have an uneven distribution which can result in great variation in the number of organisms present between replicate aliquots, often due to oocysts forming aggregates (Bukhari *et al.* 1995; Drozd and Schwartzbrod 1996).

Our unpublished observations demonstrate that the coefficient of variation (%cv) in oocyst numbers counted between replicate aliquots of an oocyst suspension is commonly greater than 10% (data not shown). Consequently, it is essential to determine the number of oocysts within a large number of replicates prior to the seeding of the system. In this way, the range of possible oocyst doses can be calculated and subsequently, the range of recovery/removal efficiencies of the system determined. Perhaps one of the greatest criticisms of many previous recovery/removal studies is that very few replicate analyses of the seed dose were performed, or the authors failed to document the number of replicates analysed (Klonicki *et al.* 1997). As a result, it is not possible for subsequent researchers to determine the variability of the dose added and therefore assess the validity of the findings. In this laboratory, the number of oocysts within 10 replicate aliquots of an oocyst suspension are routinely enumerated prior to the seeding of the system. However, the recovery data generated is often very difficult to interpret as the range of possible efficiencies of the system is commonly very large. In order to generate reliable data, therefore, the recovery/removal study must be repeated many times in an attempt to account for the variation expected within the seed dose. However, even when this is done, any intrinsic variability of a test system to remove/isolate oocysts can be difficult to identify because of the variability of the seed dose.

The present findings show that the inefficiencies of current techniques to determine the number of oocysts within seed doses may be overcome through the use of flow cytometry. This technique has the capability to sort and isolate a defined number of oocysts with very high precision. Although some variation around the target dose of 100 organisms was observed, it was extremely low (%cv = 1·1), with oocyst counts ranging from 96 to 102 organisms ($n = 100$). Present observations indicate that such low variation between doses cannot be achieved using conventional enumeration techniques. For example, when enumerating a seed dose of approximately 100 oocysts within aliquots of a stock suspension, it is not uncommon to obtain counts ranging from approximately 80–120 organisms (data not shown). Consequently, the use of oocyst seed doses produced using flow cytometry for recovery studies would allow far more accurate recovery or removal data to be generated. In addition, such studies would allow a clear determination of any intrinsic variability associated with the system under examination.

During this study, however, not all doses produced by the flow cytometer contained exactly the target number of organisms. The reason why fewer than 100 oocysts were recovered in some doses is not known. However, it could be due to several factors, such as inefficiency of the capture apparatus, loss of oocysts within the sort line, the erroneous sorting (and enumeration) of particulate material, or loss of oocysts during the labelling procedure. In 11% of the samples analysed, more than 100 oocysts were recovered; in all cases, this was due to the sorting of oocyst aggregates. Although the sort parameters were designed to avoid this, it would appear that slight modifications should be made.

In this investigation, a Becton Dickinson FACScalibur flow cytometer–cell sorter was employed, which is one of a new generation of such instruments designed to be much easier to use than previous models. This instrument required less than 20 min to set up and subsequently, each oocyst seed dose could be produced in less than 20 s. In this evaluation, the instrument was programmed to provide a dose of 100 oocysts. However, this technique can be used for the production of seed doses containing any number of organisms. Indeed, as the system has the capacity to sort more than 300 organisms per second, even very high doses may be produced rapidly.

It is widely recognized that the methods currently used for the isolation of oocysts from water concentrates are highly inefficient (Fricker 1995; Whitmore and Nazir 1997). Conventional techniques for the separation of oocysts from interfering debris often involve the use of flotation procedures. However, these can yield very variable oocyst recoveries (Bukhari *et al.* 1995; Fricker *et al.* 1995). For instance, studies performed by the US Environmental Protection Agency (USEPA) indicated that the flotation technique typically results in oocyst recoveries of less than 40% (Anon. 1996). In addition, much particulate material can remain after treatment which subsequently interferes with microscopic examination (Fricker *et al.* 1997). In view of these problems, many laboratories use flow cytometry to isolate oocysts from water concentrates. Using this instrumentation, FITC-mAb labelled organisms are separated from background debris on the basis of their size, shape and fluorescence characteristics. The sorted material can then be examined using epifluorescence microscopy to confirm the presence of oocysts. Although recovery efficiencies in excess of 95% have been reported using this system (Vesey *et al.* 1993a; Veal *et al.* 1997), it is extremely time consuming and consequently, the number of samples which can be analysed is limited. Furthermore, it

has been found that such recoveries cannot be obtained consistently from all water matrices.

In this study, the use of IMS to isolate oocysts from three different water matrices was investigated using flow cytometry to produce the seed doses (mean seed dose = 99·3 ± 1·2). Results demonstrated a mean oocyst recovery of 84·3%, with no significant difference between the recoveries obtained from the different matrices ($P > 0{\cdot}05$). While such high recoveries using this IMS kit have been previously reported for both fresh and aged oocysts (Campbell *et al.* 1997; Fricker *et al.* 1997; Bukhari *et al.* 1998; Rochelle *et al.* 1999), no study has demonstrated the efficiency of this system with such high precision. In all previous investigations, conventional techniques were used to determine the seed dose and consequently, their findings only give an indication of the possible recovery efficiencies. For example, in the study performed by Bukhari *et al.* (1998), percentage recoveries ranging from 35 to 99% were reported. However, as the range of counts obtained within the seed dose were between 525 and 870 oocysts, these data can be rather difficult to interpret.

Although data are presented here which indicate that IMS may be used for the efficient isolation of oocysts from water concentrates, care must be taken when choosing a suitable IMS system. This is because not all commercially available kits yield similarly high results (Bukhari *et al.* 1998; Rochelle *et al.* 1999). This appears to be due to the effect of sample turbidity on the ability of the antibody–bead complex to capture oocysts (Anon. 1990; Anon. 1996; Bukhari *et al.* 1998). In this study, the manufacturer of the system recommended that no more than 500 μl (1050 *g*, 10 min) of sample should be analysed, as greater volumes may affect the recoveries obtained. However, such packed pellet volumes may only represent small volumes of the original raw water (in this investigation, sample volumes ranged from 2·8 to 4·0). Therefore, if a routine monitoring programme dictates that a larger volume of sample should be examined (for instance 10 l), several aliquots must be analysed. This is of concern as the cost of such IMS systems is relatively high. However, studies indicate that a greater volume of sample may be analysed using the Dynal IMS system without significantly affecting the recoveries obtained (Bukhari *et al.* 1998; Rochelle *et al.* 1999). These investigations have only been performed on a small number of concentrates and therefore, more work is required to determine the effect of larger volumes of a variety of different water matrices.

In this series of investigations, the number of FITC-mAb labelled oocysts present on membrane filters was determined using a novel laser scanning device (Chem*Scan*) which proved to be a highly reliable instrument for the enumeration of these organisms. Although previous studies in this laboratory (data not shown) have demonstrated the efficiency of this system, comparison of counts obtained using laser scanning with those using manual microscopic examination indicated that the instrument had detected all labelled organisms present. Indeed, in two instances, manual microscopy failed to give precise counts. This clearly demonstrates one of the fundamental disadvantages of manual microscopic techniques in that they are highly reliant upon the competency of the operator and consequently are open to human error.

Conventional techniques for the examination of IMS supernatant fluids for the presence of oocysts rely on fixing the sample on microscope well slides prior to labelling with FITC-mAb, and examination using epifluorescence microscopy. In our experience, the microscopic examination of each sample requires approximately 15–20 min, with the time required increasing as subsequent preparations are analysed (this is due to operator fatigue). In contrast, examination of sample preparations using the Chem*Scan* system may be completed in less than 5 min. This includes analysis of the sample by the instrument, followed by the microscopic validation of the results. In this assay operator fatigue is not encountered and consequently, analysis time does not vary significantly. In addition, during the manual microscopic examination of samples, many laboratories routinely use a magnification of × 200 as this allows samples to be analysed at a much faster rate than at higher magnifications. However, it is our experience that oocysts in environmental samples often do not stain efficiently with FITC-mAb and consequently, can be difficult to detect under such low magnifications. Using the Chem*Scan* system, the magnification used during the validation procedure does not affect the speed of analysis and consequently, a very high magnification of × 630 is routinely used which allows visualization of even very badly labelled organisms. Perhaps the only drawback of the Chem*Scan* RDI system is that the analysis is performed on membranes which do not permit oocyst confirmation using Nomarski differential interference microscopy (DIC). This technique necessitates the illumination of samples from below. However, the membranes used in this study do not allow the transmission of light in such a manner and consequently, work is on-going in this laboratory to identify membranes which would permit the use of DIC microscopy.

Although these findings indicated that the Chem*Scan* RDI system has the capability to detect all labelled oocysts on a membrane surface, not all fluorescent events detected by the instrument were confirmed as being *Cryptosporidium* oocysts. The interfering fluorescent events observed appeared to be particulate or cellular material to which the FITC-mAb had bound non-specifically. Similar findings have been reported previously in which monoclonal antibodies developed to be specific to *Cryptosporidium* sp. have cross-reacted with particulate material and algal/yeast cells (Rodgers *et al.* 1995). Nevertheless, the number of such events detected by the system was low and consequently did not significantly affect the analysis time.

In conclusion, conventional techniques for determining the number of *Cryptosporidium* oocysts in seed doses to be used in recovery/removal studies are very inefficient and consequently, can result in the calculation of incorrect data. The present findings showed that flow cytometry may be used for the production of suspensions of *Cryptosporidium* oocysts in which the number of oocysts can be precisely determined. The use of such suspensions to seed test systems would subsequently allow precise recovery/removal data to be calculated. Furthermore, using flow cytometry to produce seed doses, the data suggest that IMS may be used for the efficient isolation of oocysts from water concentrates. However, further work is required in order to increase the amount of sample which may be analysed using this assay.

During these studies a laser scanning instrument (Chem*Scan* RDI) was used for the rapid detection and enumeration of oocysts. This instrument was found to be extremely reliable, having the capability to detect all oocysts present on a membrane surface. In addition, the number of fluorescent events detected, and their characteristics, can be stored electronically. The Chem*Scan* RDI system therefore offers a reliable and rapid procedure for the detection and enumeration of oocysts in water samples, and is a suitable alternative to direct epifluorescence microscopy.

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