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Cryptosporidia in Water (PECD 7/7/357)

DECLARATION

THE WORK DESCRIBED IN THIS FINAL REPORT ON THE SURFACE BIOLOGY AND VIABILITY OF *CRYPTOSPORIDIUM* SP. OOCYSTS TO THE DEPARMENT OF THE ENVIRONMENT IS ORIGINAL WORK PERFORMED AT THE SCOTTISH PARASITE DIAGNOSTIC LABORATORY BETWEEN APRIL 1990 AND MARCH 1991, UNDER CONTRACT NO. 10550

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FINAL REPORT on the DoE SPONSORED RESEARCH ON *CRYPTOSPORIDIUM* SPP. OOCYSTS UNDERTAKEN AT THE SCOTTISH PARASITE DIAGNOSTIC LABORATORY, STOBHILL GENERAL HOSPITAL, GLASGOW G21 3UW.

OBJECTIVES

THE TWO OBJECTIVES ADDRESSED IN THIS FINAL REPORT ARE:

1) TO DEVELOP MORE SENSITIVE RAPID METHODS FOR DETECTING *CRYPTOSPORIDIUM* SP. OOCYSTS, AND

2) TO DEVELOP METHODS FOR ASSESSING THE VIABILITY OF INDIVIDUAL *CRYPTOSPORIDIUM* SP. OOCYSTS.

SUMMARY AND RECOMMENDATIONS.

1) Rapid detection techniques

a) Rapid concentration and detection using magnetisable particles is capable of recovering over 75% of oocysts from seeded samples. The efficiency of concentration of small numbers of oocysts from seeded and environmental samples requires further investigation, as does the role of the detergents, used in the standard method for the isolation of oocysts from water, on the interaction between antibody paratope and oocyst epitope. High affinity monoclonal antibodies (McAbs) should overcome this latter problem.

b) The automated microscopic screening for fluorescently labelled oocysts is impractical at present.

c) Automatic rapid detection, using a cooled slow-scan CCD, is impractical at present only because of the need to design suitable computer software. These impracticalities are seen as technological difficulties rather than scientific impossibilities, and should be relatively easy to overcome. In addition, it should be possible to screen samples using a fluorescence activated cell sorter (FACS). This is an expensive machine (c. £130,000). Collaboration with the SPDL to develop these techniques is recommended.

d) Enhanced chemiluminescent detection of *Cryptosporidium* oocysts is capable of detecting between 5 - 10 oocysts in a sample and appears to be reproducible. We do not feel able to

recommend the enhanced chemiluminescent detection of *Cryptosporidium* oocysts as a preliminary screening procedure using the commercially available McAbs as we are undecided which is the best way to incorporate this technology into routine screening procedures. However, should a threshold level for the number of oocysts in a water sample be set, this technique could prove to be both useful and rapid. Consultation between the SPDL and other interested parties could address these points. The production of purified, high affinity McAbs raised against defined epitopes expressed on *Cryptosporidium* oocysts would improve this technique considerably.

e) The capability of a cooled slow-scan CCD to detect *Cryptosporidium* oocysts, indicates the enormous potential of these instruments in a sensitive, reliable, rapid detection technique.

2) Viability Assay

Inclusion/exclusion of the fluorescent vital dyes DAPI and PI provides a method of assessing the viability of *C.parvum* oocysts that is sensitive, reproducible, and "user friendly". For these reasons, and because it can be applied to small numbers of oocysts and individual oocysts, it is considered superior to the more traditional method of *in vitro* excystation for assessing viability. This fluorogenic viability assay is based on the principle that dead oocysts, but not viable oocysts, will include PI which fluoresces red [PI(+)], whereas viable oocysts will not only exclude PI [PI(-)], but will also include DAPI which fluoresces sky-blue [DAPI(+)/PI(-)]. Using the *in vitro* excystation protocol described herein, oocysts which exclude both fluorescent dyes [*i.e.* DAPI(-)/PI(-)] are not considered to be viable and they will not excyst over 4 h *in vitro*. However, following a further stimulus (for example, pH), DAPI(-)/PI(-) oocysts can be converted to DAPI(+)/PI(-) oocysts. DAPI(+)/PI(-) oocysts are viable and are capable of excystation *in vitro*. Thus both DAPI(+))/PI(-) and DAPI(-)/PI(-) oocysts should be regarded as being potentially infective. However, knowledge of the correlation between viability and infectivity is minimal and further research in this area is indicated.

3) Aging of *Cryptosporidium* oocysts under various environmental pressures

a) *Cryptosporidium* oocysts are resistant to the majority of environmental pressures that they are likely to encounter, including sea water.

b) While desiccation appears to be 100% lethal, a small proportion will survive long periods of being frozen. Neither frozen food nor ice should be considered without risk in the contraction of cryptosporidiosis.

c) Water treatment processes, while being of importance in the removal of oocysts from water, are unlikely to kill any oocysts at the pH used.

d) Faeces appear to confer some measure of protection on oocysts. Rapid dispersal of faecal material is recommended to maximise aging and vulnerability of oocysts.

4) Biochemical analysis of *Cryptosporidium* oocysts

a) The outer surface of *Cryptosporidium* oocysts expresses few proteins and is predominantly carbohydrate in composition. A polysaccharide containing GalNAC is hypothesised to be the major constituent present in oocyst walls.

b) Putative biochemical markers for viability have been identified and require further research.

Further work is necessary to draw together the strings of this DoE funded research, especially in areas such as:

a) magnetisable particle technology,

b) the verification of numbers,

c) the usefulness of these techniques in waters of varying qualities.

d) the relationship between viability as defined by fluorogenic dye inclusion/exclusion and infectivity, and the use of such a viability assay in identifying potential agents and processes which might be capable of rendering viable oocysts, as defined by this assay, non-viable.

Whereas it appears that the above techniques (a, b and c) can be included into routine benchwork (and have been at the SPDL), further collaboration between the SPDL and other interested parties in the water industry is recommended. The incorporation of the viability assay with a detection assay, either fluorogenic or luminescent; and the use of FACS or CCD machines in the detection of oocysts should be considered. The SPDL with its contacts in biotechnology, and its in depth knowledge of the viability assay would be pleased to be involved further in this research programme.

ACKNOWLEDGEMENT

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INTRODUCTION

Despite the wealth of scientific literature generated about the coccidian parasite *Cryptosporidium* spp. since the first cases of human cryptosporidiosis were reported (Miesel *et al*, 1976; Nime *et al*, 1976) it was not until 1990 that the public health significance of infection was officially recognised in the UK when an expert committee (the Badenoch Committee) recommended to the British Government that cryptosporidiosis should be a "reportable" disease (HMSO, 1990). In Scotland, both human cryptosporidiosis and giardiasis became repaortable diseases in January 1989.

The global impact of cryptosporidiosis, in both human and economic terms, must be enormous. In well-nourished, immunocompetent individuals the disease is self-limiting. In malnourished, immunosuppressed individuals the disease is life-threatening and there is no known pharmaceutical cure. In countries where malnutrition is rife the cumulative and synergistic misery caused by this parasitic infection is incalculable. Wealthier nations also cannot afford to be complacent, especially with the world-wide spread of HIV infection. Despite this, much basic research on *Cryptosporidium* has yet to be accomplished.

In the last ten years, the importance of waterborne transmission of cryptosporidiosis has been accepted worldwide. Six outbreaks of waterborne cryptosporidiosis have been documented, four of which occurred in the UK and two of which occurred in the USA (HMSO, 1990), and in one an estimated 13,000 persons were affected, indicating the large numbers of consumers at risk of contracting disease from contaminated potable water. In two of the outbreaks, post-treatment contamination was assumed to be the cause, but two outbreaks have occurred when water treatment works produced final water which met the accepted standards of water quality for that country (Smith and Rose, 1990). In three of these outbreaks, anthropozoonotic transmission from slurry or muck spreading has been tentatively suggested. Oocysts can gain access into potable water systems by a variety of routes, and documented waterborne outbreaks have demonstrated that they can cause infection following the consumption of filtered chlorinated water, indicating that they can pass through filtration systems currently in use and are resistant to the standard chlorination regimes used in the water industry.

That oocysts can be detected in chlorinated final water in the absence of bacteria such as *Escherichia coli*, which is often used as an indicator organism of faecal contamination by the water industry, is of concern, and points to the fact that conventional bacterial indicator organisms are of doubtful use to the water industry as indicators of

parasitic protozoan contamination. Although oocysts are excreted in large numbers by infected mammals, their dilution and settlement in bodies of water, and their incomplete removal by water treatment processes indicate that oocysts normally occur in low numbers in potable water (Smith and Rose, 1990). The identification of protozoan cysts and oocysts is a skill that is being encouraged in most water supply microbiological laboratories, at present. Because the minimum infective dose to human beings is thought to be small (between 10 - 100 oocysts, and possibly less than 10 oocysts), it is important to be able to identify small numbers of oocysts accurately.

Detection of *Cryptosporidium* oocysts in water supplies is, at present, a time-consuming, labour-intensive and tedious job. The standard method involves filtering between 500-1000 litres through a cartridge filter of 1 μm nominal pore size, and to release the entrapped oocysts, the filter matrix is cut, teased apart and eluted with up to four litres of weak detergent solution. The resulting suspension is centrifuged, the pellet clarified by a flotation technique using a 1.18 specific gravity sucrose solution on which oocysts float. Oocysts extracted this way can be visualized microscopically following the addition of fluorescently labelled monoclonal antibodies specific for epitopes on/in the oocyst wall. Confirmation of the putative oocyst is accomplished by the identification of organelles (*e.g.* sporozoites, nucleii) within the oocyst by Nomarski differential interference contrast microscopy (DIC). The method requires not only dedicated equipment but full-time, well-trained personnel. Development of methodology that would reduce the potential for inaccuracies inherent in the present detection system, the man-hours, the level of necessary personnel training, and the turn-around time for reporting results would be invaluable.

Current detection methods rely on physical concentration and separation of oocysts, and involve concentrating putative oocysts ten thousand-fold, by centrifugation, from an original filter-eluate volume of 4 to 5 litres to approximately 500 microlitres, which is time-consuming. Alternative concentration methods, such as magnetisable particles, could prove to be as efficient, if not more so, and more rapid than centrifugation. Magnetisable particles are uniformly sized polystyrene particles which contain Fe_2O_3 throughout their core. This bestows superparamagnetic properties upon the particles and allows consistent and reproducible reaction to a magnetic field. Antibody can be covalently-linked to the surface of magnetisable particles to enable the immuno-magnetic separation of either solid-phased (*e.g.* cellular) or soluble antigens. Immuno-magnetic separation, based either on positive or negative selection of the target cell, can provide from 60% to 99.98% enrichment for certain target cells. Oocysts in water-related samples are identified following the application of a monoclonal or polyclonal antibody

which recognises epitopes on/in the oocyst wall, and which is labelled either directly or indirectly (*via* a second antibody) to a fluorogen such as fluorescein isothiocyanate (FITC). Such fluorescent antibody stains have proven invaluable in the detection of waterborne infectious disease agents and their inclusion into standard protocols has increased the sensitivity of detection of such protocols. Colorimetric enzyme-labelled antibody stains are as sensitive and robust as fluorescent antibody stains however, enzyme-labelled antibody stains offer the possibility of increased sensitivity when light-emitting substrates or luminogens rather than colorimetric substrates or chromogens are used. Rapid and sensitive quantifications of the widely used enzyme, horseradish peroxidase, is possible with several chemiluminescent and bioluminescent systems (Thorpe and Kricka, 1986). A modified, enhanced chemiluminescent assay, developed at SPDL (Smith and Campbell, 1991, Smith *et al*, 1991), has been used in an attempt to identify small numbers of oocysts without microscopic analysis.

A fluorescent monoclonal antibody (FITC-McAb), reactive with epitopes on the oocyst wall, can be bound onto oocysts and a secondary (biotinylated anti-FITC) McAb attached to the primary McAb. This step is an amplification stage which does not impair the fluorescence emission of the primary (FITC labelled anti-*Cryptosporidium*) McAb. An enhanced chemiluminescence reaction involving streptavidin-peroxidase and the sequestration of luminol to emit photons of light can be employed. The light emitted can be subsequently detected using X-ray film, photomultipliers and charge couple devices (CCD). Environmental waters containing oocysts have been tested to assess the usefulness of these systems.

Determination of the viability of the transmissive stages (cysts and oocysts) of waterborne protozoan parasites has received some attention in recent years. The viability of *Giardia* spp. cysts has been assessed by the methods of a) *in vitro* excystation (Bingham and Meyer, 1979; Rice and Schaefer, 1981), b) exclusion/inclusion of various vital dyes (Bingham *et al*, 1979; Hudson *et al*, 1988; Jones and Senft, 1985; Kasprzak and Majewska, 1983; Schupp and Erlandsen, 1987; Smith and Smith, 1989) and c) infectivity of animal models (Roberts-Thomson *et al*, 1976; Schupp and Erlandsen, 1987).

Until more recently, determination of the viability of *Cryptosporidium* has received less attention. However, with the occurrence of documented outbreaks of cryptosporidiosis in the USA and UK (HMSO, 1990; Smith and Rose, 1990), more attention has been focussed upon the development of a reproducible, sensitive, "user-friendly" assay which can be applied to small numbers of oocysts. As the number of oocysts detected following outbreaks of cryptosporidiosis associated with potable water has been low (Smith, 1990) the application of such an assay to

small numbers of, or individual, oocysts is important. Such an assay would not only have an obvious applied use in the water industry, but would also be a valuable tool in further research.

Other than infectivity studies, which as well as being cumbersome and expensive, are inappropriate for assessing the viability of small numbers of oocysts, the viability of *Cryptosporidium* spp. oocysts has been determined by quantifying excystation *in vitro* under a defined excystation protocol. Unfortunately this method has been demonstrated to give variable results for the same pool of oocysts (Smith *et al*, 1989a). This is likely to be because quantification of *in vitro* excystation can be difficult and time consuming, and determination may be subjective. Likewise, accurate quantification of sporozoite numbers post-excystation is difficult due to their extreme fragility. Slight differences in excystation solutions might also influence excystation efficiency.

In our research, excystation time trials were initially conducted in order to determine the dynamics of *in vitro* excystation. A range of fluorogenic vital dyes have been tested on an inclusion/exclusion & morphological basis to investigate whether any of these correlated with maximised excystation *in vitro*. It is important that any correlation should remain valid even if the viability of the oocysts is altered by harsh treatments.

Although the hardiness and resistance of *Cryptosporidium* oocysts is widely acknowledged (Blewett, 1989), there is a paucity of detailed studies of the effects of various environmental pressures on the aging of oocysts. By following the aging of oocysts under stresses such as freezing, processes used by the water industry, and by placing oocysts in "natural situations" rather than laboratory mock-ups, the duration of potential threats of infection can be assessed realistically and measures by which this might be reduced can be identified.

In most parasitic infections, the parasite outer surface plays an important role in the survival of the organism. This vital interface maintains the integrity of the parasite against attack from various destructive host mechanisms and degradation by the environment. Therefore, the study of the organisation and function of the outer surface of *Cryptosporidium* spp. oocysts is particularly relevant. Complex carbohydrates play a crucial role in biology by conferring specificity to structures in which they occur. Antigens expressed on the surface or plasma membrane of parasites have been considered important for the induction of a protective immune response, as target molecules for cytotoxic immune mechanisms and drugs and in diagnostic tests. In addition, cell surface glycoconjugates are considered to be of prime importance in a variety of surface-associated phenomena such as intercellular adhesion (Yamada and Pastan, 1975; Hynes, 1974) and growth control (Vaheri *et al*, 1972). A more detailed knowledge of

carbohydrate expression on oocyst outer surfaces should lead to a more critical understanding of the function of these surface moieties. Some biochemical properties of the oocyst wall have been characterised with respect to the carbohydrate and protein containing components.

Biotin is a 244 Dalton vitamin which can be conjugated to many compounds, and conjugated compounds can react with several molecules of biotin. The biotin molecules, in turn, can bind to avidin, a glycoprotein in egg white, or streptavidin, a similar reacting protein from *Streptomyces avidinii* which contains no carbohydrate. Each streptavidin molecule can bind up to four biotin molecules. This phenomenon greatly increases the sensitivity of many assay procedures. The avidin-biotin interaction is the strongest known non-covalent biological reaction (K_D = 10^{-15} M) between protein and ligand. The bond formation between biotin and avidin is very rapid and once formed is unaffected by extremes of pH, organic solvents and other denaturing agents.

N-hydroxysuccinimide (NHS) esters react with unprotonated primary amines (Anderson *et al*, 1964). In biological networks these are exposed principally as lysine epsilon groups and the NHS ester of biotin has been employed to label exposed primary amines expressed within components of parasite surfaces (LaRochelle and Frochner, 1986; Gardiner *et al*, 1987). Periodate treatment of exposed sugars oxidises vicinal dihydrols of the reducing terminal of an oligo- or poly-saccharide. These oxidised carbohydrates can be labelled with biotin using biotin hydrazide (BHZ). Detection of these biotinylated surface molecules using avidin conjugated horseradish peroxidase, substrate and chromogen, following Western blotting, has been reported to be as sensitive as comparable radiolabelling methods (Alvarez *et al* 1989). We have covalently linked biotin molecules to exposed primary amines or terminal sugars expressed on *Cryptosporidium* oocysts, analysed the spectrum of the surface exposed components, following western blotting and exposure to X-ray film using an enhanced chemiluminescent detection system (the BEC system) developed at SPDL (Smith and Campbell, 1990; Smith *et al*, 1990). The biotin labelling methods used yields stable compounds which can be viewed *in situ* following the addition of avidin-fluorochromes.

Lectin binding and biotin labelling analysis of oocysts from various sources have been used to determine whether compounds could be selective enough to identify any biochemical difference between oocyst "strains" and to determine whether a surface marker for viability exists.

MATERIALS & METHODS

Source of oocysts

C.parvum oocysts used in this study were obtained from the following sources:-

1) Purified cervine/ovine oocysts (c/o oocysts): purchased from the Moredun Institute (M.I), Edinburgh, where they had been purified. This strain, originally isolated from deer faeces, has been passaged in sheep by the M.I.

2) Human oocysts in faecal samples: isolated from different human faecal samples submitted for routine examination to SPDL, Stobhill Hospital. The oocysts were not pooled but kept as separate isolates.

3) Bovine oocysts in faecal samples: isolated from bovine faecal samples obtained from either a study farm by Glasgow Veterinary School or from the M.I.

4) Purified bovine oocysts: purchased from the M.I. in Edinburgh where they had been purified. This isolate has been passaged in calves by the M.I.

Purification of oocysts

Oocysts purchased from the M.I. had been purified using a semi-automated method. This method involves incubation in 1% SDS and both acid sedimentation and sucrose flotation (S.Wright, pers.comm.). The oocysts were obtained suspended in phosphate buffered saline, pH 7.2 (PBS) containing 100 U penicillin/100μg streptomycin per ml.

Oocysts obtained from bovine and human faecal samples were purified at the SPDL, as follows. Faecal samples (~5g) were made up to 50ml with reverse osmosis (RO) water, thoroughly vortexed on a whirlimixer, and centrifuged at 900*g* for 5 min. The supernatant was discarded by aspiration. This washing procedure was repeated between 3-5 times until the supernatant was clear. The pellet was then resuspended in 10ml RO water and overlaid with an equal volume of diethyl ether. After thorough mixing by shaking, the samples were centrifuged at 900*g* for 5 min, and the supernatant and fat layers discarded by aspiration. The pellet was washed twice in RO water as above, to remove traces of diethyl ether. Further purification of oocysts was performed by resuspending the pellet in 10ml of RO water which was underlayed with a sucrose solution of a specific gravity 1.18 when cold, and centrifuging at 900*g* for 15 min. The interface was recovered, diluted with RO water and washed. Oocyst purification on sucrose was repeated until the oocyst suspension was considered to be free from excess contaminating matter. All oocyst suspensions were stored at 4°C in RO water, and routinely plated out for bacterial

and fungal contaminants.

Rapid Detection Techniques

Concentration of oocysts using magnetisable particles

a) Concentration of oocysts using *anti-Cryptosporidium* McAb-coated magnetisable particles

Monosized polystyrene particles containing Fe_2O_3, 4.5 µm diameter, were purchased from Dynal (UK) Ltd. 500 µl of a homogeneous suspension of M-450 beads were incubated with 500 µl of *anti-Cryptosporidium* McAb (IgM isotype) at a concentration of 150 µg/ml in 20 mM Borate buffer pH 9.5. Beads and antibody were incubated at room temperature for 24 h by slow end-over-end rotation on a Matburn rotator. The magnetic particles were concentrated by the application of a magnetic field, the supernatant discarded and the magnet removed. Particles were resuspended in PBS (0.50 mM, pH7.2) containing 0.1% bovine serum albumin (PBS-BSA), rotated for 5 min, magnetised and the supernatant discarded. This procedure was repeated once, then the beads were resuspended twice in PBS-BSA buffer and rotated each time for 30 min. The beads were finally suspended in PBS-BSA to a final concentration of approximately 4×10^8. 6×10^6 antibody-coated beads were incubated with 1.5×10^5 suspended oocysts (a ratio of 40 : 1; beads to target cells), incubated at 37°C for 30 min, magnetised and the supernatant discarded. This washing step was repeated twice in order to separate oocysts which had bound onto magnetisable particles from unbound oocysts. The efficiency of attachment was assessed by counting the number of oocysts recovered attached onto the magnetisable particles under Nomarski optics at a magnification of x 400.

b) Concentration of oocysts using anti-FITC McAb-coated magnetisable particles and FITC-labelled *anyi-Cryptosporidium* McAb-coated oocysts

A suspension of oocysts (10^5) in 500 µl PBS was reacted with 500 µl of FITC-labelled *anti-Cryptosporidium* McAb, diluted optimally, at 37°C for 30 min and the excess FITC-McAb removed by thorough washing.

Biotinylation of anti-FITC McAb: A monoclonal antibody raised against fluorescein isothiocyanate (FITC) was obtained as a gift from DMRQC, PHLS, Colindale, London, UK. and biotinylated using a modified method of Pohlit *et al* (1979).

6×10^6 (8.57 µl) of streptavidin-coated beads, 2.8 µm diameter, (M-280, Dynal (UK) Ltd.) were incubated with 2 µl biotinylated anti-FITC McAb (1 mg protein/ml) at 37°C for 30 min and the excess biotinylated McAb removed from the magnetisable beads by magnet. FITC-McAb-labelled oocysts were mixed, in suspension, with biotinylated

anti-FITC-McAb-coated beads at a ratio of 40 : 1 (6 x 10^6 beads to 1.5 x 10^5 target cells) and incubated at 37°C for 10 min with gentle shaking. The magnetisable particles were concentrated by magnet and the supernatant discarded. This washing step was repeated twice in order to separate oocysts which had bound onto magnetisable particles from unbound oocysts. Bound oocysts were resuspended in 1 ml PBS and the efficiency of attachment assessed by counting the number of fluorescent oocysts recovered which were attached onto the magnetisable particles.

Fluorescent imaging using charge couple devices

Charge couple device (CCD) image intensifying systems are capable of detecting very low light emissions over a broad spectral range. Two commercially available CCD image intensifying systems were assessed for their ability to detect low numbers of *Cryptosporidium* oocysts. The systems tested were the Astromed 2200 system, a cooled slow scan CCD (Astromed Ltd., Cambridge, U.K.) and the Photonic Darkstar 800 system (Photonic Science Ltd., Sussex, U.K.) Both systems were coupled to an Olympus BH2 fluorescent microscope and the fluorescent emission computer-imaged using their respective software.

Purified oocysts were labelled with a commercial fluorescein labelled monoclonal antibody (FITC McAb) (Northumbria Biologicals Ltd., Cramlington, UK) by incubating approximately 10^4 oocysts in 100 µl PBS with 50 µl of the stock FITC McAb for 30 min at 37°C . The FITC McAb labelled oocysts were then washed three times in 50 mM PBS.

Oocysts stained with the vital dyes DAPI and PI as described in the viability assay to enhance the oocyst morphology were examined in conjunction with the FITC-McAb in a fresh wet mount.

The fluorescent oocysts were focused onto either CCD both containing an array of approximately 385 x 578 picture elements (pixels) each having dimensions 22 µm x 22 µm. The Astromed 2200 system was cooled to approximately 140 K with liquid nitrogen. The Photonic system was not cooled but consisted of a more automated system including CCD camera with an auto focus and computer controlled microscope stage. In both cases the image was computer generated and displayed on a video monitor.

Both systems were tested to determine the minimum magnification necessary to definitively detect fluorescently labelled oocysts.

The feasibility of automating the screening for fluorescently labelled oocysts was assessed using the Photonic system.

Enhanced chemiluminescent detection

Primary Antibody labelling: Purified oocysts were labelled with the Northumbria FITC McAb as described above.

Anti-FITC multiplication step: The monoclonal antibody raised against fluorescein (obtained as a gift from DMRQC, Colindale) was biotinylated using a modified method of Pohlit *et al* (1979). The anti-FITC McAb was incubated at the optimum dilution (1:200 of 1µg/µl) for 30 min at 37°C with the FITC McAb labelled oocysts. Following incubation the oocysts were washed three times in PBS.

Streptavidin-Peroxidase labelling: The above FITC/Anti-FITC-biotin labelled oocysts were labelled with streptavidin conjugated to horseradish peroxidase (STR-HRP) by incubating in 1µg STR-HRP in PBS for 30 min at room temperature. The oocysts were then washed three times in PBS. Oocysts were heat fixed onto multi-spot slides by air-drying followed by passing the slide six times through a blue flame. Earlier research at SPDL has shown heat-fixation to be the optimum method for ensuring the retention of oocysts onto slides (McRae, 1990).

Visual confirmation of the numbers of oocysts retained on slides: The FITC fluorescence from the Northumbria McAb is not quenched by the subsequent labelling steps. The wells were viewed for fluorescent oocysts using an Olympus BH2 microscope, equipped with a blue filter block (490 nm excitation, 520 emission) and the numbers observed noted.

Chemiluminescence detection system for HRP conjugates: A modified 4-iodophenol-enhanced chemiluminescence detection system as described by Schneppenheim and Rautenberg (1988) was used. This was prepared by adding 40 mg luminol to 100 ml of 150 mM NaCl in 50 mM Tris-HCl, pH 8.0, to which was added 10 mg 4-iodophenol dissolved in 1 ml DMSO. Immediately before use 0.01% H_2O_2 (32 µl of 30%) was added.

The method is described visually in Figure A, overleaf.

The effectiveness of three types of imaging systems was assessed for their effectiveness in identifying low numbers of *Cryptosporidium* oocysts. These were:

1. X-ray film
2. Photomultiplier tube
3. Cooled slow scan CCD camera

X-ray film: Labelled oocysts were aliquoted onto wells of slides in known numbers, and air-dried before the addition of 50 µl the chemiluminescent detection system. The slides were then placed directly onto X-ray film in a

custom built film-holder for varying periods of time from 10 sec to 5 min.

Photomultiplier: A flow microplate luminometer (loaned by Flow Labs UK Ltd) was used which could detect light emission from individual wells of a standard 96 well ELISA plate. Serial dilutions from 100 labelled oocysts were made in a white 96 well luminometer ELISA plate. 100 µl of the chemiluminescent detection system was injected automatically and the light emission detected was recorded at the peak light emission time, typically 30 sec. The test was repeated 11 times and the mean theoretical number of oocysts present calculated and plotted against the mean number of arbitrary light units detected. Background luminescence was automatically subtracted from the light units measured.

CCD camera imaging: Chemiluminescence was imaged electronically using the Astromed 2200 imaging system. Four slides with air dried FITC/anti-FITC-biotin labelled oocysts in each well, were analysed at Astromed (UK) Ltd. There the STR-HRP labelling step was performed as above. 50 µl of the chemiluminescent system was applied to each well before the whole slide was focused onto the CCD. The average photon counts over the light emitting area of the well was recorded.

Environmental samples: 21 water samples from a variety of sources, including raw water from rivers and reservoirs and final treated water from a variety of treatment works and storage reservoirs within Strathclyde region were obtained. These samples had previously been confirmed positive or negative for *Cryptosporidium* spp. oocysts by the SPDL, using the method of Smith *et al* (1989b).

25 µl aliquots from samples numbered 1-13 were heat fixed. The endogenous peroxidase activity of contaminating micro-organisms was then destroyed by immersing the slide in acid methanol for 10 min. The labelling procedure above was performed using 25 µl of the appropriate ligand, diluted in PBS with 0.01 % tween-20 which contained 5 % dried skimmed milk (PBS-T-DSM) (37°C, 30 min) in a moist chamber. Three immersions in PBS was used for the washing step. The slides were air dried before the addition of the chemiluminescent detection system as before.

Samples numbered 14-21 were labelled as described for the purified oocyst preparations but in PBS-T-DSM in all incubation steps.

All environmental samples were imaged using X-ray film.

Viability Assay

Testing of vital dyes

Initially a range of fluorogenic vital dyes (Table 1) were tested on an inclusion/exclusion and morphological basis to investigate whether any of these correlated with maximised excystation. Of these dyes, only DAPI and PI are discussed in detail in this report as we were unable to correlate any of the other dyes with excystation.

Table 1. Vital dyes tested as indicators of viability

Acridine orange
7-Aminoactinomin D
1-Arginine 4-Methoxy-β-naphthylamide
Auramine O
Nα-Benzoyl-1-arginine 7-amino-4-methylcoumarin
Calcein blue
5(6)Carboxyfluorescein diacetate
Coumarin 151
Dansyl lysine
4'6-Diamidino-2-Phenylindole (DAPI)
Dihydroethidium
Ethidium bromide
Ethidium monoazide
Fluorescein diacetate
Fluorescein dilaurate
Fluorescein isothiocyanate
Fluorescein isothiocyanate diacetate
Hoescht 33258
Hoescht 33342
N-Hydroxysuccinimidobiotin
4-Methylumbelliferone heptonate
Merocyanine 540
Propidium iodide (PI)
Rhodamine 123
Rhodamine 6-G

Oocyst Incubation with DAPI & PI

PI is not considered to be able to traverse intact cell membranes. It has been reported that only cells with disrupted or broken membranes can be stained with PI (Horan and Kappler, 1977).

DAPI is an AT selective DNA stain. Binding of DAPI to DNA occurs with about a 20-fold fluorescence enhancement (Kubista *et al*, 1987).

Working solutions of both PI and DAPI were stored at 4°C in the dark.

100 µl suspensions of oocysts in Hank's balanced salt solution (HBSS) (2 x 10^4 oocysts/µl) were incubated

simultaneously with 10 µl working solution PI and 10 µl working solution DAPI at 37°C in the dark.

Before viewing by fluorescence microscopy oocysts were washed twice in HBSS and once in Eagle's minimum essential medium containing Hank's salts (HMEM).

Microscopy

10 µl samples of the oocyst suspension were viewed under both epifluorescence and DIC (Nomarski) optics using an Olympus BH2 microscope, equipped with a UV filter block for DAPI and a green filter block for PI.

The proportion of ruptured (ghost), PI positive (PI(+)), DAPI positive-PI negative (DAPI(+)/PI(-)), DAPI negative-PI negative (DAPI(-)/PI(-)), oocysts were quantified by enumerating over 100 oocysts per aliquot sample.

Ghost oocysts were easily visualised under Nomarski optics, being non-refractile apart from the residual body.

PI(+) oocysts fluoresced bright red under the green filter block. Oocysts were considered to be DAPI(+) only if the nuclei of the sporozoites fluoresced a distinctive sky-blue under the UV filter block, and were not PI (+). Those oocysts which were not PI(+) and showed either an even fluorescence, a rim fluorescence or an absence of fluorescence when viewed under the UV filter block were considered to be DAPI(-) (Fig 1).

Excystation protocol

After the dyes had been washed out of the oocyst suspension, the oocysts were resuspended in 100 µl HMEM.

The following excystation protocol was then carefully followed. Fresh solutions of bovine bile (10mg bile/ml HMEM,) and sodium hydrogen carbonate (11 mg $NaHCO_3$/2.5 ml RO water) were made up and 200 µl of the bile solution and 50 µl of the $NaHCO_3$ solution were added to the oocyst suspension which was mixed well before incubation at 37°C.

Time trial

10 µl aliquots of the excystation suspensions were sampled at regular time intervals and viewed by fluorescence and Nomarski microscopy. The proportion of a) ghost (ruptured or totally excysted), b) partially excysted, c) PI(+), d) non excysted DAPI(+)/PI(-) and e) non excysted DAPI(-)/PI(-) oocysts were quantified by the enumeration of over 100 oocysts per sample aliquot. Partially excysted oocysts were easily discernible by the fluorescence of 1 to 3 DAPI stained sporozoite nuclei within the oocyst.

Acid/alkali pretreatment

In order to follow the effect of acid/alkali pretreatments on DAPI(-)/PI(-) and DAPI(+)/PI(-) oocysts, oocysts from

two sources were pooled, one batch being predominantly DAPI(-) (low viability) and the other being predominantly DAPI (+) (high viability). In order to differentiate between the isolates within the pool, the outer walls of the oocysts with the low percentage viability were labelled with avidin fluorochromes.

100 µl suspensions of pooled oocysts were subjected to 1 ml of either 0.1M NaOH or 0.1M HCl at room temperature for 1h. To the control suspension was added 1 ml RO water. Following three washes in HBSS, the DAPI/PI staining protocol was followed as described above. In quantifying the PI/DAPI staining of oocysts, at least one hundred fluorescent oocysts (low viability pool) and one hundred non-fluorescent oocysts (high viability pool) were counted. Similarly, following a subsequent 4 h excystation protocol described above, over one hundred fluorescent oocysts and one hundred non-fluorescent oocysts were counted.

Aging of *Cryptosporidium* oocysts under various environmental pressures

Freezing

100 µl aliquots of oocysts (10^6 oocysts/ml RO water) contained in micro-cryotubes at 4° C were either snap-frozen by being immersed in liquid nitrogen or frozen slowly by being placed in a freezer set at -22°C. In the "slow"-freezing method, 19 oocyst aliquots from the same initial oocyst suspension were frozen periodically over a period of 800 h and defrosted simultaneously to assess the effect of freezing over time.

Oocysts, either snap-frozen or frozen to -22°C in the freezer were thawed at 4°C for between 1 and 2 h. Oocysts were resuspended in HBSS and their viability assessed with DAPI and PI. Randomly selected oocyst aliquots were excysted as described above to ascertain that the viability predicted by the inclusion/exclusion of DAPI and PI correlated with *in vitro* excystation. The viability of the initial, unfrozen oocyst suspension was also assessed.

Dehydration/desiccation

50 µl aliquots of an oocyst suspension (10^6 oocysts/ml RO water) were placed on glass slides and air dried at room temperature (between 18 and 20°C). At noted time intervals over a period of 8 h the oocysts were washed off the glass slides by agitated soaking in 50ml tubes of RO water. The oocysts were pelleted by centrifugation and resuspended in 100 µl of HBSS. The viability of the oocysts was assessed by DAPI and PI. Randomly selected oocyst aliquots were excysted as described above to ascertain that the viability predicted by the inclusion/exclusion of DAPI and PI correlated with *in vitro* excystation. The viability of the initial oocyst suspension was also assessed.

In stools

Stools from three individuals with cryptosporidial infections were stored at 4°C in the dark. At intervals over the course of the investigation, a small portion of the faeces was taken and suspended in 100 µl of HBSS and the viability of the oocysts assessed by inclusion/exclusion of DAPI and PI.

In order to identify oocysts amongst the contaminating faecal material, oocysts were labelled with Northumbria FITC-McAb. *In vitro* excystations were not conducted to correlate with vital dye inclusion/exclusion as this was considered to be impractical.

In laboratory models of water treatment processes

Details (contact times, pH, temperature etc.) of some water treatment processes, in particular liming and alum and ferric floccing, were obtained from Central Scotland and Grampian water boards. Oocyst suspensions (10^6 oocysts/ml RO water) were subjected to scaled down laboratory models of these processes. In some instances the processes were exaggerated (for example increasing contact time and/or concentration of the active agent) so that oocysts were exposed to conditions greatly in excess of those that they would normally encounter in a water treatment plant.

In all cases, oocyst viability was assessed following the treatment process by resuspending the oocysts in 100 µl HBSS and incubating with DAPI and PI. Randomly selected aliquots were excysted, as previously described, in order to ascertain that the viability predicted by inclusion/exclusion of DAPI and PI correlated with *in vitro* excystation. For every treatment investigated, the viability of an appropriate control in RO water was similarly assessed, in triplicate.

Alum floccing: Suspensions of oocysts were subjected to the following treatments and their viability assessed.

1) Aluminium sulphate (5% aluminium) for 1 h at room temperature.

2) Aluminium sulphate (5% aluminium) corrected to pH 6 with lime (10 mM calcium hydroxide), for 1 h at room temperature.

3) Aluminium sulphate (5% aluminium) corrected to pH 6 with 100 mM sodium hydroxide, for 1 h at room temperature.

4) Aluminium sulphate (1.5 ppm and 0.975 ppm aluminium) for 7 min at room temperature and 4°C. This was repeated in triplicate for oocyst suspensions of both high and low viability.

5) Aluminium sulphate (1.5 ppm and 0.975 ppm aluminium) corrected to pH 6 with lime, for 7 min at room temperature and 4°C.

6) Aluminium sulphate (1.5 ppm and 0.975 ppm aluminium) corrected to pH 6 with lime and the polyelectrolyte Wispofloc N., for 7 min at room temperature and 4°C.

Liming and polyelectrolytes: Suspensions of oocysts were subjected to the following treatments and their viability assessed.

1) 0.2% lime for 1 h at room temperature.

2) 0.2% lime corrected to pH 6 with 200 mM hydrochloric acid, for 1 h at room temperature.

3) 5% Wispofloc N for 1 h at room temperature.

Ferric floccing: Suspensions of oocysts were subjected to the following treatments and their viability assessed.

1) Ferric sulphate (5% iron) for 1 h at room temperature.

2) Ferric sulphate (5% iron) corrected to pH 6 with lime, for 1 h at room temperature.

3) Ferric sulphate (5% iron) corrected to pH 6 with sodium hydroxide, for 1 h at room temperature.

4) Ferric sulphate (3.5 ppm and 16 ppm iron) for 1 h and 5 h at room temperature and 4°C.

5) Ferric sulphate (3.5 ppm and 16 ppm iron) corrected to pH 6 and pH 9 with lime, for 1 h and 5 h at room temperature and 4°C.

In semi-permeable containers in selected environments

To enable oocysts to be placed in defined environments with minimal risk of bacterial contamination and to facilitate sampling at regular time intervals, a semi-permeable container was developed. The first container was a heat-sealed triangular semi-permeable membrane (0.2 μm nominal porosity) with a resealable silicone bung glued firmly into one of the corners of the triangle. Although it performed well in laboratory-based experiments it was not sufficiently robust for environmental work. A second model was developed and this container comprised of a solid cylindrical plastic support of approximately 60 mm height and 25 mm diameter. Each side of the cylinder was cut away leaving a frame-like structure of essentially two rings joined to each other by two struts. Either end of the cylindrical frame was sealed with silicon bungs (Neville and More Ltd, Southwater, UK) that were self sealing on

puncture with a hypodermic needle. The cylinder was then wrapped in a single layer of a semi-permeable membrane (Vokes Ltd, Guildford, UK) of nominal porosity 0.2 μm. All joins were thoroughly sealed with a silicon based sealant. Contents of the container would thus be in contact with the environment through the semi-permeable membrane. In addition the container would be easy to sample repeatedly from by accessing the cylinder from either end through the silicon bungs using a hypodermic syringe. Before oocysts were injected into these containers, the containers were thoroughly sterilised in a 1% hypochlorite solution which was then washed out by repeated immersion in RO water.

Oocyst suspensions of both high and low viability were treated overnight with an antibiotic solution before being injected into the containers. The antibiotic solution (4μl gentamycin of 40μg/μl; 20μl flucytosine of 10 μg/μl; 50μl penicillin-G of 1μg/μl; per ml oocyst suspension) had been previously tested and found not to affect the viability of oocyst suspensions. Plating out of aliquots of the untreated oocyst suspension onto blood agar plates with wells containing the antibiotic solution and incubating the plates at 37°C indicated the efficacy of the antibiotic solution. Approximately 2.8×10^7 oocysts of either high or low viability were injected into each container which were then placed in defined environments. Control oocysts were taken from both the low and high viability suspensions and injected into containers which were stored in 4 L of RO water at 4°C in the dark. The RO water was changed at fortnightly intervals.

Four environments were selected which were considered to be of interest in terms of how they would affect the viability of *Cryptosporidium* oocysts over time.

1. Tap water: A light-proof tank was plumbed into the mains water supply, with water entering at the bottom of the tank, and the outflow being at the top of the tank. This enabled a constant throughput of mains tap water to be maintained.

2. River water: The containers of oocysts were placed in a 4 L plastic jar each side of which had been drilled with 10 holes of 20 mm diameter. The 4 L jar was weighted with stones and submerged in a small river. Retrieval of the oocyst containers from the 4 L jar was facilitated by tethering the jar to a convenient tree. Attempts to record temperature by inclusion of a maximum/minimum thermometer within the 4 L jar were thwarted by the thermometer being broken before the first sample was taken.

3. Sea water: Two attempts were made to immerse the oocysts in the sea. A 4 L jar similar to that described for the river samples was utilised on each occasion. However, despite the use of a boat anchor and a shackle embedded in

rock for securing the jar, on both occasions the jar was destroyed by stormy seas.

The viability of oocysts in sea water was assessed by suspending 50 µl of a suspension of oocysts (10^6 oocysts/ml RO water) in 1 ml sea water. After 48 h at 4°C the oocysts were resuspended in HBSS and their viability assessed by inclusion/exclusion of DAPI and PI. The viability of a control (48 h at 4°C in RO water) was assessed in triplicate.

4. Cow faeces: Approximately 25 L of semi-solid cow faeces were obtained from Glasgow Veterinary School. The faeces were stored in a plastic bucket in the dark in an outside store. The containers of *Cryptosporidium* oocysts were buried in the faeces with retrieval being facilitated by placing the containers prior to burial in a cage of tensile stainless steel wire. An electronic temperature probe was also attached to the cage.

At approximately fortnightly intervals samples were taken from each of the containers of oocysts, including the control containers. The containers were retrieved from their particular environments and shaken vigorously to ensure thorough mixing and to reduce the adhesion of the oocysts to the walls of the containers. Approximately 0.25 ml of the oocyst suspension was taken from each container by hypodermic syringe through the self-sealing silicon bungs. The samples were kept as close to 4°C as possible until all the samples had been collected, including the controls. The samples were then resuspended in 100 µl HBSS and their viability assessed on the basis of inclusion/exclusion of DAPI and PI.

Biochemical analysis of *Cryptosporidium* oocysts

Fluorescent lectin binding analysis

Approximately 10^6 purified oocysts of c/o, bovine and human origin were labelled with the vital dyes DAPI and PI as previously described. These oocysts were then incubated with 50 µg/ml of the following lectins in PBS for 30 min at 20°C; wheat germ agglutinin (rhodamine (TRITC) conjugated); *Arachis hypogaea* agglutinin (PNA); *Dolichos biflorus* agglutinin; *Glycine max* agglutinin (SBA); *Lens culinaris* agglutinin (LCA); *Phaseolus limensis* agglutinin; *Ricinus communis* agglutinin (all fluorescein conjugated) and Concanavalin A; Succinyl-Concanavalin A; *Bandeiria simplicifolia* I_4 agglutinin (all biotinylated). These biotinylated lectins require the presence of 0.1 mM Ca^{+2} and 0.1 mM Mn^{+2} for binding. The oocysts were washed three times in PBS. Oocysts treated with the biotinylated lectins were then incubated in 20 µg/ml STR-FITC for 30 min in PBS, before being washed three times in PBS and viewed along with the other lectin treated oocysts. At least 100 oocysts were examined in a fresh

wet mount and assessed for binding of the fluorescent lectins under the blue filter block (green for TRITC-wheat germ agglutinin). Any labelled oocyst had its viability determined by inclusion/exclusion of DAPI and PI. The specificity of lectin binding was studied by incubating the lectins with 100 mM of their competing monosaccharide for 30 min at 20°C before incubating with the oocysts.

The lectin analysis described above was also performed on oocysts (c/o isolate) that had been subjected to an excystation protocol.

Surface biotinylation

Oocyst suspensions of both high and low viability and oocysts that had been freeze-thawed and heat-killed were utilised in this study. Before the labelling procedure oocysts were treated for at least 48 h with the antibiotic solution described for oocysts placed in selected environments.

Surface expressed primary amines were labelled using N-hydroxysuccinimide esters of biotin (NHS-biotin) and biotinaminidocaproate (NHS-lc-biotin). Stock solutions of both these NHS-esters were made up by dissolving 1 mg ml^{-1} in DMSO. Between 10^7-10^8 purified oocysts were incubated with 0.1 mg ml^{-1} of either biotin ester in PBS for 30 min, before being washed five times with PBS.

Periodate-sensitive carbohydrate moieties expressed on the oocysts were oxidised by incubating with 100 mM sodium periodate in PBS for 10 min at 4°C in the dark after which the oxidised trophozoites were washed three times in PBS. The oxidised carbohydrates were labelled with biotin by incubating the oocysts with 10 mM biotin-lc-hydrazide (BHZ) dissolved in PBS for 30 min before being washed five times with PBS. A control experiment, in which the sodium periodate oxidation step was omitted, was included.

D-galactosyl or D-galactosaminyl residues expressed on the oocysts were oxidised using 10 U ml^{-1} galactose oxidase in PBS (37°C, 30 min). The oxidised products were derivatized using 10 mM BHZ, as above.

Fluorescent labelling of biotinylated moieties on oocysts

As a visual check for the successful biotinylation of the oocysts following the above procedures, a proportion of the labelled oocysts were incubated with streptavidin-FITC (STR-FITC) at a concentration of 20 µg ml^{-1} in PBS, (4°C, 30 min), washed three times in PBS before being examined under a Olympus BH2 fluorescent microscope equipped with blue filter block.

A second aliquot of the above biotin-labelled oocysts were DAPI/PI labelled, before the addition of the STR-FITC, to assess any correlation with viability, and viewed as before. In all cases at least 100 oocysts were examined in a

fresh wet mount.

SDS-PAGE

Biotinylated oocyst preparations were boiled in sample buffer which contained 2.5% sodium dodecyl sulphate (SDS) and 5% β-mercaptoethanol, for 5 min, and analysed on SDS-PAGE comprising of a 5% stacking and a 12% resolving gel using the discontinuous buffer system of Laemmli (1970).

These biotinylated moieties were solubilised by two methods:

a. Snap-freezing the oocysts followed by pelleting by centrifugation, and boiling both the supernatant and the oocyst pellet for 5 min.

b. Boiling the oocysts for 5 min in sample buffer.

Solubilised extracts from between 10^6 and 10^7 biotinylated oocysts were loaded on to each track of the gel.

DAPI stained oocysts often exhibit bright rim fluorescence. The nature of the compounds to which DAPI binds was examined as above. After SDS-PAGE the gels were removed from their cassettes and placed on a u.v. light box (Transilluminator), with maximum emission wavelength of 302 nm.

Western blot analyses

Samples fractionated on SDS-PAGE were electrophoretically transferred to pre-wetted Immobilon PVDF Transfer membrane (Pluskal *et al*, 1986) in a Bio-Rad Trans blot-cell at 30 V for 16 h, with cooling coils, following which the blots were blocked in PBS-T-DSM for 1 h at room temperature.

Biotin labelled components were detected following the addition of STR-HRP. Typically, blots were placed in 1 μg ml^{-1} STR-HRP in PBS-T-DSM for 30 min at room temperature. The blots were then washed five times in PBS prior to the addition of the chemiluminescence detection system. All procedures were performed with constant shaking. The relative mobility of each band detected was calculated.

Chemiluminescence detection system for horseradish peroxidase conjugates

A modified 4-iodophenol-enhanced chemiluminescence detection system described by Schneppenheim and Rautenberg (1988) was used (Smith and Campbell, 1991). This was prepared by dissolving 40 mg luminol in 100 ml of 150 mM NaCl in 50 mM Tris-HCl, pH 8.0, to which was added 10 mg 4-iodophenol dissolved in 1 ml DMSO. Immediately before use 0.01% H_2O_2 (32 μl of 30%) was added.

Blots probed with STR-HRP were immersed in the chemiluminescence detection system for 1 min, excess solution

drained off, and the wet blot placed either in a self sealing translucent polythene bag, or wrapped in a translucent polythene sheet and exposed to Dupont Cronex X-ray film for varying lengths of time from 10 sec to 10 min depending on the intensity of the signal.

RESULTS

Rapid detection Techniques

Concentration of oocysts using magnetisable particles

The *anti-Cryptosporidium* McAb-coated magnetisable particles bound an average of 75% of seeded oocysts, whereas the anti-FITC McAb-coated magnetisable particles bound an average of 87% of seeded *anti-Cryptosporidium* McAb-coated oocysts in three separate consecutive trials. No further trials were performed because of the scarcity of the *anti-Cryptosporidium* McAb.

Fluorescent imaging using charge couple devices

The most sensitive image intensifying system was the Astromed cooled slow scan CCD. Oocysts could be detected easily at a final magnification of x 31.25, using the FITC fluorescence. The Photonic system needed a magnification of at least x 62, before the oocysts could be identified. DAPI and PI stained oocysts could readily be imaged using the Astromed 2200 system (100-500 ms exposure). Nuclear staining could not be identified within DAPI(-) oocysts, even on long exposures (up to 10 min).

The Photonics system successfully imaged DAPI, but not PI. The CCD was not sufficiently sensitive for a thorough test of the ability of the automated microscope system to screen for *Cryptosporidium* oocysts.

Enhanced chemiluminescent detection

X-ray film: The results from the multi-spot slide assay for *Cryptosporidium* oocysts detected by FITC/anti-FITC-biotin enhanced chemiluminescence (the BEC system) are shown in table 2. The lowest number of oocysts that could be detected using the BEC system was 3 oocysts.

Table 2. Sensitivity of X-ray film in detecting small numbers of *Cryptosporidium* oocysts by enhanced chemiluminesence.

THEORETICAL DILUTIONS						CONTROL	
50	10	5	2.5	1	0.5	0	
47*	6*	6*	3*	0	0*	0	0
45*	9*	2	2*	0	0	0*	0
30*	11*	3*	0*	0*	0*	0*	0

Numbers stated in the table are the actual numbers observed on subsequent examination of the slides by microscopy. Those marked by an asterisk were detected to be positive by enhanced chemiluminescence. Those without an asterisk were found to be negative. It should be noted that of the controls, known to be negative, two of them were read as positive by chemiluminescence. In subsequent testing of controls of RO water, 6 out of 21 were read as positive by chemiluminescence.

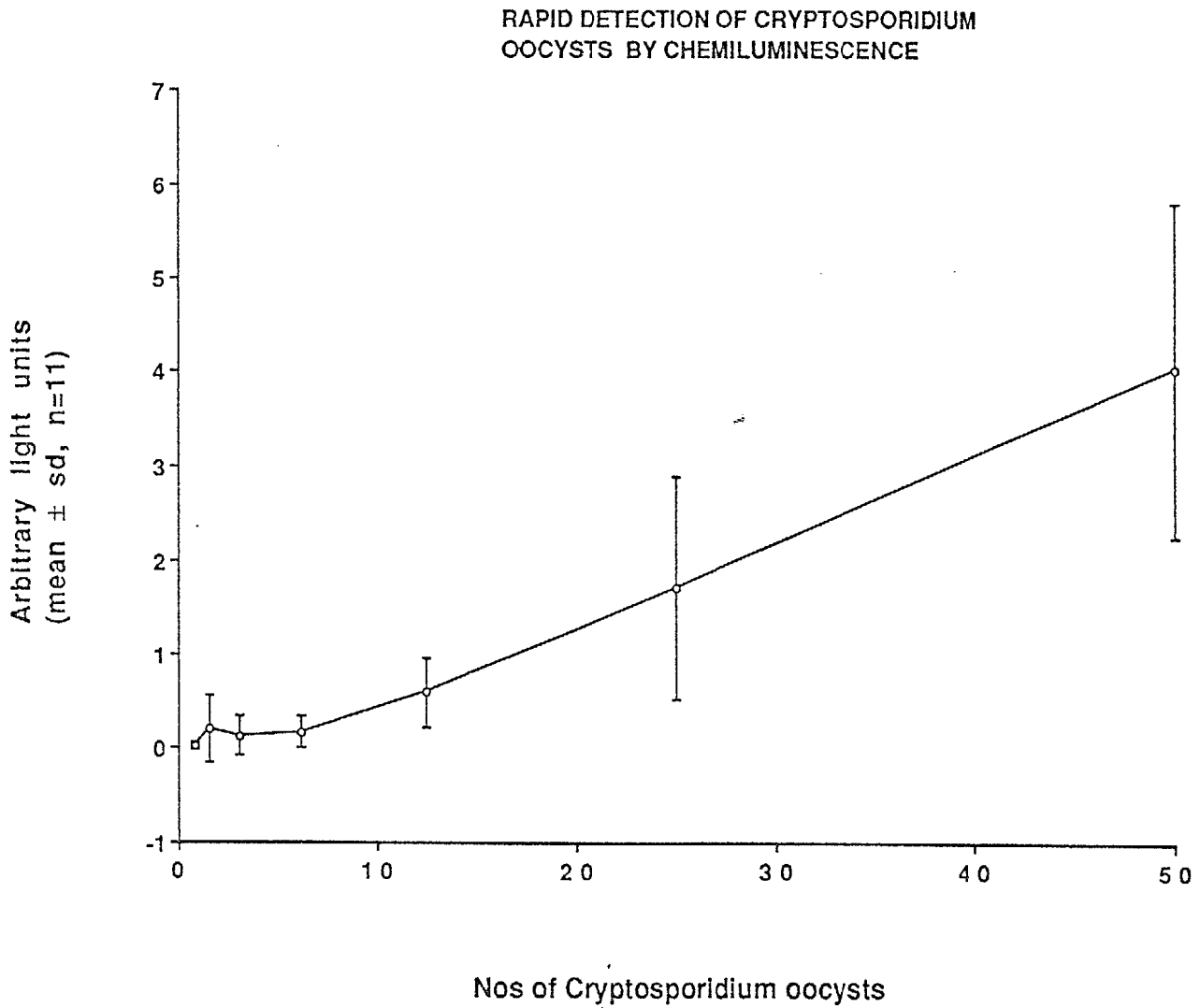

RAPID DETECTION OF CRYPTOSPORIDIUM
OOCYSTS BY CHEMILUMINESCENCE
Arbitrary light units
(mean ± sd, n=11)
7
6
5
4
3
2
1
0
-1
0
10
20
30
40
50
Nos of Cryptosporidium oocysts

Figure 1

Of six controls treated with the labelling protocol, two were detected as positive by the BEC system. In a total of 13 wells where no oocysts were detected by microscopy 6 were detected as positive by BEC.

At the theoretical dilutions of 0.5 and 1 oocyst no fluorescent oocysts could be detected on subsequent microscopy nevertheless, two of these assays were positive by BEC.

At the theoretical dilution of 2.5 oocysts, all 3 wells were positive by BEC however, on subsequent microscopic examination fluorescent oocysts were only detected in 2 of the wells.

At the higher theoretical dilutions (5-50 oocysts per well) all but one of the wells were positive by the BEC system. In this one well which was negative by BEC two putative oocysts were detected on subsequent microscopy. Although both objects were between 4-6 μm in diameter and exhibited weak rim fluorescence no suture or double membrane was discernible by fluorescence microscopy. In addition, the characteristic *Cryptosporidium* oocyst morphology, as seen with Nomarski DIC microscopy (*i.e.* sporozoites and their organelles), was not discernible.

Photomultiplier: The theoretical number of oocysts in each well was plotted against the mean of the light units detected over the area of the well. A theoretical minimum of 6.25 oocysts could be detected reproducibly (Figure 1).

Cooled slow scan CCD: The results of the slide assay suggest a degree of non-specific binding, however there was a graduation in light levels within slides which corresponded to the numbers of FITC labelled oocysts present. The theoretical numbers of FITC/anti-FITC-biotin labelled *Cryptosporidium* oocysts air dried onto wells of multispot slides are shown in table 3. On 2 occasions a single oocyst could be detected. The average photon counts over the area of the well, following STR-HRP labelling and chemiluminescent detection are listed below each number. The actual numbers observed, after subsequent microscopy, are given in parentheses.

Table 3. Chemiluminescence of *Cryptosporidium* oocysts detected by CCD camera.

	Numbers of oocysts per slide well Theoretical dilution number (Actual number counted by microscopy)			
	Well 1	Well 2	Well 3	Well 4
Slide A	20 (21)	20 (27)	10 (12)	10 (9)
CCD reading	17967	16292	15963	13292
Slide B	0 (0)	8 (9)	4 (3)	2 (2)
CCD reading	5954	12403	8667	7070

Slide C	4 (1)	2 (1)	1 (2)	0 (0)
CCD reading	3698	3534	3847	1959
Slide D	10 (17)	5 (2)	2.5 (0)	0 (0)
CCD reading	11816	11491	12696	10757

CCD readings are the average photon counts over the area of the well.

A photograph of the computer generated luminescent image from slide B (table 3) is shown in figure 2 , with the average photon counts next to each well.

Environmental samples: Of the 21 environmental samples examined, 8 were final water and 13 were raw water. Only 7 out of 13 samples screened as negative by fluorescence microscopy at SPDL were found to be negative also by BEC. However, all those samples which were considered to be positive by fluorescent microscopy were also considered positive by BEC, (Table 4). No significant difference was noted between fixed (1-13) and liquid (14-21) samples, and there was also no apparent difference between raw and final water.

Table 4. Rapid detection of *Cryptosporidium* oocysts in environmental samples by enhanced chemiluminesence.

Sample number	Water Type	Oocyst Concentration	Triplicate Assay Result		
Fixed Samples			1	2	3
1	Raw	0.16/L	+	+	+
2	Raw	1.04/L	+	+	+
3	Raw	negative	+	-	-
4	Final	negative	-	-	-
5	Raw	0.09/L	+	+	+
6	Final	0.03/L	+	+	+
7	Final	0.22/L	+	+	+
8	Final	0.72/L	+	+	+
9	Raw	negative	-	-	-
10	Final	negative	-	-	-
11	Final	negative	-	-	-
12	Raw	negative	+	+	+
13	Final	negative	+	+	+
Liquid Samples					
14	Raw	negative	-	-	-
15	Raw	negative	+	+	+
16	Raw	negative	-	+	+
17	Final	negative	-	-	-
18	Final	negative	-	+	-
19	Final	negative	-	-	-
20	Raw	0.52/L	+	+	+
21	Final	0.32/L	+	+	+

Viability assay

It was hypothesised that the inclusion/exclusion of DAPI and PI could be used to predict the viability of *C.parvum* oocysts; the proportion of DAPI(+)/PI(-) oocysts being the predicted viability and the observed or actual viability being calculated from the maximised excystation. Oocysts were considered viable if they ejected at least one sporozoite (totally or partially excysted) under the *in vitro* excystation protocol.

Photographs show PI(+) (Figure 3), DAPI(+) and DAPI(-) (Figures 4 and 5) oocysts. Figure 5 is a CCD image.

For human, bovine and c/o isolates of oocysts, the excystation efficiency maximised after 4 h. The predicted viability appeared comparable to maximum excystation efficiency (Figure 6).

Maximum excystation efficiency (observed viability) correlated with predicted viability gave a correlation coefficient of 0.997. Linear regression analysis provided a mathematical relationship between predicted viability (x) and observed viability (y) of $y = 1.85+0.936X$ (R-squared adjusted for D.F. = 99.3%). Analysis of co-variance demonstrated no significant difference between this relationship ($y = 1.85 + 0.936X$) and the simpler mathematical relationship $y=x$ (Figure 7).

Since a simple correlation between predicted viability (percentage DAPI(+)/PI(-) oocysts using the fluorogenic vital dyes) and observed viability (maximal *in vitro* excystation efficiency) had been derived, it was suggested that those oocysts which excysted over a 4 h excystation protocol would be those which were DAPI(+)/PI(-). Results from the excystation time trial showed that for all oocyst isolates there was a marked decrease in the proportion of DAPI(+)/PI(-) oocysts with time. The proportion of DAPI(-)/PI(-) oocysts however, although fluctuating, did not significantly decrease with time (Figure 8).

Treatment of oocysts with acid and alkali resulted in a marked alteration in oocyst viability (Figure 9). In those oocysts with a relatively high percentage viability, initial treatment with alkali resulted in a marked reduction in viability whereas treatment with acid had a much weaker effect. In those oocysts with a relatively low control viability both acid and alkali treatments increased the percentage viability.

For both oocyst sources both acid and alkali treatment assessed by *in vitro* excystation correlated well with the viability predicted by inclusion/exclusion of DAPI and PI.

Figure 2. Photomicrograph of a computer-generated image from slide B in Table 3. The average photon counts appear next to each of the four wells.

Figure 2

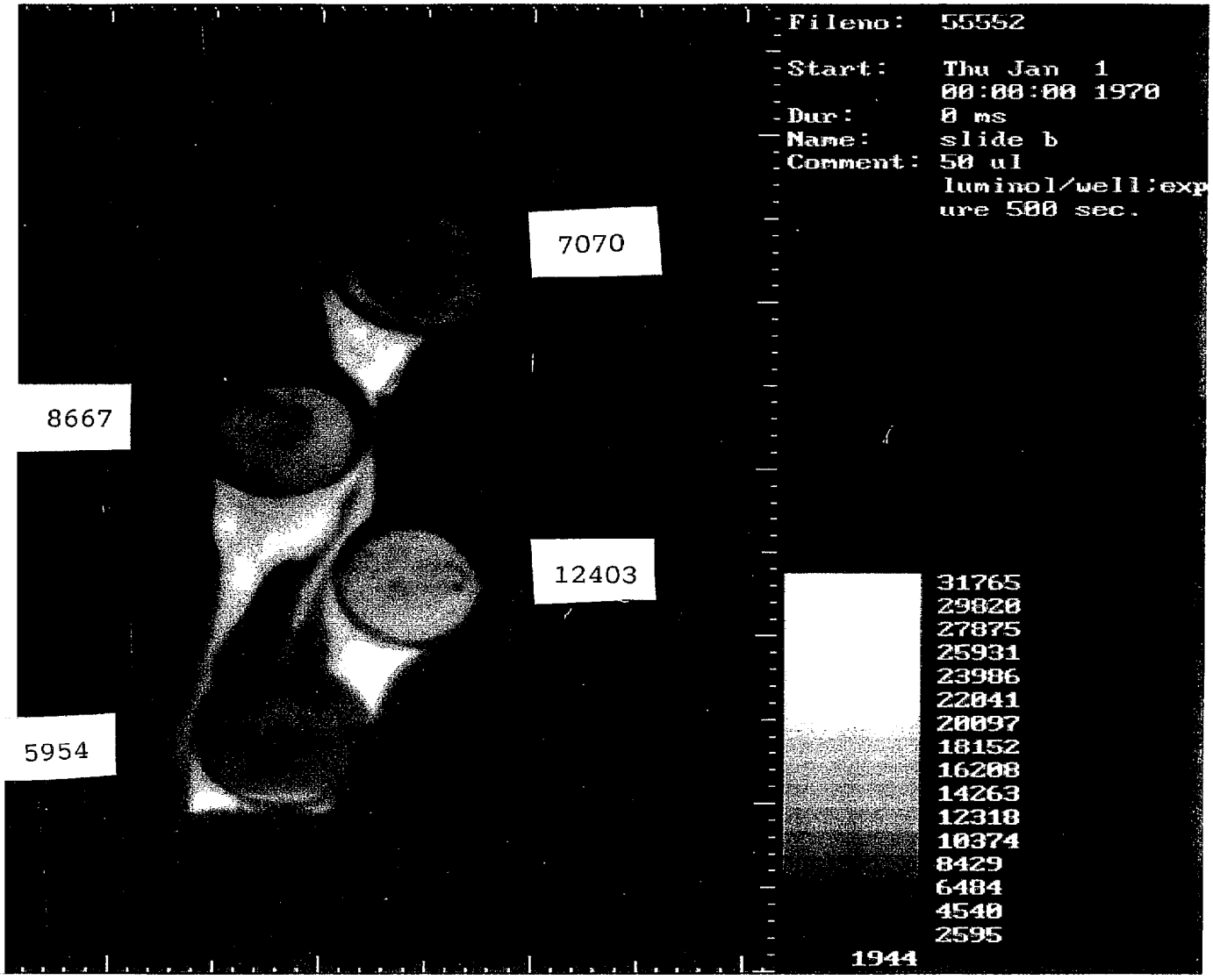

Fileno: 55552
Start: Thu Jan 1 00:00:00 1970
Dur: 0 ms
Name: slide b
Comment: 50 ul luminol/well;exp ure 500 sec.
7070
8667
12403
5954
31765
29820
27875
25931
23986
22041
20097
18152
16208
14263
12318
10374
8429
6484
4540
2595
1944

Figure 3. Photomicrograph of PI(+) oocyst.

Figure 3

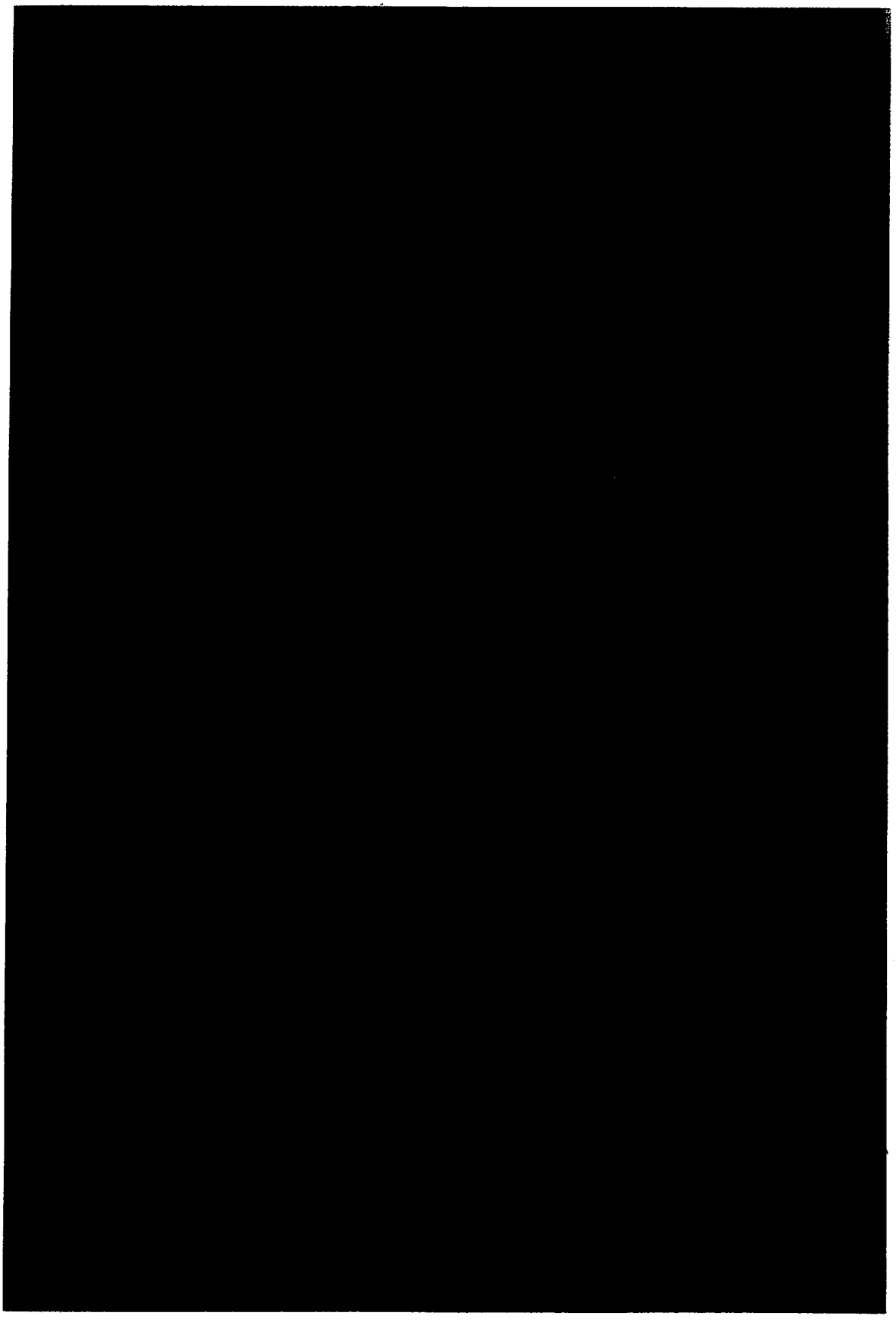


Figure 4. Photomicrograph of both DAPI(-) and DAPI(+) oocysts. Note the pin-point fluorescence emitted by the nuclei of the sporozoites in the DAPI(+) oocyst.

Figure 4

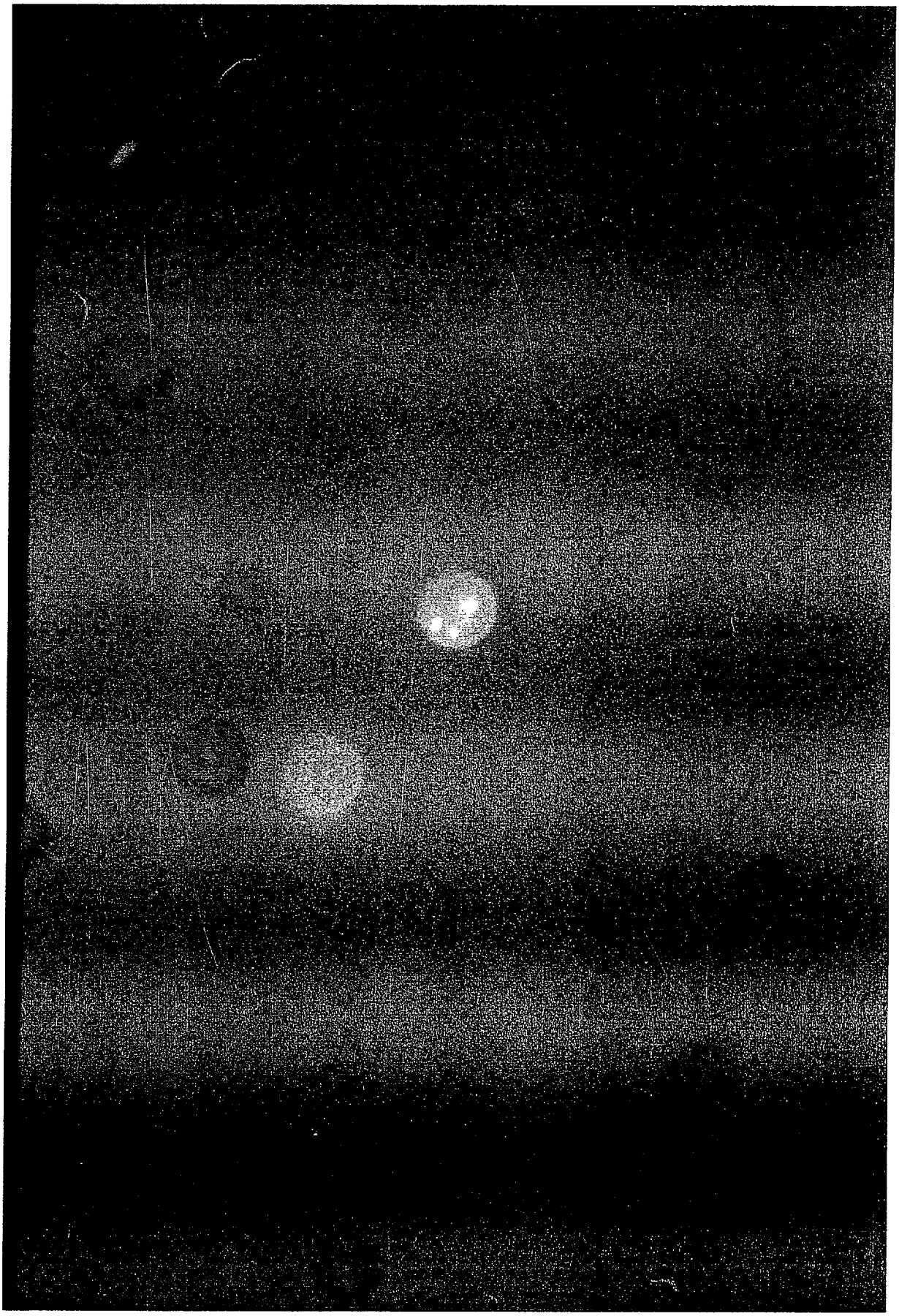


Figure 5. Photomicrograph of a computer-generated image of a DAPI(+) oocyst detected by a cooled slow scan charge couple device. Note the nuclei of the four sporozoites contained within the oocyst fluorescing sky-blue.

Figure 5

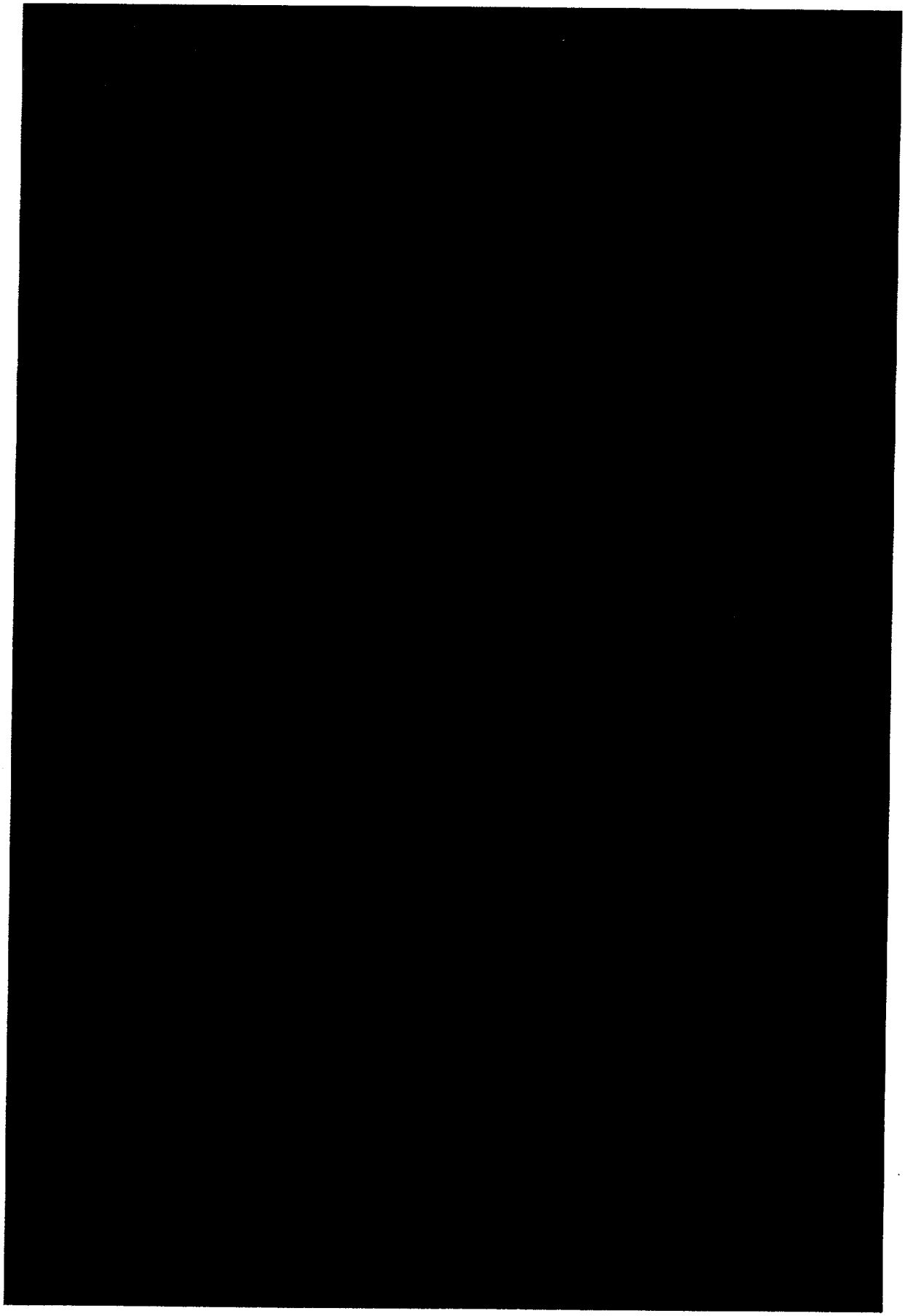


Figure 6

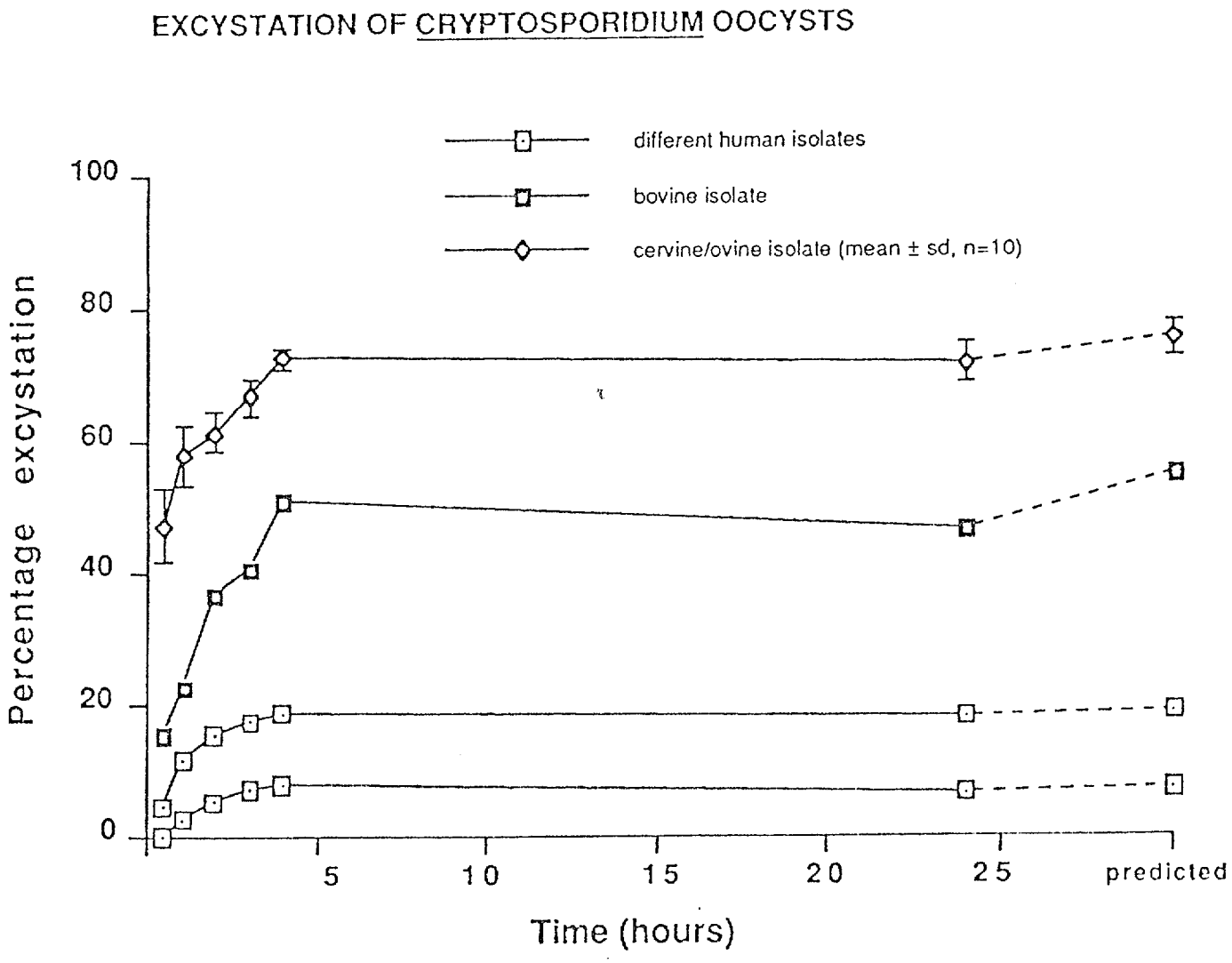

EXCYSTATION OF CRYPTOSPORIDIUM OOCYSTS
different human isolates
bovine isolate
cervine/ovine isolate (mean ± sd, n=10)
Percentage excystation
100
80
60
40
20
0
5
10
15
20
25
predicted
Time (hours)

Figure 7

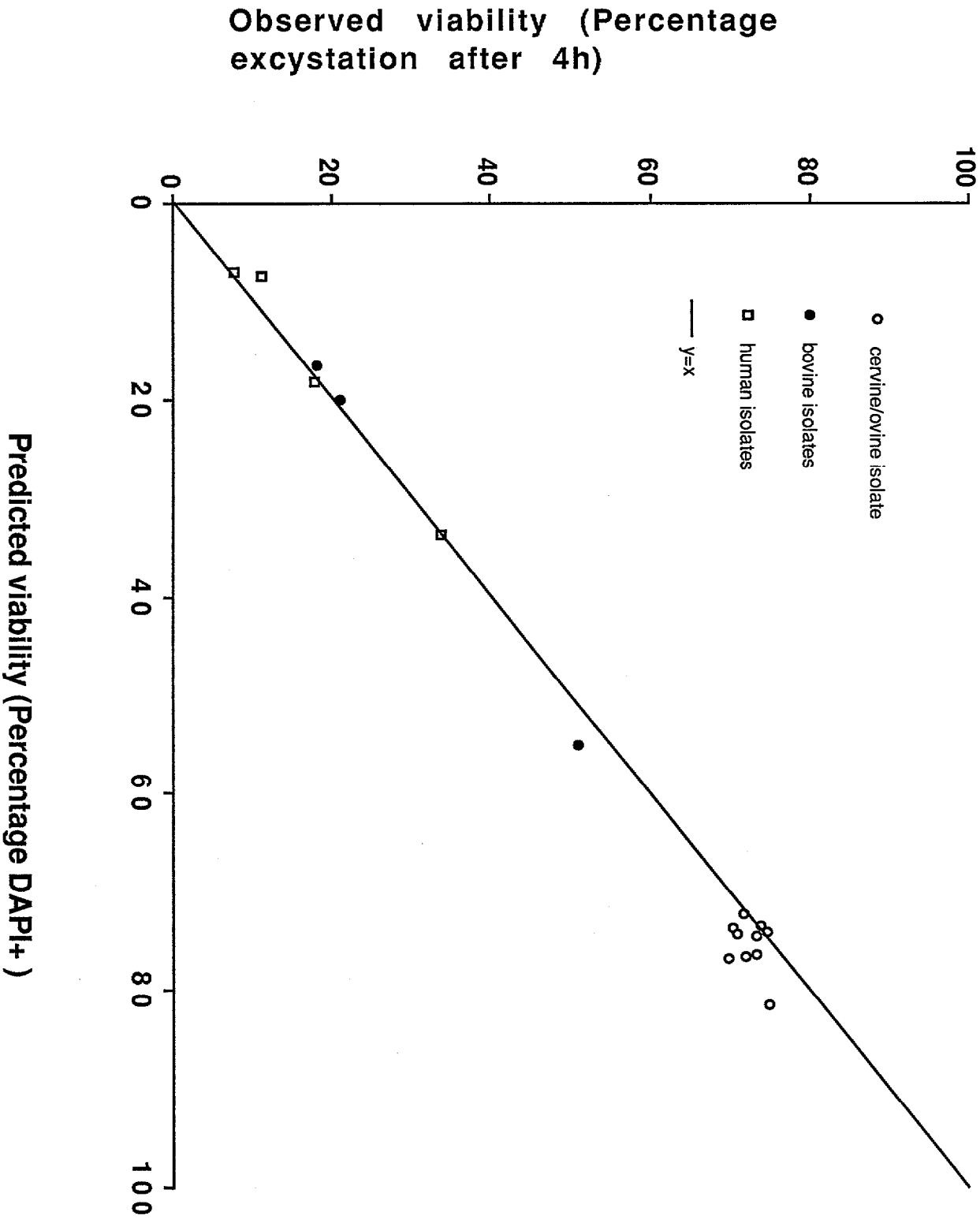
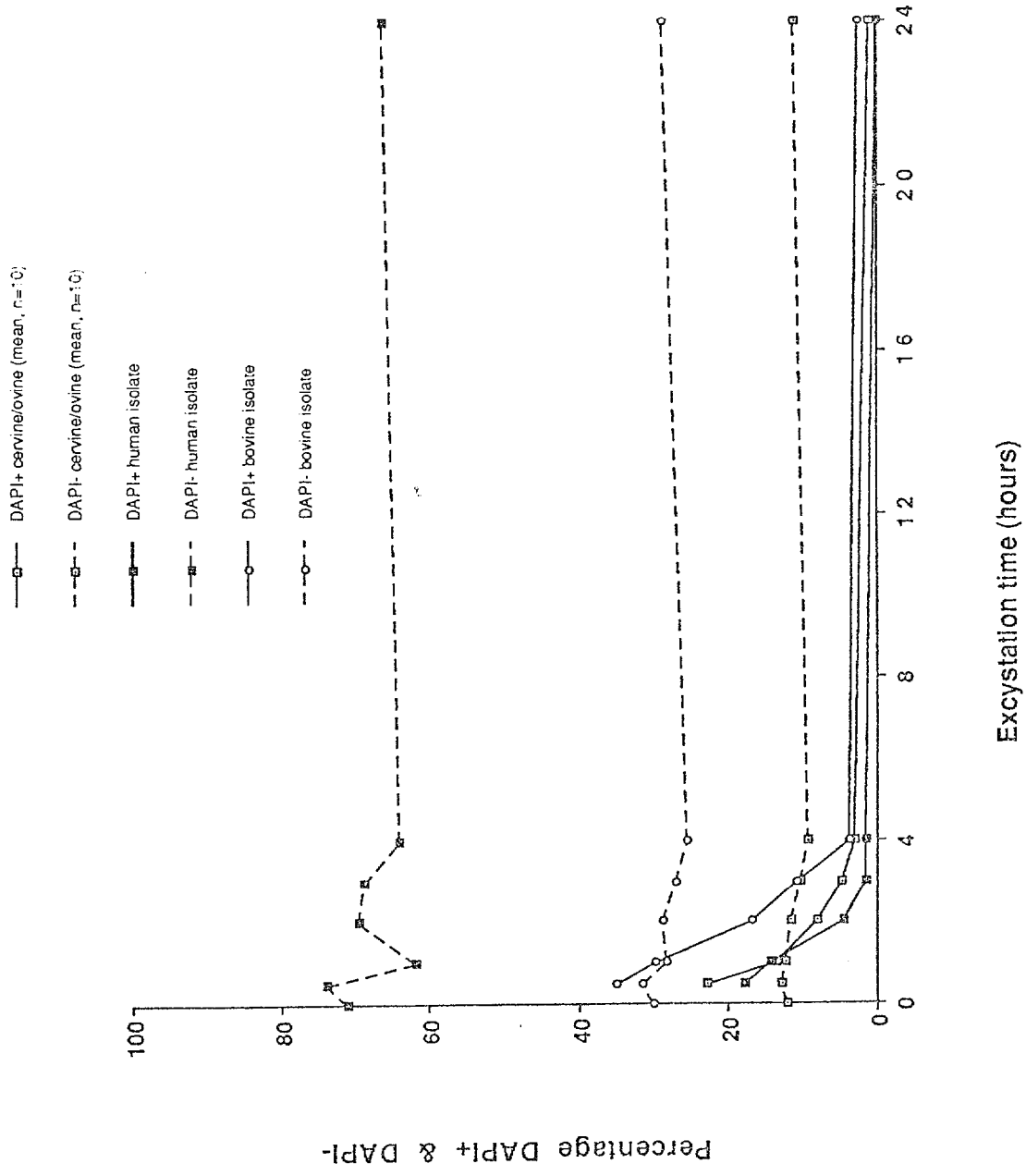

Observed viability (Percentage excystation after 4h)
Predicted viability (Percentage DAPI+)
0
20
40
60
80
100
cervine/ovine isolate
bovine isolates
human isolates
y=x

Figure 8


DAPI+ cervine/ovine (mean, n=10)
DAPI- cervine/ovine (mean, n=10)
DAPI+ human isolate
DAPI- human isolate
DAPI+ bovine isolate
DAPI- bovine isolate
Percentage DAPI+ & DAPI-
100
80
60
40
20
0
0
4
8
12
16
20
24
Excystation time (hours)

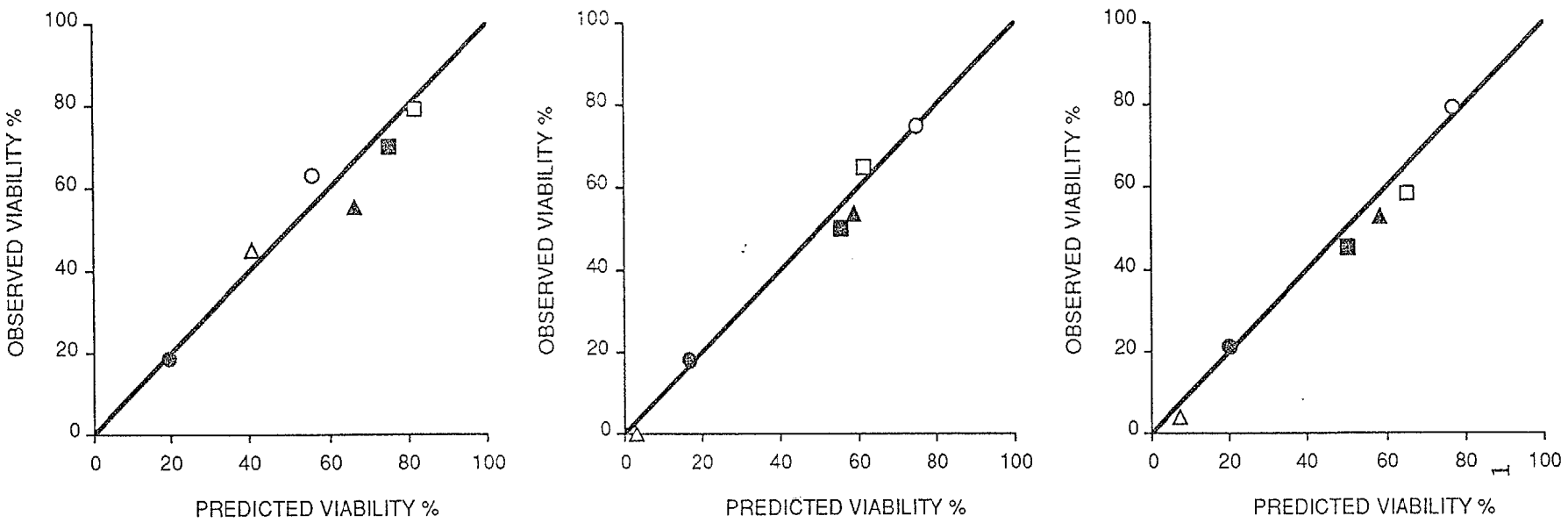


Figure 9

● ○ = RO H_2O

■ □ = acid

▲ △ = alkali

LOW HIGH
VIABILITY

——— X = Y

Aging of *Cryptosporidium* oocysts under various environmental pressures

Freezing

Snap-freezing of oocysts resulted in 100% death of the oocysts (either ghosts or PI (+)), slow freezing, however, was less effective at killing oocysts (Figure 10).

After 21 h at -22°C, only 67% of the oocysts had been killed, and despite this increasing to over 90% after 152 h, even after as much as 799 h at -22°C a small proportion of oocysts were still potentially infective. After 173 h at -22°C DAPI(-) oocysts were not detected.

In vitro excystation correlated with the results obtained by inclusion/exclusion of DAPI and PI.

Dehydration/desiccation

Although after only 2 h of air drying, 3% of oocysts were still viable, slightly longer periods of drying resulted in 100% death of the oocysts (Table 5). *In vitro* excystation correlated with results obtained by exclusion/inclusion of PI and DAPI.

Table 5. Effect of drying/dessication on the viability of *Cryptosporidium* oocysts

HOURS DRYING	% DEAD (GHOSTS & PI)	% DAPI(+)	% DAPI(-)	% POTENTIALLY INFECTIVE (DAPI(+) & DAPI(-))
0	23.7	72.8	3.5	76.3
2	97.0	3.0	0	3.0
4	100.0	0	0	0
6	100.0	0	0	0
8	100.0	0	0	0

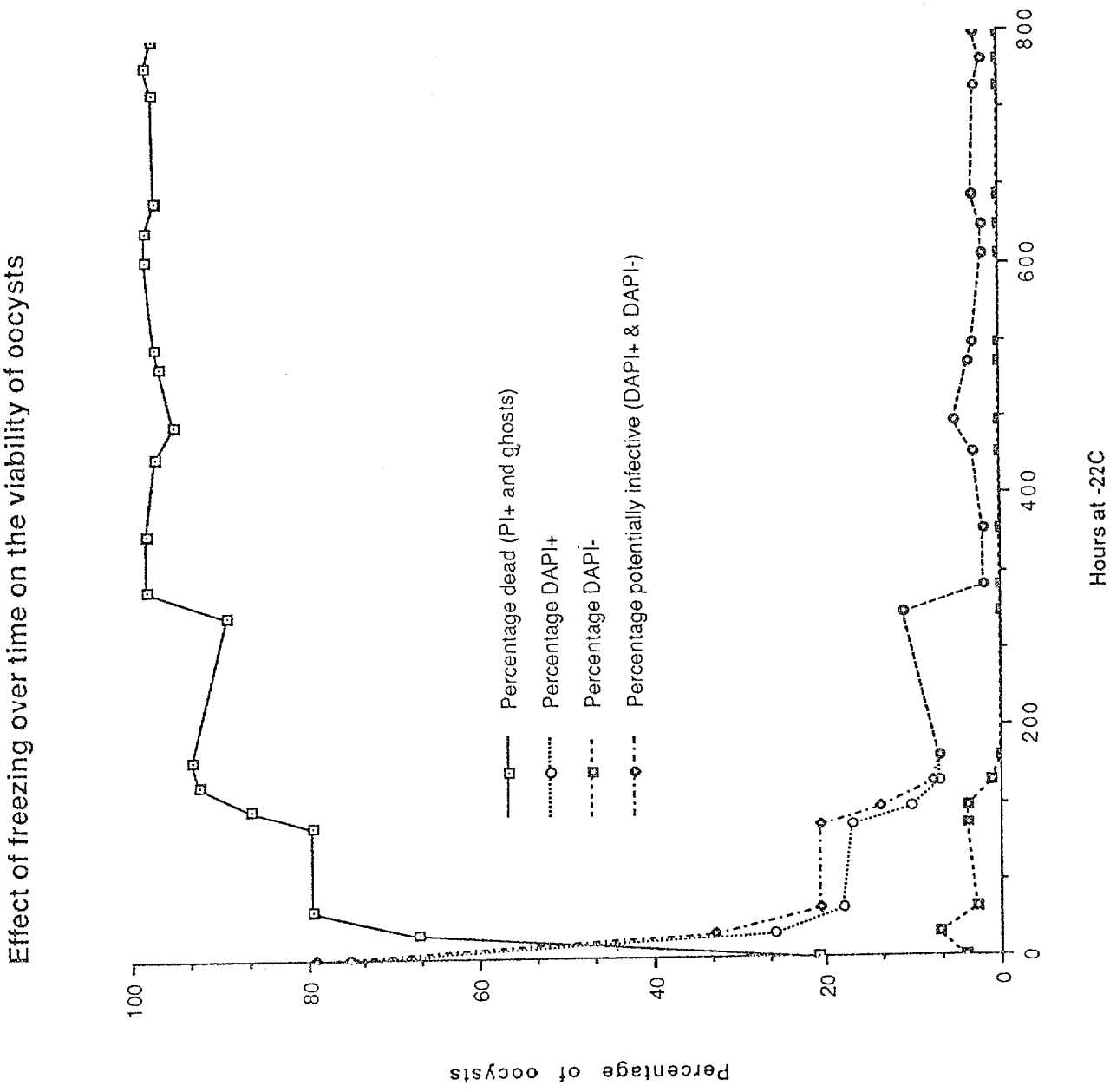
In stools

Despite there being some fluctuation in percentage of DAPI(+)/PI(-) and DAPI(-)/PI(-) oocysts over time, the percentage of dead oocysts and potentially infective oocysts in the stool samples varied only slightly over the 48 days of investigation. Percentage death tended to increase with time, and there was a corresponding decrease in the proportion of potentially infective oocysts (Figure 11).

In laboratory models of water treatment processes

Alum flocculation: Although contact with high aluminium concentrations for prolonged periods killed oocysts (Table 6), alum flocculation at the pH, concentration and contact time used in the water industry appeared to have no impact on the viability of *Cryptosporidium* oocysts (Tables 7 & 8). Also, even prolonged contact times with high

Figure 10


Effect of freezing over time on the viability of oocysts
Percentage dead (PI+ and ghosts)
Percentage DAPI+
Percentage DAPI-
Percentage potentially infective (DAPI+ & DAPI-)
Percentage of oocysts
Hours at -22C
0
20
40
60
80
100
0
200
400
600
800

Aging of *Cryptosporidium* oocysts in faecal samples

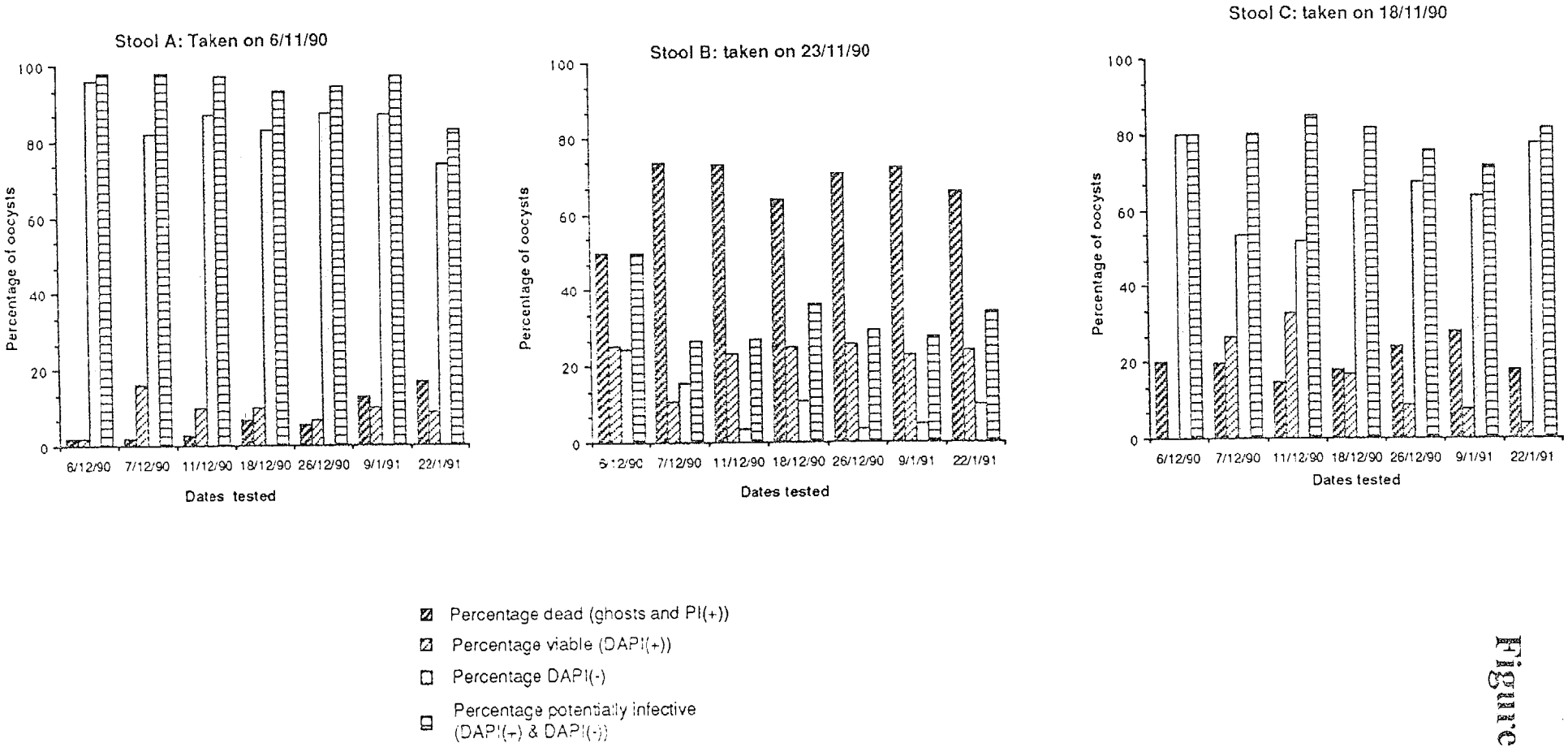


Fig. 11

aluminium concentrations did not affect oocyst viability if pH was corrected (from approximately 1.5 to approximately 6) with either lime or sodium hydroxide (Table 9).

Table 6. Effect of 5% aluminium (aluminium sulphate) or 5% Wispofloc N (polyelectrolyte, WfN) for 1 h at room temperature on the viability of *Cryptosporidium* oocysts.

	% DEAD (GHOSTS & PI)	% VIABLE (DAPI(+))	% DAPI(-)	% POTENTIALLY INFECTIVE (DAPI(+) & DAPI(-))
CONTROL MEAN	20.1	75.4	4.5	79.9
± SD (n=3)	±2.7	±2.6	±1.1	±2.6
5% AL	42.3	54.1	3.6	57.7
5% WfN	22.3	72.8	4.9	77.7

Table 7. Effect of 0.975 or 1.5 ppm Aluminium (aluminium sulphate) for 7 minutes at room temperature or 4°C on the viability of two isolates of *Cryptosporidium* oocysts.
All results are given as means ± SD. n=3.

Isolate A (high initial viability)

	% DEAD (PI & GHOSTS)	% VIABLE (DAPI(+))	% DAPI(-)	% POTENTIALLY INFECTIVE (DAPI(+) & DAPI(-))
Room Temperature				
Control	14.9±3.6	77.0±4.5	8.1±6.2	85.1±5.6
1.5ppm Al	18.3±4.1	75.9±5.6	5.8±2.6	81.7±4.1
1.0ppm Al	23.6±6.8	70.6±5.4	5.8±1.6	76.4±3.8
4°C				
Control	20.0±7.1	72.5±7.5	7.5±3.9	80.0±7.1
1.5ppm Al	22.0±5.3	73.5±4.2	4.5±2.3	78.0±5.3
1.0ppm Al	24.0±5.9	71.8±5.2	4.2±1.1	76.0±5.9

Isolate B (low initial viability)

Room temperature				
Control	16.2±2.7	52.1±4.6	31.6±6.1	83.7±2.5
1.5ppm Al	18.9±6.1	58.4±6.3	22.7±1.0	81.1±6.1
1.0ppm Al	16.3±2.3	60.3±5.3	23.4±2.1	83.7±4.1
4°C				
Control	15.8±2.0	52.9±5.7	31.3±5.1	84.2±2.0
1.5ppm Al	15.9±5.4	56.2±5.7	27.8±5.4	84.0±5.4
1.0ppm Al	22.5±6.0	52.3±4.0	25.2±3.0	77.5±5.2

Table 8. Effect of 0.975 ppm and 1.5 ppm Aluminium (aluminium sulphate) (corrected to pH 6 with lime and in the presence or absence of the polyelectrolyte Wispofloc N (WfN)) for seven minutes at room temperature or 4°C on the viability of *Cryptosporidium* oocysts.

	% DEAD (PI & GHOSTS)	% VIABLE (DAPI(+))	% DAPI(-)	% POTENTIALLY INFECTIVE (DAPI(+) & DAPI(-))
Room Temperature				
Control	15.0	81.0	4.0	85.0
Control pH6	21.4	72.3	6.3	78.6
Control & WfN	14.0	82.0	4.0	86.0
Control pH6 & WfN	17.6	77.8	4.6	82.4
0.975ppm Al pH6	21.0	76.0	3.0	79.0
0.975ppm Al pH6 & WfN	22.5	71.6	5.9	77.5
1.5ppm Al pH6	21.0	75.0	4.0	79.0
1.5ppm Al pH6 & WfN	19.8	74.3	5.9	80.2
4°C				
Control	17.8	77.2	5.0	82.2
Control pH6	20.0	72.3	7.7	80.0
Control & WfN	15.4	79.8	4.8	84.6
Control pH6 & WfN	17.6	77.5	4.9	82.4
0.975ppm Al pH6	18.0	75.0	7.0	82.0
0.975ppm Al pH6 & WfN	22.6	70.8	6.6	77.4
1.5ppm Al pH6	22.0	74.0	4.0	78.0
1.5ppm Al pH6 & WfN	21.9	74.9	4.2	79.1

Table 9. Effect of pH corrected aluminium (5% aluminium sulphate) and pH corrected lime (0.2% calcium hydroxide) for 1 h at room temperature on the viability of *Cryptosporidium* oocysts.

	% DEAD (PI & GHOSTS)	% VIABLE (DAPI(+))	% DAPI(-)	% POTENTIALLY INFECTIVE (DAPI(+) & DAPI(-))
Control (mean ± SD, n=3)	28.5 ± 4.6	40.4 ± 1.6	31.1 ± 4.2	71.5 ± 4.5
5%Al, pH6 lime	23.6	41.5	34.9	76.4
5%Al, pH6 NaOH	27.2	39.8	33.0	72.8
lime, pH6 HCl	32.0	41.0	27.0	68.0

Liming and polyelectrolytes: High concentrations of lime over prolonged periods of time affected the viability of *Cryptosporidium* oocysts, significantly increasing the percentage of dead oocysts, with a correspondingly significant decrease in the proportion of DAPI(+) and DAPI(-) oocysts (Table 10). However, if the lime was pH corrected (from approximately 10.5 to approximately 6) by the addition of 1% HCl, the viability of oocysts appeared to be unaltered (Table 9).

Table 10. Effect of lime (0.2% calcium hydroxide) for 1 h at room temperature on the viability of *Cryptosporidium* oocysts

	% DEAD (PI & GHOSTS)	% VIABLE (DAPI(+))	% DAPI(-)	% POTENTIALLY INFECTIVE (DAPI(+) & DAPI(-))
Control (mean ± SD, n=3)	28.0 ± 0.9	42.1 ± 1.2	29.9 ± 2.0	72.0 ± 0.9
+ lime	49.5	25.7	24.8	50.5

High concentrations of the polyelectrolyte Wispofloc N (pH 6 without correction) did not appear to affect the viability of *Cryptosporidium* oocysts (Table 6).

Ferric flocculation: Although, as with alum, high concentrations of iron killed oocysts, when the pH was corrected (from approximately 1.5 to approximately 6) by either sodium hydroxide or lime, there was no impact on the viability of *Cryptosporidium* oocysts (Table 11). The concentrations of ferric used by the water industry only affected the viability off oocysts if the pH was not corrected (which does not occur in the water authorities consulted for this investigation) or if the pH was corrected to pH 9 (Tables 12 & 13).

For all laboratory models of water treatment processes *in vitro* excystation correlated well with results obtained by inclusion/exclusion of DAPI and PI.

Table 11. Effect of 5% iron (Ferric sulphate) on the viability of *Cryptosporidium* oocysts.

	% DEAD (PI & GHOSTS)	% VIABLE (DAPI(+))	% DAPI(-)	% POTENTIALLY INFECTIVE (DAPI(+) & DAPI(-))
Control (mean ± SD, n=3)	27.8 ± 3.5	70.2 ± 2.1	2.0 ± 3.4	72.2 ± 3.6
5%Fe	97.0	3.0	0.0	3.0
5%Fe, pH6 lime	26.3	68.4	5.3	73.7
5%Al, pH6 NaOH	31.4	64.4	4.2	68.6

Table 12. Effect of 3.5 ppm and 16 ppm iron (ferric sulphate) for 1 h or 5 h at room temperature on the viability of *Cryptosporidium* oocysts. Lime has been used for pH correction where indicated.

	% DEAD (PI & GHOSTS)	% VIABLE (DAPI(+))	% DAPI(-)	% POTENTIALLY INFECTIVE (DAPI(+) & DAPI(-))
One hour contact time				
Control (mean	32.5	65.7	1.9	67.5
± SD, n=3)	± 7.2	± 7.3	± 0.9	± 6.2
3.5ppm Fe	31.4	63.8	4.8	68.6
3.5ppm Fe, pH6	39.0	58.2	2.8	61.0
3.5ppm Fe, pH9	41.4	57.7	0.9	58.6
16ppm Fe	45.2	50.9	4.0	54.9
16ppm Fe, pH6	37.6	59.6	2.8	62.4
16ppm Fe, pH9	50.0	48.2	1.8	50.0
Five hour contact time				
Control (mean	32.0	65.2	2.7	67.9
± SD, n=3)	± 4.4	± 7.9	± 2.4	± 8.7
3.5ppm Fe	58.4	36.6	5.0	41.6
3.5ppm Fe, pH6	38.0	57.0	5.0	62.0
3.5ppm Fe, pH9	76.2	21.9	1.9	23.8
16ppm Fe	49.6	46.1	4.3	47.4
16ppm Fe, pH6	40.5	57.0	2.5	59.5
16ppm Fe, pH9	56.9	37.6	5.5	43.1

Table 13. Effect of 3.5 ppm and 16 ppm iron (ferric sulphate) for 1 h or 5 h at 4°C on the viability of *Cryptosporidium* oocysts. Lime has been used for pH correction where indicated.

	% DEAD (PI & GHOSTS)	% VIABLE (DAPI(+))	% DAPI(-)	% POTENTIALLY INFECTIVE (DAPI(+) & DAPI(-))
One hour contact time				
Control (mean	36.9	60.1	2.9	63.1
± SD, n=3)	± 3.0	± 3.1	± 2.0	± 3.0
3.5ppm Fe	44.7	51.8	3.5	55.3
3.5ppm Fe, pH6	34.0	63.1	2.9	66.0
3.5ppm Fe, pH9	53.8	42.3	3.9	46.2
16ppm Fe	43.6	54.5	1.9	56.4
16ppm Fe, pH6	39.8	59.2	1.0	60.2
16ppm Fe, pH9	41.0	56.2	2.8	59.0
Five hour contact time				
Control (mean	42.5	54.4	3.1	57.5
± SD, n=3)	± 4.3	± 2.4	± 1.3	± 3.3
3.5ppm Fe	46.9	49.6	3.5	53.1
3.5ppm Fe, pH6	36.9	51.2	2.9	54.1
3.5ppm Fe, pH9	52.6	44.0	3.4	47.4
16ppm Fe	52.5	44.6	2.9	47.5
16ppm Fe, pH6	43.4	53.3	3.3	56.6
16ppm Fe, pH9	57.4	38.6	4.0	42.6

In semi-permeable containers in selected environments

In all environments, including the RO water control the proportion of dead oocysts increased over time (Figure 12). Death was most rapid in oocysts placed in river and tap water. The proportion of potentially infective oocysts (DAPI(+) and DAPI(-)) decreased correspondingly with the increase in dead oocysts (Figure 13). However, the proportion of viable (DAPI(+)) and DAPI(-) oocysts followed a pattern which appears to be related both to the particular environment in which the oocysts were placed and to the initial viability of the oocyst suspension (Tables 14 & 15). Both isolates of oocysts which were placed in the cow faeces experienced a marked drop in the percentage of DAPI(+) oocysts with a corresponding increase in the proportion of DAPI(-) oocysts. In the other environments, those oocysts of initially a high viability appeared to experience a conversion from DAPI(+) to DAPI(-), and those oocysts of an initial low viability appeared to experience the opposite, with a decrease in the proportion of DAPI(-) oocysts.

Table 14. Alterations in the proportion of DAPI(-) oocysts over time in various environments.

	HIGH VIABILITY ISOLATE				LOW VIABILITY ISOLATE			
	PERCENTAGE DAPI(-)							
Date of sampling	Control (RO Water)	Tap Water	River Water	Cow Faeces	Control (RO Water)	Tap Water	River Water	Cow Faeces
9/12/90	3.3	3.3	3.3	3.3	39.2	39.2	39.2	39.2
20/12/90	3.9	5.0	3.8	9.5	21.4	15.6	20.0	41.9
28/12/90	6.7	5.4	5.0	23.4	35.4	18.6	37.0	55.1
11/01/91	4.8	10.1	10.8	45.5	17.9	17.8	29.0	63.8
25/01/91	9.9	10.5	7.7	44.9	20.5	13.0	14.7	55.0

Table 15. Alterations in the proportion of viable (DAPI(+)) oocysts over time in various environments.

	HIGH VIABILITY ISOLATE				LOW VIABILITY ISOLATE			
	PERCENTAGE DAPI(+)							
Date of sampling	Control (RO Water)	Tap Water	River Water	Cow Faeces	Control (RO Water)	Tap Water	River Water	Cow Faeces
9/12/90	74.4	74.4	74.4	74.4	44.6	44.6	44.6	44.6
20/12/90	71.3	70.0	81.6	63.8	49.5	56.3	55.0	41.1
28/12/90	67.0	72.1	42.0	45.8	46.9	40.2	44.1	28.4
11/01/91	69.5	59.6	44.9	21.5	53.8	42.6	31.0	13.4
25/01/91	67.6	53.9	55.2	16.8	45.5	38.0	30.4	12.0

Alterations in the proportion of dead (PI(+) and ghost) oocysts over time in various environments.

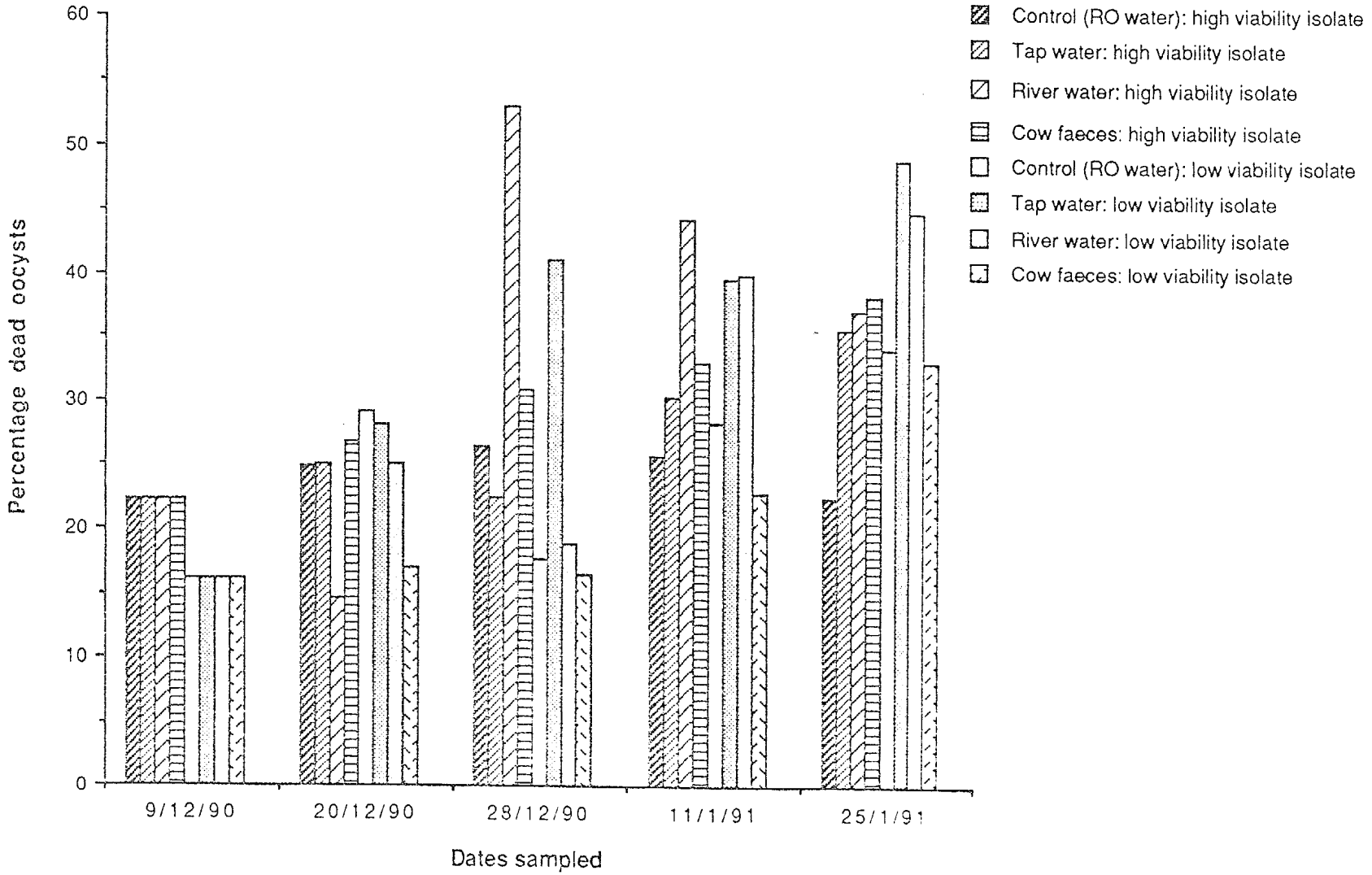


Figure 12

Alterations in the proportion of potentially infective (DAPI(+) & DAPI(-)) oocysts over time in various environments.

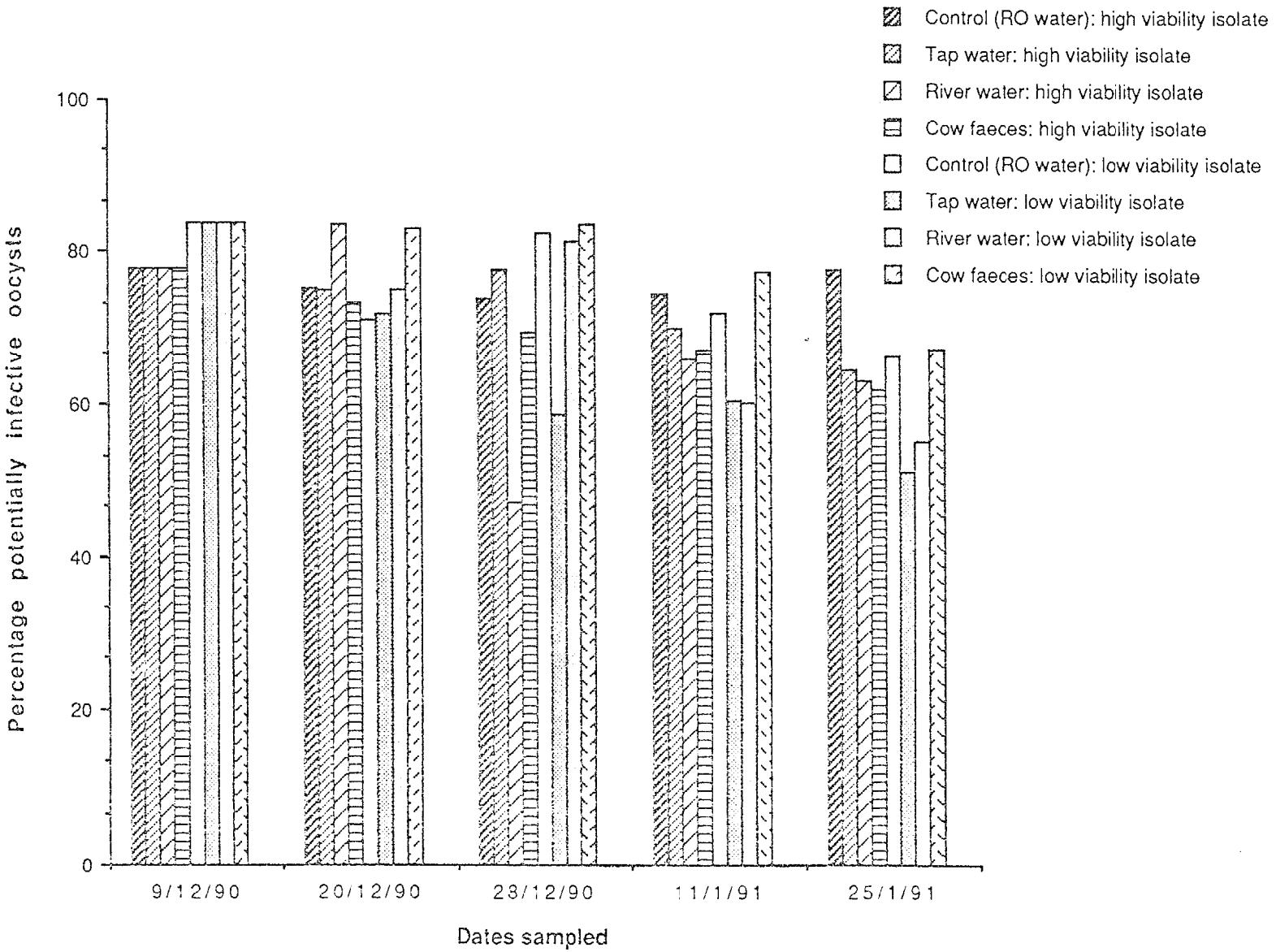


Figure 13

Sea water (48 h at 4°C) had no effect on the viability of *Cryptosporidium* oocysts (Table 16).

Table 16. Effect of sea water (48h at 4°C) on the viability of *Cryptosporidium* oocysts.

	% DEAD (PI & GHOSTS)	% VIABLE (DAPI(+))	% DAPI(-)	% POTENTIALLY INFECTIVE (DAPI(+) & DAPI(-))
Control (mean	36.1	57.0	6.9	63.9
± SD, n=3)	± 3.3	± 2.4	± 1.0	± 3.3
Sea water	37.0	57.0	6.0	63.0

Biochemical analysis of *Cryptosporidium* oocysts

Fluorescent lectin binding analysis

Of all the lectins tested only SBA and LCA were selective enough to bind glycoconjugates of *C.parvum* oocysts. Both of these were only observed to bind to oocysts that had ruptured cell membranes (PI(+)). Lectin binding was never observed on intact oocysts, whether DAPI(-) or DAPI(+).

SBA has an affinity for the monosaccharide N-acetyl-D-galactosamine (GalNAc), whereas LCA has an affinity for either D-mannose or D-glucose. Fluorescence due to SBA binding could be inhibited by pre-incubating the lectin with GalNAc, whereas fluorescence due to LCA binding was not inhibited using either of its competing monosaccharides (D-mannose and D-glucose).

The pattern of lectin reactivity was the same for all the isolates tested (c/o, bovine and 2 human isolates).

When the oocysts (c/o isolate) were subjected to an *in vitro* excystation protocol followed by lectin binding analysis, lectin reactivity associated with oocysts did not increase.

SBA selectively bound the outer wall of ghost oocysts.

LCA staining was associated predominantly within PI (+) oocysts.

Motile sporozoites were observed to bind PNA, which has an affinity for the disaccharide D-Galactose-β-(1→3)-D-GalNAc. Binding of PNA could be inhibited by this disaccharide unit.

Surface biotinylation

Biotinylated moieties on *C.parvum* oocysts were visualized after the addition of STR-FITC. The fluorescent profiles obtained after the various labelling methods, in conjunction with DAPI/PI staining, are summarized in table 17.

Biotin labelling procedures did not affect the viability of the oocysts.

Intact, potentially infective oocysts (DAPI (+) and DAPI (-)) expressed significantly less primary amines than dead (PI(+) and ghost) oocysts. No detectable difference in labelling intensity was observed between the two biotin NHS esters.

Table 17. Fluorescent profiles of DAPI/PI stained *Cryptosporidium* oocysts obtained by biotinylation.

	Intensity of fluorescence after STR-FITC labelling of biotinylated moieties			
	Ghost	PI(+)	DAPI(+)	DAPI(-)
NHS-biotin	+++	+++	+	+
NHS-lc-biotin	+++	+++	+	+
$NaIO_4$/BHZ	+++	+++	+++	+++
Gal.oxidase/BHZ	+++	++	-	+++
BHZ	-	+	-	-

Intensity of fluorescence is indicated here on an arbitrary scale from - (absence of fluorescence) to +++ (peak intensity of fluorescence).

Periodate oxidised oocysts treated with BHZ exhibited the strongest fluorescent signal of all the oocysts studied. This label was associated with the oocyst outer wall in all cases.

The biotin label was shown to be highly stable. It was detectable on oocysts even after harsh chemical treatments (incubation with 0.1 M HCl or NaOH), and was retained for more than 2 months on oocysts stored at 4 °C.

Ghost oocysts and DAPI (-) oocysts exhibited very intense rim fluorescence using galactose oxidase/BHZ.

PI(+) oocysts only exhibited weak fluorescence, with much the same intensity as observed in the periodate control (BHZ only). However, DAPI (+) oocysts were never observed to exhibit fluorescence, rim or otherwise, with galactose oxidase/BHZ.

The NHS-esters and the periodate/BHZ labelling regimes were >90% successful. However, with the galactose oxidase/BHZ regime the proportion of DAPI(-) oocysts which labelled with STR-FITC ranged from 67.7 % (c/o isolate) to 12.5 % (bovine isolate). This label was lost from DAPI(-) oocysts over a 48 h period at 4 °C in PBS.

All isolates tested (4 human, 2 bovine and c/o), exhibited the same pattern of fluorescence after the various biotin labelling regimes.

SDS-PAGE/Western blot analyses

After the gel run had been completed, DAPI stained oocysts fractions resolved in the gel were visualised using a transilluminator. The only staining that was observed was a fast migrating band which ran immediately in front of the tracking dye.

The table of results displays the relative mobilities of the major bands and the approximate molecular weight range in kiloDaltons (kD). More accurate estimates of molecular weights could not be made as of the 5 commercial biotinylated molecular weight standards tested, not one was found to be satisfactory.

The relative mobilities of the major bands identified after surface labelling oocysts of the c/o isolate with NHS-lc-biotin followed by the BEC detection system are shown in table 18.

Table 18. Relative mobilities of major bands identified after labelling isolates with NHS-lc-biotin and detected using the BEC system.

RELATIVE MOBILITIES			APPROXIMATE
LIVE ISOLATES		DEAD ISOLATE	MOLECULAR WEIGHT
Human	c/o	c/o	(kD)
		0.85	10
		0.75	
		0.65	50
0.53	0.57	0.60	50
0.50	0.55	0.55	
0.42	0.45	0.46	80
		0.385	
		0.34	
		0.27	
		0.24	120
		0.22	120
0.20		0.20	
	0.145	0.17	200
0.04	0.06	0.08	200-400

A labelling difference was not observed between the c/o isolate and a purified human isolate, both of which were predominantly DAPI (+). However, there were many more bands visualised in the dead c/o isolate. This would be expected as the fluorescence intensity after the labelling step was much greater with dead oocysts.

Bands were detected only with extracts from NHS-lc-biotin treated oocysts, and not from control, untreated oocysts. This shows that the banding pattern observed on X-ray film with the BEC system was a direct consequence of the selective binding of STR-HRP to the biotin groups introduced into the oocysts.

When intact (DAPI(+) and DAPI(-)) oocysts were studied, approximately $5x10^6$ oocysts per track of gel were required, whereas only $2x10^6$ dead oocysts were needed before a suitable level of intensity was reached.

Periodate oxidation followed by BHZ treatment incorporates a biotin label into expressed glycoconjugates. The relative mobilities of the major bands identified using this surface labelling method on oocysts from a bovine isolate and detected by the BEC system are shown in table 19. In oocyst preparations which were snap frozen, then

boiled in sample buffer a polydisperse smear containing multiple bands from 400 kD - 20 kD was observed even after loading the equivalent of as few as 10^6 oocysts.

The bands observed from both boiled and snap frozen supernatants preparations of dead (PI(+)) oocysts were compared to bands obtained from high viability (DAPI(+)) oocysts prepared by the same methods.

Using boiling as the method of solubilising biotinylated moieties, as in the NHS-lc-biotin luminograms, more bands were observed in the extracts from dead oocysts, than in the extracts obtained from the high viability preparations.

The supernatant obtained after snap freezing the high viability oocyst preparations contained several bands (Table 19). Only one of these bands (200-400 kD) could be detected in the supernatant obtained from dead oocyst preparations.

Table 19. Relative mobilities of major bands identified after surface labelling periodate oxidised bovine oocysts with biotin hydrazide and detected using the BEC system.

RELATIVE MOBILITIES				APPROXIMATE
High viability DAPI(+)/PI(-)		Dead PI(+)		MOLECULAR WEIGHT (kD)
A	B	A	B	
0.60	0.62			50
0.55	0.56	0.52		
		0.45		75
		0.40		75
		0.36		
0.30		0.28		
0.24	0.24	0.24		120
		0.16		120-200
0.05	0.05	0.045	0.045	200-400

A: labelled oocysts boiled in sample buffer for 10 min. The supernatant was collected and loaded onto the gel.
B: labelled oocysts were snap frozen then pelleted. The supernatant was collected and boiled in sample buffer then loaded onto the gel.

No bands could be resolved in the gel after labelling galactose or galactosaminyl residues with BHZ then analysing the extracts using the BEC system. However using a population of oocysts which were predominantly DAPI(-) BHZ was incorporated into fast migrating glycoconjugates at the gel front.

In the bands resolved, BHZ specifically labels the periodate generated aldehydes, as no biotin label could be

detected in blots after treating oocysts with BHZ without prior oxidation with periodate.

DISCUSSION

Rapid detection techniques

Concentration of oocysts using magnetisable particles

Immuno-magnetic particles have been used to separate and concentrate a variety of target cells from various biological fluids, and one of this technique's major advantages is that enrichment for a specific target cell requires no centrifugation step. Two procedures were assessed namely, a) initial conjugation of the *anti-Cryptosporidium* McAb onto 4.5 µm diameter magnetisable particles followed by incubation of the coated particles with seeded oocysts, and b) initial conjugation of a biotinylated anti-FITC McAb onto streptavidin-coated 2.8 µm diameter magnetisable particles followed by incubation of the coated particles with previously coated FITC-labelled oocysts. Both systems were capable of removing 75% or greater of oocysts from seeded samples. However, the number of trials performed was small because of the scarcity of both the *anti-Cryptosporidium* McAb (obtained as ascites fluid, and purified at SPDL) and the biotinylated anti-FITC McAb (obtained as ascites fluid, purified and biotinylated at SPDL), and an insufficient quantity was available to perform further tests on magnetisable particles as well as for the techniques discussed below. Although standard protocols were followed for the conjugation of both the *anti-Cryptosporidium* McAb onto 4.5 µm diameter magnetisable particles and the biotinylated anti-FITC McAb onto strepavidin-coated 2.8 µm diameter magnetisable particles, the exact ammount of antibody required for optimal coating of beads will vary according to the affinities of the McAb's to be coated. As for any immunological technique, its effectiveness is dependent upon both the specificity and avidity of the primary (coating) antibody in question, and certain antibodies may have reduced binding capacity when coated onto M450 beads (pers. comm. Dynal (UK) Ltd.). In an attempt to overcome such a potential problem, the second (attachment of biotinylated anti-FITC McAb onto strepavidin-coated 2.8 µm diameter magnetisable particles followed by incubation of the coated particles with previously coated FITC-labelled oocysts) protocol was adopted, and a marginally higher percentage recovery was obtained in preliminary studies. Little is known of the affinity of the paratope of either McAb for its respective epitope however, it can be concluded that the higher the affinity, the better the binding. One advantage of the second protocol was that oocysts bound onto the anti-FITC coated beads were already bound by the FITC-labelled *anti-Cryptosporidium* McAb, which made identification, based on morphometrics, easier. Such a system has the inherent advantage of rapid visual confirmation, by fluorescence microscopy, of putative oocysts isolated

from environmental samples.

Because of the inclusion of detergent solutions in the standard procedure for the isolation of oocysts, species-specific, high affinity McAb's could, with further research, make magnetic separation an useful technique for the concentration of oocysts from water samples.

Fluorescent imaging using charge couple devices

CCD cameras incorporate high quantum efficiency and excellent geometrical accuracy and repeatability with optical ruggedness.

The Astromed cooled, slow-scan image intensifying system readily detected all fluorescent emissions. The limiting factor for oocyst detection was the magnification required for visualisation. Although this was x 30, by using the enhanced oocyst morphology obtained with DAPI, this could probably be lowered to approximately x 20.

The Astromed CCD system was found to be superior to the Photonics system for screening for oocysts. This is largely because the Astromed system is cooled and incorporates slow-scan read out.

By cooling the CCD, the dark current (thermally generated signals mimicking light falling on the device) is greatly reduced. Consequently, the signal charge collected by the CCD is produced almost entirely by the incident light, even with very long exposure times and low light intensities.

The slow-scan readout means that readout noise (electronic noise associated with measuring the signal charge) is much less than for a video rated camera. This allows the signal charge collected by the CCD to be more accurately measured.

Theoretically, it would be possible to screen a slide using a computer controlled microscope system however, the sophisticated software required for image recognition is not available at present.

Enhanced chemiluminescent detection

X-ray film: In the slide assay of purified oocysts exposed to X-ray film, many false positive results were obtained (6 out of 13) by BEC. However, only on one occasion was a sample detected as negative, which on subsequent microscopy was considered to be positive. In this instance the two putative oocysts exhibited only weak rim fluorescence and neither the suture nor double membrane was visible. The decision to classify these fluorescent objects as *Cryptosporidium* oocysts was based upon their size (4-6 μm) but not on other morphological grounds, and because the sample should have been seeded with oocysts.

Photomultiplier: By using a photomultiplier tube on a 96 well format microtiter plate, the minimum theoretical number of oocysts that could be reproducibly detected was 6.25. Light could, however, be still detected from some wells seeded with a theoretical 0.78 oocysts. This is probably due to the artifact of clumping of purified oocysts which would cause the distribution of oocysts between the slide wells to be unequal. The actual numbers of *Cryptosporidium* oocysts counted on serially diluted multi-well slides rarely corresponded to the theoretical numbers.

CCD camera imaging: The cooled slow-scan CCD was more sensitive than both the X-ray film and the photomultiplier tube.

In 3 out of 4 slides, individual oocysts could be detected and the results obtained by BEC were comparable to the numbers counted upon subsequent microscopy. In the slide assay, background luminescence was the main problem. In all the wells of the CCD imaged slide, light was found to emanate from the whole area of the dried sample rather than from the specific site of the oocysts.

Environmental samples: Over 50% of the negative samples screened were found to be positive for light emissions by BEC. This quantity of false positives makes the use of this assay at present an inefficient option.

The main cause of the false positive results in all three techniques was probably the affinity of the primary McAb (the FITC-McAb) for epitopes other than those on the surface of *Cryptosporidium* oocysts. Numerous researchers have observed the binding of the FITC-McAb to objects such as pollen, yeast and algal cells. The STR-HRP and anti-FITC biotin McAb are both high affinity labelling steps.

The FITC McAb also contains a relatively large amount of fluorescent inorganic matter which might perhaps bind the anti-FITC biotin McAb.

A further purification step by affinity chromatography, to give a McAb with a more defined and avid reactivity towards *C.parvum* expressed epitopes is required. To ensure that oocysts are not inadvertently missed the CCD imaging system would then be the optimum method of detection.

Viability assay

Oocysts incubated with DAPI and PI could be categorised on the basis of inclusion or exclusion of these two vital dyes. Those oocysts which included PI (PI(+)) have disrupted or broken membranes and are considered dead. During the course of our investigations motile sporozoites were never seen to include PI. Oocysts are also considered dead if they have ruptured with the loss of contents (ghost oocysts).

Those oocysts which are permeable to DAPI but not to PI (DAPI(+)/PI(-)) will excyst in the 4 h excystation protocol reported. This relationship still holds if the permeability of the oocysts to DAPI and PI is altered by harsh treatments with acid or alkali. Thus DAPI(+)/PI(-) oocysts are described as viable and their incubation with DAPI and PI provides a reproducible, sensitive, "user-friendly" method for assessing the viability of *Cryptosporidium* oocysts.

The permeation of viable oocysts with DAPI results in the nuclei of the sporozoites fluorescing a characteristic sky-blue colour when viewed under a UV filter block. This visualisation of the sporozoite nuclei also provides a useful adjunct for definitive identification of *C.parvum* oocysts.

Those oocysts which are neither DAPI(+) nor PI(+) nor ghosts are not considered viable at assay. However, these DAPI(-)/PI(-) oocysts appear by Nomarski microscopy to be morphologically intact and undamaged. It is proposed that while DAPI(-)/PI(-) oocysts are not viable at the time as assay (as defined by *in vitro* excystation), neither are they dead. Perhaps these DAPI(-)/PI(-) oocysts require a further stimulus not provided in the excystation protocol described in order to be capable of excystation. Alternatively, perhaps they are temporally or biophysically immature oocysts which, in time, or after exposure to chemical and/or physical stimuli, may mature to become DAPI(+)/PI(-) viable oocysts. The latter hypothesis is supported by results from the harsh treatments with acid or alkali.

In one batch of oocysts these acid and alkali treatments tended to reduce the percentage viability with a reduction in the percentage of DAPI(+)/PI(-) oocysts and a corresponding increase in percentage PI(+) oocysts. However, in the batch of oocysts with a low viability initially, these treatments increased the percentage viability considerably, with an increase in the proportion of oocysts permeable to DAPI and a corresponding reduction in the proportion of DAPI(-)/PI(-) oocysts. This suggests that permeability to DAPI should be considered in interpreting results of environmental and disinfection studies (Blewett, 1989; Campbell *et al*, 1982; Korich *et al*, 1990; Peeters *et al*, 1989). That these alterations in viability predicted by inclusion/exclusion of DAPI and PI correlated with the viability assessed by *in vitro* excystation provides further evidence of the validity of utilising these dyes in a viability assay.

Whether viability correlates with infectivity is an important issue that is presently unanswered, although a correlation between *in vitro* excystation and infection of mice with large inocula (10^5 oocysts/mouse) of

Cryptosporidium oocysts has been reported (Blewett, 1989).

It is possible that digestive processes may mature DAPI(-)/PI(-) oocysts *in vivo* making them capable of excystation and infection. Thus the DAPI(-)/PI(-) oocysts, although non-viable in *in vitro* excystation, are potentially infective. However, extrapolation from the results of harsh acid or alkali treatments suggests that although DAPI(-)/PI(-) oocysts may be matured by digestive processes, similarly DAPI(+)/PI(-) oocysts may be killed by the digestive processes experienced prior to the intestine where excystation and infection would occur. Our excystation efficiency could doubtless be improved by conversion of DAPI(-)/PI(-) oocysts to DAPI(+)/PI(-) oocysts. For example, a low pH or enzymatic induction step as utilised by Blewett (1989) could be incorporated although this might kill some DAPI(+)/PI(-) oocysts.

In conclusion, although simultaneous DAPI and PI staining is an excellent indicator of viability as defined by *in vitro* excystation it is unclear how accurately any *in vitro* excystation protocol correlates with infection *in vivo*.

Aging of *Cryptosporidium* oocysts under various environmental pressures

The robust nature of coccidian oocysts has long been recognised. They are well known to be resistant to many forms of environmental stress that would prove lethal to other species of infectious agents. Desiccation and freezing have apparently been noted to be the exceptions (Blewett, 1989). However, although data presented here supports the former theory, we have also demonstrated that a small proportion of oocysts are capable of surviving at least 799 h at -22°C. However, whether these oocysts were infective was not investigated, although, in our opinion, they should be considered to have that potential despite data presented by Sherwood *et al* (1982) in which a number of cryopreservative techniques (including freezing to -20°C for 14 days in PBS followed by a slow thaw) apparently destroyed the infectivity of *Cryptosporidium* oocysts to mice. Tzipori (1983) likewise claims that oocyst infectivity is destroyed by exposure to temperatures below freezing, but data is not provided to support this. Nevertheless, an anecdotal report (Nichols and Thom, 1985) describes the infection of an individual with *Cryptosporidium*, apparently as a result of accidental ingestion of previously frozen tripe contaminated with *Cryptosporidium* oocysts. Our data suggest that it may be unwise to assume that freezing renders *Cryptosporidium* oocysts incapable of inducing infection.

Investigation of laboratory models of water treatment processes on the viability of *Cryptosporidium* oocysts suggest that although contact with lime, ferric and alum individually kills oocysts, this is a phenomenon of alkalinity (lime) or acidity (alum and ferric). If the pH is corrected to approximately pH 6, then none of these

treatments appears to have any significant impact on the viability of *Cryptosporidium* oocysts.

Results from following the viability of oocysts placed in various environments reemphasises their resistance to the pressures that they are likely to encounter in a non-laboratory setting.

Relatively short term immersion in sea water had no impact on the viability of oocysts. Unfortunately we were not able to investigate whether longer periods of contact with sea water would kill oocysts. However, this result suggests that the risk of contracting cryptosporidiosis from accidentally ingesting oocysts while swimming from those areas of coast contaminated by sewage cannot be dismissed. Further work ir required to determine whether immersion in sea water for longer peroids of time is detrimental to oocysts.

Previous results from the effect of acid and alkali on oocyst viability suggested that it is likely to be DAPI(-)/PI(-) oocysts rather than DAPI(+)/PI(-) oocysts that are more resistant to deleterious pressures. Although this appears not to be supported by the results obtained here, with death occurring more rapidly in the oocyst suspension of a lower initial DAPI(+)/DAPI(-) ratio, this could be an artifact of strain difference.

Apart from the oocyst suspensions stored in the cow faeces, there appears to be a rapid conversion from DAPI(-) to DAPI(+) in the oocyst suspension of initial low viability, and it is probable that it is these DAPI(+) oocysts that then succumb to environmental pressures and die.

The oocysts stored in cow faeces demonstrate an interesting conversion from DAPI(+) to DAPI(-) for both isolates. Death rate is also comparable to that of the controls. This relatively high DAPI(-)/DAPI(+) ratio is also demonstrated in the 3 stools from infected individuals that were sampled over a longer period of time.

These results imply that a component of faeces, possibly a mucopolysaccharide or similar, might insert into the oocyst wall, converting them from DAPI(+) to DAPI(-). This faecal component would afford the oocyst further protection from environmental stresses, but be easily lost in digestive processes so that oocysts reaching the intestine would be viable (DAPI(+)) and thus be capable of excystation and infection. The possible identity of such a component is considered in the biochemical analysis of *Cryptosporidium* oocysts.

Biochemical analysis of *Cryptosporidium* oocysts

The environmentally stable nature of oocysts suggest that there must be a carbohydrate based polymer present on their outer surfaces. This is likely to be similar in construction to the cell walls of environmentally hardy organisms like yeasts (chitin, a polymer of N-acetyl glucosamine (GlcNAc)) (Calib, 1981) or *Giardia* spp. cysts (a polymer of

GalNAc) (Jarroll *et al*, 1989).

Lectins of known sugar specificity were used as probes to analyse the expressed carbohydrate moieties of *Cryptosporidium* oocysts. Lectins with an affinity for various residues of GalNAc (*Dolichos biflorus* agglutinin, SBA, *Phaseolus limensis* agglutinin, wheat germ agglutinin and *Bandeiria simplicifolia* I_4 agglutinin), GlcNAc (wheat germ agglutinin), galactose (PNA, *Ricinus communis* agglutinin and *Bandeiria simplicifolia* I_4 agglutinin) and mannose or glucose (Concanavalin A, Succinyl-Concanavalin A and LCA) all failed to react with the surface of *Cryptosporidium* oocysts.

This hypothetical structural polysaccharide is unlikely to be either chitin or the polymer of GalNAc believed to be present in *Giardia* spp. cysts, as neither wheat germ agglutinin nor *Phasolus limensis* agglutinin bound to the oocysts. This could perhaps, however, be a configurational incompatibility.

Evidence that the oocyst wall is composed of a polysaccharide which contains GalNAc is demonstrated by the binding of SBA to ruptured (dead) oocysts and to oocysts that have excysted. The fluorescence of freshly excysted oocysts with SBA is considerably greater than the fluorescence associated with SBA binding to the dead oocysts. This could possibly be due to enzymic processes degrading the terminal sugars.

Galactose might be surface expressed on sporozoites; only motile sporozoites were observed to bind to the lectin PNA.

Mannose and/or glucose is present within dead oocysts, as demonstrated by the binding of LCA. The LCA could not be competed off by either of the free monosaccharides. This is probably because the affinity of a lectin for a monosaccharide is often several orders of magnitude less than its affinity for the poly ligand (Goldstein and Hayes, 1978), and glycogen or a similar storage polysaccharide is certain to be present.

Despite none of the lectins tested being selective enough to bind to the outer oocyst wall, evidence that the main component of the outer wall is likely to be a carbohydrate is provided by the biotin labelling regimes.

Intact (DAPI(+) and DAPI(-)) oocysts express few primary amines on the outer oocyst wall as determined by le-biotin incorporation. The major constituents of these oocyst walls are carbohydrate based as determined by periodate oxidation.

Periodate oxidation followed by BIIZ labelling gives minimal information regarding structure or composition on its own. Most sugars at the terminal reducing end of a polysaccharide have available vicinal dihydrols which can be oxidised by periodate, therefore this technique is a very good indicator for the presence of such moieties.

Structural detail of the expressed carbohydrates is only available after labelling the oocysts with BHZ following specific oxidation with the enzyme galactose oxidase. Rim fluorescence was observed with PI(+) and ghost oocysts (*cf* SBA). GalNAc residues are available for both lectin binding and enzyme oxidation on the inside of oocysts only.

We hypothesise that the oocyst wall is composed mainly of a polysaccharide that contains GalNAc. Because of our inability to detect exposed galactose residues on the outer surfaces of viable intact (DAPI(+)/PI(-)) oocysts either with lectins or following galactose oxidase oxidation, whereas galactose was readily detected on non-viable (DAPI(+)/PI(+)) oocysts using both these methods, we speculate that the configurational shape must be such that any GalNAc exposed ends of the polysaccharide chains are directed towards the inside of the oocyst wall. In addition, if any galactose residues are present on the outer surface they must be organised in such a manner that makes them inaccessible to either lectin binding or enzyme catalysis.

Dead (PI(+) or ghost) oocysts express many more primary amines than intact (DAPI(+) and DAPI(-)) oocysts. This is not solely due to the proteins expressed by sporozoites, as dead oocysts exhibit very bright rim fluorescence after NHS-lc-biotin labelling. There also were many more biotin-labelled protein containing components visualised on a BEC luminogram. Those proteins specific to the outer oocyst wall that might provide a useful surface marker for loss of viability have yet to be defined.

Tilley *et al* (1990) has reported the presence of up to 18 discrete proteins on the outer wall of *Cryptosporidium* oocysts ranging from 15-300 kD. The bands obtained after ^{125}I labelling might not be from the outer oocyst wall, as is stated by the authors, as no estimation of oocyst viability was performed, either microscopically or by *in vivo* infection. The oocysts were also stored in a strong oxidising agent for an undefined period of time. It is therefore possible that at least some of the bands described were derived from dead oocysts and are not surface associated. It has been previously shown that the biotin labelling methods employed in our study is as sensitive as comparable radio-labelling techniques (Alvarez *et al*, 1989).

Many carbohydrate containing compounds can be visualised on a BEC luminogram following periodate-BHZ labelling. After freeze thawing then boiling the oocysts too many components appear for them to be individually recognised. This is possibly due in part to some degradation occurring as a result of freezing, thawing and boiling. The best methods for solubilising oocyst components were found to be a) boiling the labelled oocysts without

freeze-thawing, and b) freeze-thawing oocysts, and boiling the SDS soluble fractions in the supernatant of the freeze-thaw preparation. These loosely associated SDS soluble fractions offer the best hope for a surface marker for viability. More bands were observed in this type of preparation from viable oocysts than were observed in similarly prepared dead oocyst fractions. This could be an artifact of the method of killing the oocysts - either snap freezing in liquid nitrogen or heating to 60°C for two 10 min periods. Further research is required to optimise this system.

The galactose oxidase sensitive component of DAPI(-) oocysts observed following BHZ treatment then labelling with avidin fluorochromes could not be resolved by the BEC system. This component is likely to be a mucopolysaccharide or glycolipid. It is readily lost from DAPI(-) oocysts over a 48 h period and DAPI(+) oocysts are readily converted to DAPI(-) by faecal material (*cf* results from faecal environmental samples).

BHZ was observed to bind to some PI(+) oocysts without prior oxidation. This label was not associated with the oocyst outer wall, and was not resolved using the BEC system.

Biotin labelling methods used were unable to distinguish any difference between the strains of oocysts examined. However, the system is not yet optimised and further work is required.

Further work is required to elucidate the fundamental components associated with viable and non-viable *Cryptosporidium* oocyst surfaces. In particular, the possibility that the marked contrast between the amounts of protein available for labelling on dead (PI(+) and ghost) oocysts and the carbohydrate content of intact (DAPI(-) and DAPI(+)) oocysts could be utilised, should be explored.

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