# DEVELOPMENT OF A RAPID TEST KIT FOR *CRYPTOSPORIDIUM* AND *GIARDIA*

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# DEVELOPMENT OF A RAPID TEST KIT FOR
## *CRYPTOSPORIDIUM* AND *GIARDIA*

by

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# EXECUTIVE SUMMARY

## Background and motivation

The development of an efficient but simple detection method for South Africa for the parasitic protozoa was determined to be one of two priorities discussed at a Cryptosporidium Workshop organised by the Water Research Commission and held at Umgeni Water in February 1996. A need to establish a simpler and cheaper method for concentrating and quantifying *Cryptosporidium* oocysts and *Giardia* cysts from water had been discussed in previous meetings between Drs Macario (Wadsworth Center, New York State Department of Health), Dr Howgrave-Graham (University of Natal) and Mr Bailey (Umgeni Water). The Drs Macario were world leaders in Slide Immuno-Assay (SIA) technology and offered their expertise in a collaborative project to provide a test kit for use in developing countries.

During the planning of this project, immunomagnetic separation (IMS) for *Cryptosporidium* or *Giardia* was not commercially available and it was hoped to developed this in the project. Unfortunately however, during this period rapid development had taken place in the US and UK making IMS the standard clarification method in those countries. Therefore, the project emphasis shifted to taking IMS a step further by attempting to replace the commonly used concentration techniques with the development of a continuous IMS capture system from flowing water. Owing to the rapid development of methods for the detection of these protozoa, this project also served to test some of the most common methods of analysis available, under South African conditions. An in-depth literature review of these was also provided.

Since the mid-1970s, both *Cryptosporidium parvum* and *Giardia lamblia* have been recognised as important waterborne pathogenic protozoa of man, with the potential to cause diarrhoeal disease, in developed and developing countries. Faecal contamination of waterways has led to massive outbreaks such as that experienced in Milwaukee, Wisconsin, USA. Over 1.5 million consumers were exposed to the *Cryptosporidium* pathogen, of which 403 000 became ill and many of those who were immuno-compromised died (Lisle and Rose, 1995). In addition, this outbreak was estimated to cost the community millions of dollars (Lisle and Rose, 1995). More recently *Cryptosporidium* and *Giardia* made headline news in the wake of the award of the year 2000 Olympics host city, when a water utility in Sydney, Australia was alerted to their presence in the potable water supply. Whilst the incidence did not lead to a major outbreak, it did result in the resignation of senior water utility management (Hayward, 1998).

## Project objectives as specified in contract

1. Develop slide immunoenzymatic assay (SIA) reagents and technology targeting *Cryptosporidium* and *Giardia*, or antigens from these organisms and compare these to commercially available antibody test kits which will be optimised for use on turbid water samples.
2. Develop a multiple solid phase kit with SIA technology suitable for the detection of *Cryptosporidium* oocysts and *Giardia* cysts or their antigens.

3. Manufacture and standardise a neodymium magnet or electromagnet apparatus for concentrating oocysts.
4. Field-test the above system on South African rivers and dams and faecal samples to determine their suitability for turbid environmental water samples and pathological samples.

## Study design

An economical system for detecting *Cryptosporidium* oocysts and *Giardia* cysts in water concentrates using the slide immunoenzymatic assay (SIA), which is based on the principles of the enzyme-linked immunosorbent assay (ELISA), was developed. SIA was carried out in a reaction circle, delimited by an epoxy-Teflon coating, of a glass slide. This allowed rapid contact of reagents in the drop with the reagent anchored on the circle and enhances the specificity and sensitivity of SIA. A multiple solid-phase SIA test that combined liquid and solid phases (prefixed, dry ready-for-use reagents), for laboratory and field use, was optimised for use with water samples of varying turbidity.

Three commonly used methods used to concentrate *Cryptosporidium* oocysts or *Giardia* cysts from water, namely wound cartridge filtration, membrane dissolution and calcium carbonate flocculation combined with an immuno-fluorescence assay for detection were evaluated. They were then compared with the IMS and SIA detection techniques described above.

The detection of *Cryptosporidium* and *Giardia* in pre-concentrated water samples is often hampered by extraneous debris, which can mask the oocysts and cysts and immunomagnetic separation (IMS) can be used for clarification. Commercially available anti-*Cryptosporidium* or anti-*Giardia* coated paramagnetic beads (Dynabeads) were evaluated for their ability to recover (oo)cysts. Owing to cost implications, uncoated paramagnetic beads (Dynabeads) and tosylactivated Dynabeads were coated with monoclonal antibodies against *Cryptosporidium* or *Giardia* and compared with the commercially available Dynabeads.

A neodymium magnet was developed for "continuous" immunomagnetic capture of (oo)cysts from flowing water and was evaluated using commercially available Dynabeads. Polypropylene beads were coated in-house with antibodies specific to *Cryptosporidium* or *Giardia* and compared with these Dynabeads for their ability to recover (oo)cysts from "batch" and flowing waters.

## Brief summary of results and conclusions

Multiple-phase SIA was found to produce optimal colour reactions when the primary and secondary antibody slides were in solid phase at the optimal dilutions and the substrate used was provided as a liquid.. A 1:50 dilution of primary antibody against *Cryptosporidium* and a 1:5 dilution of anti-*Giardia* antibody were required to produce a colour reaction that could be seen by the naked eye while a 1:50 dilution of secondary antibody was used. Using these dilutions, the minimum number of oocysts and of cysts present in the samples giving a visibly detectable yellow reaction was 154 and 74 per 10 µl of concentrated sample respectively, although 30 and 37 respectively were possible with more concentrated reagents. Lower numbers of oocysts and cysts

could be detected spectrophotometrically, or with improved purity and specificity of antibodies, but would increase the cost. A simple low-cost spectrophotometer was constructed, but initial evaluation of the system showed that the instrument was not sufficiently sensitive. If normal flocculation techniques, like flocculation are used, then it would be possible to detect 30/10 liters, even from turbid waters.

SIA is potentially a simple, economical means of detecting *Cryptosporidium* and *Giardia* in pre-concentrated water at R 30-00 per sample, compared to other techniques such as IFA (which uses a fluorescence microscope), which would cost between R420-00 and R85-00 per sample (depending on the volume of concentrate stained and the accuracy required). However, a centrifuge would also be required.

Calcium carbonate flocculation proved to have the best recovery of (oo)cysts compared to membrane dissolution or wound cartridge filtration with a 66.5% average recovery of oocysts and a 70.0% average recovery of cysts. Although recovery of oocysts improved with IMS, following concentration by membrane dissolution or cartridge filtration, this was not the case following flocculation, owing to interferences.

The in-house coated tosylactivated beads had the highest recoveries of oocysts (95.5%) and cysts (80.1%) compared to the ordinary in-house coated beads and the commercially available Dynabeads. However, the cost was higher (R 409-89) than the commercially available beads (R 364-80).

Larger 10mm polypropylene beads, coated with high concentrations of antibody (anti-*Cryptosporidium*: 1:10 dilution and anti-*Giardia*: undiluted) had recoveries of oocysts ranging from 14.3 - 90.3%, whilst cyst recovery ranged from 28.0 - 83.6%. Following continuous capture from flowing water, up to 27.2% of oocysts and up to 41.9% of cysts were recovered using seven polypropylene immuno-beads contained within a filter-housing unit. With environmental waters and a flow rate of 9-11 $\ell$ min-1, using a maximum of 95 immuno-beads, the recovery of oocysts increased from 11.8% to 74.8%. The cost of producing these beads was R 33 per anti-*Cryptosporidium* bead and R 165 per anti-*Giardia* bead. This makes the polypropylene immuno-bead continuous capture system (PICCS) an appropriate but expensive means of simultaneously concentrating and clarifying (oo)cysts from a variety of water types.

**Extent to which objectives were reached and actions to be taken as a result of the findings**

All but the last objective as specified in the contract were completed, to a greater or lesser extent, as described above:

- Although the operating principles were demonstrated to perform, the processes generally still need to be optimised and refined.
- Owing to the complexity of developing the SIA and PICCS concentration and detection system for recovering (oo)cysts from water samples, it was not possible to evaluate faecal

or dung specimens owing to time constraints. However, as (oo)cysts are usually present in much higher numbers in infected faeces and dung, SIA should be able to recover (oo)cysts from such samples.

- It was also not possible to field test the system as such, but some environmental samples were tested, however. Indeed the apparatus could work well in a simple laboratory, but a concentration step would be required eg. flocculation, which would then also need a centrifuge, unless the PICCS system was additionally used. However, the usual fluorescence microscope and FIA reagents are not required.
- In its present un-optimised form the SIA technique could be used to detect and monitor oocysts and cysts at contamination sources where the protists are in high quantities. These could include run-off points from livestock farms, informal settlement areas or especially for monitoring sewage effluents.
- A comprehensive literature review has been provided of the commonly used concentration methods, together with a comparative study of these.

## Recommendations for future research and technology transfer

- The whole SIA process needs to be further optimised and refined for greater sensitivity, but shows great potential.
- An SIA colour chart correlating colour intensity with (oo)cyst concentration could be developed as a guide to the direct visual quantification of (oo)cysts in samples
- The simple low cost spectrophotometer for reading of SIA slides needs to be optimised/developed.
- The PICCS needs to be optimised in terms of flow rates and size of polypropylene beads to maximises (oo)cysts recovery from flowing water.
- The SIA should be tested against (oo)cysts in faecal and dung samples, as there is great potential for this application.

This report needs to be circulated to commercial development, research and educational establishments interested in producing and marketing water testing kits, who could take this research further.

## Publications

C. Jarmey-Swan, A. J. L. Macario, I. W. Bailey and A. R. Howgrave-Graham. 1998. Preparation of kits for the detection of *Cryptosporidium* and *Giardia* in water supplies of developing countries. In *WISA proceedings*, **2**, paper 2B-1.

C. Jarmey-Swan, A. J. L. Macario, I. W. Bailey and A. R. Howgrave-Graham. 1998. A simple and economic slide immunoenzymatic assay (SIA) for detection of *Cryptosporidium* and *Giardia* in water supplies of developing countries. *IAWQ Health-related Water Microbiology Conference, Vancouver pre-prints*, **10**, 24-31.

## Capacity building

Ms Jarmey-Swan spent three months at the Wadsworth Centre, New York State Department of Health under the auspices of Dr. Macario. During this time the SIA techniques were mastered and applied to detecting *Cryptosporidium* and *Giardia* in water and used to train the staff below.

Four previously disadvantaged individuals were employed during the duration of this project namely Zola Msiska, Lungile Mthembu, Nosipho Gulwa and Melanie Naidoo. They were trained in concentration of protists in water, clarification of water sample using IMS, coating of Dynabeads and polypropylene beads and the use of SIA and IFA for the detection of (oo)cysts.

Ms. Mthembu has subsequently been employed as a permanent technician at Umgeni Water.
Ms. Gulwa is now employed as a microbiologist with Amatola Water in the Eastern Cape.
Ms. Msiska obtained a BSc and was employed by the Agricultrual Research Council
Ms. Naidoo obtained a BSc and was employed by the WITS Medical School
Ms. Jarmey-Swan obtained an MSc from the University of Natal

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| | |
|---|---|
| BSA | Bovine serum albumen |
| CCD | Cooled charge couple device |
| CCS | Continuous capture system |
| CM+ | Complete medium (Dulbecco's modified Eagle medium) |
| DAPI | 4', 6-diamidino-2-phenylindole |
| DIC | Differential interference contrast |
| DNA | Deoxyribonucleic acid |
| ELISA | Enzyme linked immunosorbent assay |
| FCCS | Flow cytometry and cell sorting |
| FISH | Fluorescent *in situ* hybridisation |
| FITC | Fluorescein isothiocyanate |
| FSC | Forward scatter |
| HCl | Hydrochloric acid |
| hsp | Heat shock protein |
| IFA | Immunofluorescence assay |
| IMS | Immunomagnetic separation |
| LED | Light emitting diode |
| LSD | Light sensing diode |
| mRNA | Messenger ribonucleic acid |
| NaOH | Sodium hydroxide |
| NTU | Nephelometric turbidity units |
| OPD | o-phenylenediamine |
| PBS | Phosphate buffered saline |
| PBS/BSA | Phosphate buffered saline / bovine serum albumen |
| PCR | Polymerase chain reaction |
| PICCS | Polypropylene immunobead continuous capture system |
| RNA | Ribonucleic acid |
| RT-PCR | Reverse transcriptase- polymerase chain reaction |
| RT | Room temperature |
| SIA | Slide immunoenzymatic assay |
| SSC | Side scatter |
| std. dev. | Standard deviation |
| USA | United States of America |

## INTRODUCTION

Since the mid-1970s, both *Cryptosporidium parvum* and *Giardia lamblia* have been recognised as important waterborne pathogenic protozoa of man, with the potential to cause diarrhoeal disease, in developed and developing countries.

Faecal contamination of waterways has led to massive outbreaks such as that experienced in Milwaukee, Wisconsin, USA. Over 1.5 million consumers were exposed to the *Cryptosporidium* pathogen, of which 403 000 became ill and many of those who were immuno-compromised died (Lisle and Rose, 1995). In addition, this outbreak was estimated to cost the community millions of dollars (Lisle and Rose, 1995). More recently *Cryptosporidium* and *Giardia* made headline news in the wake of the award of the year 2000 Olympics when a water utility in Sydney, Australia was alerted to their presence in the potable water supply. Whilst the incidence did not lead to a major outbreak, it did result in the resignation of senior water utility management (Hayward, 1998).

Suspected sources of contamination in outbreaks have included raw sewage, runoff from cattle grazing areas or agricultural land, treated wastewater from boats, faults in operational procedure or mechanical and operational deficiencies at water treatment plants (Hayes *et al.*, 1989). Properly operated conventional treatment (coagulation, sedimentation and filtration) can remove 99.0% or more oocysts and cysts (Nieminski, 1994). The commonly used water treatment disinfectant, chlorine, has been shown to be ineffective against *Cryptosporidium* oocysts (Korich *et al.*,1990; Ransome *et al.*, 1993)., Ozone has emerged as one of the most promising alternatives for inactivation of *Cryptosporidium* and *Giardia*, although disinfection by-products may form (Joret *et al.*, 1997; Ozekin and Westerhoff, 1998).

It is therefore important that the water industry monitor waters for the presence of protozoa to ensure public health safety. Routine analysis for *Giardia* and *Cryptosporidium* in environmental samples includes: (1) a concentration step leading to a reduction of the volume from 10-1000 $\ell$ to 0.5 m$\ell$, (2) a purification step which separates the (oo) cysts from the interfering particles and (3) a detection step. Speciation (usually by molecular biology techniques such as PCR) and viability assessment are more specialised techniques used at this time only by research laboratories.

Methods of concentration and detection are generally notoriously laborious, inaccurate and expensive which is probably the reason most countries do not standard procedures or legislation governing the presence of the organisms in water. However, since mid-1999, the United States of America's Environmental Protection Agency (EPA) and the United Kingdom Drinking Water Inspectorate (DWI) have issued standard operating procedures for the concentration and detection of *Cryptosporidium* and *Giardia* from water using filtration (Envirocheck cartridges or Genera Filta-Max cartridges respectively), immunomagnetic separation (IMS) and the immunofluorescence assay (IFA).

## 1.1 Objectives

The objectives of the project were to:

1. Develop slide immunoenzymatic assay (SIA) reagents and technology targeting *Cryptosporidium* and *Giardia*, or antigens from these organisms and compare these to commercially available antibody test kits which will be optimised for use on turbid water samples.

2. Develop a multiple solid phase kit with SIA technology suitable for the detection of *Cryptosporidium* oocysts and *Giardia* cysts or their antigens.

3. Manufacture and standardise a neodymium magnet or electromagnet apparatus for concentrating oocysts.

4. Field-test the above system on South African rivers and dams and faecal samples to determine their suitability for turbid environmental samples and pathological samples.

## 2    LITERATURE SURVEY

### 2.1    Concentration techniques

A variety of different techniques have been evaluated for their ability to recover oocysts and cysts from potable and turbid water samples. Unlike conventional bacteriology, the techniques involve large volumes of water, which can either be filtered on-site or collected and filtered or flocculated in the laboratory. The most popular methods are described below.

### 2.1.1    Cartridge filters

The most commonly used procedures include the filtration of large volumes of water (20 - 1000 $\ell$) through a cartridge filter, elution of the entrapped oocysts and cysts from the filter matrix with further concentration and possibly clarification, prior to detection by staining and microscopy. A number of different media type cartridge filters, namely polypropylene, cotton, borosilicate glass microfibre and Vokes polyfil, have been evaluated for their recovery efficiency of oocysts and cysts. Recoveries of oocysts from seeded tap water samples ranged from 0.4 to 129.0% (Rose et al., 1986; Musial et al., 1987; Gilmour et al., 1991; Whitmore and Carrington, 1993; LeChevallier et al., 1995; Nieminski et al., 1995) while cyst recoveries from seeded tap water samples ranged from 9.8 - 68% (Gilmour et al., 1991). Recovery of oocysts and cysts from seeded turbid water samples are generally much lower and ranged from 3.9 – 14.2% and 9.8 – 20.4% respectively (Shepherd and Wyn-Jones 1996).

The Genera filters (£30.00), which are currently used in the UK standard procedure, are comprised of multiple layers of reticulated open foam discs, compressed to act as a filter. Envirocheck cartridges, however, have been shown to be suitable for a range of water matrices with excellent recoveries. Using the Envirocheck cartridges, Cryptosporidium recovery from tap, synthetic turbid water and surface water samples averaged 91.8%, 74.0% and 55.0% respectively while the average recovery of Giardia was 89.5%, 95.0% and 67.0% for each water type analysed (Clancy et al., 1997). Other authors reported an average recovery rate of 25% for Cryptosporidium in surface water samples (turbidity around 10 NTU) (Cervantes et al., 1997).

Of the cartridge filters available, the Gelman Envirocheck cartridges have the best recovery of oocysts and cysts from a variety of water types although the cost of these cartridges ($ 80.00) may hinder their routine use.

### 2.1.2    Membrane filters

In membrane filtration a certain volume of water is passed through a membrane medium of specified pore size and the retained material removed from the surface and collected for further processing. Differences in filter matrices have been noted to affect the elution of the (oo)cysts and their subsequent recovery from samples (Shepherd and Wyn-Jones, 1996). This method is not feasible for use with larger volumes of water (100 - 1 000 $\ell$) especially raw waters as the membranes are prone to blockages. In addition, each membrane costs approximately US$4.00.

Polycarbonate membrane filters had recoveries of 27.2 - 101.1% depending on the pore size used and water matrix analysed. An optimum recovery of oocysts and cysts from seeded tap and synthetic turbid (five NTU) water was obtained by Clancy et al. (1997) using a 3.0 µm pore size polycarbonate membrane. Polyethylsulfone membranes (nominal porosity 0.8 µm) were found to have much lower recoveries (around 20% for oocysts and 40% for cysts), probably the

membrane pore was too small or the membrane binding capacity had irreversibly attached many of the cysts and oocysts to the surface (Shepherd and Wyn-Jones, 1996).

Cellulose acetate membranes (1.2 µm pore size) were able to recover 32.0 – 87.0% of oocysts from three to 10 liters of seeded tap water (Aldom and Chagla, 1995; Shepherd and Wyn-Jones, 1996) whilst 4.5 – 51.3% of cysts were recovered from 10 ℓ seeded tap water (Shepherd and Wyn-Jones, 1996). The material retained on the membrane was either eluted by scraping with a rubber policeman, sonication or dissolution, which resulted in a higher recovery range of 61.0 – 87.0% (Aldom and Chagla, 1995). Cellulose-nitrate (pore size 3.0 µm) and acrylic copolymer membranes had recoveries of 10.2 –36.0% for oocysts and 22.4 – 77.6% for cysts from seeded tap water (10 ℓ) (Shepherd and Wyn-Jones, 1996).

### 2.1.3  Flocculation

Calcium carbonate flocculation (Vesey et al., 1993) has gained popularity as a means of routinely sampling and concentrating smaller volumes of water (10 - 20 ℓ). It involves the addition of calcium chloride and sodium bicarbonate which produces a floc of finely dispersed calcium carbonate in the water sample. This floc settles over a period of time (minimum of four hours), thereby causing oocysts, cysts and other particulate matter to sediment. Acid is used to dissolve the carbonate residue and the remaining debris is further concentrated by centrifugation.

This technique is simple, robust and economic (approx. US$ 2.00 per sample), and is less labour intensive than other methods (Vesey et al., 1993). It has been shown to have consistently high recoveries (71.3 - 76.0%) in deionised, tap and river water (Vesey et al., 1993; Shepherd and Wyn-Jones, 1996). A difficulty with this technique is that it concentrates all particulates (Vesey et al., 1993) and can reduce oocyst viability (Campbell et al., 1994) which may be of concern in potable water samples. Other flocculation techniques, namely ferric sulfate and aluminium sulfate, have been described by the same authors and later adapted and tested by Compagnon et al. (1999a). Ferric sulfate gave the best results, with 100% recovery for Cryptosporidium for water samples between 6 and 15 NTU and 100% recovery for Giardia for water turbidities up to 30 NTU. Ferric sulfate flocculation could therefore be the method of choice for concentrating the cysts and oocysts from surface and waste water samples. The main drawback of the technique is that only a small volume of the pellet can subsequently be analyzed; it should thus be used in association with a clarification technique eg IMS allowing the reduction of the volume of the concentrate still further.

## 2.2   Clarification of concentrated samples

The detection of Cryptosporidium and Giardia in water samples is hampered by extraneous debris, which can mask the oocysts and cysts (Smith et al., 1995) especially in concentrated samples. Immunomagnetic separation and density gradient centrifugation have been used to alleviate this problem.

### 2.2.1  Immunomagnetic separation (IMS)

Immunomagnetic separation (IMS) or antibody-magnetite, using small paramagnetic beads coated with antibodies against surface antigens of cells, has been shown to be efficient in isolating certain eukaryotic cells (Ugelstad et al., 1992) and prokaryotic organisms such as bacteria, as well as viruses (Olsvik et al., 1994) from different media including water

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(Rossomando et al., 1994). IMS allowed high recoveries for *Cryptosporidium*: 49 to 67% from raw water and 87 to 97.4% from turbid waters (Campbell et al., 1997; Stanfield et al., 1999). The method is simple, fast and reaches small volumes of concentrates. It is therefore possible to observe the entire concentrate obtained by this technique, thus bringing the detection limit to one (oo)cyst in the sampled volume. IMS is therefore today becoming the method of choice for purifying concentrates in routine analysis although it is expensive (approx. US$ 40.00 per sample).

### 2.2.2 Density gradients

A number of *Cryptosporidium* and *Giardia* concentration procedures include a density gradient to separate oocysts or cysts from debris in as they will float on the top of the gradient while heavier debris will be centrifuged to the bottom (LeChevallier et al., 1995). Various gradient solutions have been described (Rose et al. 1988). LeChevallier et al. (1995) found the 1.15 specific gravity Percoll-sucrose gradient efficient in the recovery of oocysts (100.0%) and of cysts (110.0%) from seeded tap water. The same specific gravity recovered 67.0% of oocysts and 96.0% of cysts from seeded environmental samples.

Other researchers found recoveries of oocysts were low when sucrose flotation techniques were used with seeded tap water (0.7 - 16.0%) and river water samples (0.4 - 11.2%) compared to higher recoveries without the use of the density gradient (Shepherd and Wyn-Jones, 1995). Other researchers also found that oocysts and cysts were lost during the sucrose gradient flotation step and recommend that this process be avoided when processing treated water samples (Nieminski et al 1995). These differences in recovery may, however, be affected by the concentration method used (Compagnon et al., 1999), density of the gradient used (LeChevallier et al., 1995) or the viability status of the (oo)cysts (Bukhari and Smith, 1995; LeChevallier et al., 1995). Although sucrose flotation removes interfering debris which may make microscopy easier, the discrepancy in recoveries highlights the need to evaluate the use of this technique with various concentration procedures.

### 2.2.3 Flow cytometry

Flow cytometers, which cost approximately US$ 100 000 to purchase, measure the amount of light scattered in the forward and in the 90° angle directions (side scatter). These measurements correlate to size and internal complexity respectively; in addition the instrument measures the fluorescent light emitted by each particle. Detection of *Cryptosporidium* oocysts and *Giardia* cysts by flow cytometry requires labelling with fluorescein which, when excited by laser light, emits energy which is detected by the flow cytometer's detectors, converted to electrical energy, quantitated and plotted (Hoffman et al., 1995). Campbell et al. (1993) have used flow cytometry to separate oocysts from contaminating debris in water samples. Confirmation of the oocyst identity was by a cooled charge couple device (CCD) connected to a fluorescent microscope with the appropriate excitation and emission filters. This computer controlled CCD system allowed three-dimensional visualisation of individual oocysts and could map the detailed morphology and exact size of the oocysts, thereby eliminating the need for visual confirmation by fluorescence microscopy.

As flow cytometry is unable to distinguish between oocysts and some auto-fluorescent plants, algae or mineral particles in water samples combinations using flow cytometry and cell sorting (FCCS) have been used to separate oocysts and cysts from background debris (Vesey et al.,

1993). This resulted in the recovery of oocysts and cysts seeded into river and reservoir water samples in excess of 92% (Vesey et al., 1994) or 100% in river and partially treated water samples (Compagnon et al., 1997). They found that the actual quantification or confirmation of sorted particles by microscopy was rapid and easy. In addition, fluorogenic techniques are well suited to flow cytometry and could be used simultaneously with antibodies to detect oocysts and cysts and to determine viability (Vesey et al., 1994). Disadvantages of flow cytometry include the highly trained personnel requirements for the running of the cytometer, the need to pre-concentrate, the difficulty in obtaining the equipment due to the expense and the need for confirmation of oocyst and cyst identity by microscopy (Jakubowski et al., 1996). Owing to the time and equipment needed, it cannot be regarded as a routine technique. Moreover, only a fraction of the concentrate can be analysed, leading to high detection limits.

## 2.3 Detection methods for *Cryptosporidium* and *Giardia* in water

Detection of oocysts and cysts in the water sample concentrates is generally performed by fluorescence microscopy using monoclonal antibodies bound to fluorogenic dyes thereby highlighting the oocyst or cyst wall (Smith et al., 1995).

### 2.3.1 *Microscopic observation*

Commercially available direct immunofluorescence (the antibody conjugated to the fluorochrome)(approx. US$14.00 per sample) assays use fluorescein isothiocyanate (FITC) labeled monoclonal or polyclonal antibodies specific to *Cryptosporidium* or *Giardia* antigen. The stained preparation is examined by epifluorescence microscopy and oocysts and cysts are identified using the following criteria: (a) distinct apple green fluorescence of the (oo)cyst wall; (b) shape: *Cryptosporidium* spherical; *Giardia* oval; (c) size: *Cryptosporidium* 2 - 6 $\mu$m; *Giardia* 8 - 18 $\mu$m in length and 5 - 15 $\mu$m in width; (d) (optional) the presence of internal structures under differential interference contrast (DIC). In a *Cryptosporidium* oocyst one to four crescent shaped sporozoites (motile infective stage) and a granular residual body (contains ribosomes, endoplasmic reticulum and micronemes) may be present. *Giardia* has the following internal structures: axoneme (a bundle of fibres at the centre of a flagellum), two to four nuclei and median bodies (claw-hammar shaped paired organelles found in the posterior half of the cyst).

Confirmation by DIC or phase contrast is required when examining environmental samples to avoid false positives which may occur with algae, which are similar in size, shape and fluorescence to *Cryptosporidium* and *Giardia*, due to cross-reactivity (Nieminski et al., 1995). IFA results are very dependant on the quality of the monoclonal antibodies (Zanelli et al.,1999). IFA is also labour intensive and requires an experienced microscopist (Jakubowski et al., 1996) while debris in environmental samples may also lead to false negatives as (oo)cysts can be occluded (Johnson et al., 1993). To reduce the likelihood of this occurring, the nucleic acid stain 4', 6-diamidino-2-phenylindole (DAPI) used in conjunction with the immunofluorescence assay (IFA) to provide an easy, reproducible method for the detection and identification of sporulated oocysts, as sporozoite nuclei are stained sky-blue (Grimason et al., 1994).

### 2.3.2 *Laser scanning cytometry*

Laser scanning cytometry allows the rapid automated analysis of an entire 25 mm diameter membrane within three minutes. A special machine has been developed by the Chemunex Company for the detection of FITC labelled *Cryptosporidium* from water concentrates. The data are analysed on line to determine if the fluorescent signal originates from labelled

*Cryptosporidium* oocysts or from some other fluorescent particles. At the end of the scan the results are displayed on a map, which identifies the position of the presumed oocysts on the membrane surface. Each event is then confirmed by observation under epifluorescent microscopy. The Chemscan has proved to detect more oocysts than direct microscopic observation or flow cytometry (Reynolds *et al.*, 1996; Fricker, 1998). This application is compatible with the existing concentration (flocculation, cartridge filtration) and purification (percoll-sucrose gradient, IMS) methods and is being evaluated for the enumeration of *Giardia* cysts (Zanelli *et al.*,1999). Although the price of the equipment (US$ 125 000) still prevents widespread utilisation, laser-scanning cytometry greatly facilitates microscopic observations, reducing the time of observation by four to six times and rendering the enumeration more reliable.

The techniques described above for concentration, purification of the concentrate and detection do not allow the differentiation between human pathogenic and non-pathogenic species, and do not give information on the viable/infectious state of the (oo)cysts. They are however the most commonly routinely used techniques for the enumeration of *Giardia* cysts and *Cryptosporidium* oocysts from environmental samples.

# 3 DEVELOPMENT OF A MULTIPLE PHASE KIT WITH SIA TECHNOLOGY

*Cryptosporidium* and *Giardia* detection techniques such as laser scanning cytometry combined with microscopy (Fricker, 1998) or polymerase chain reaction (PCR)(Stinear *et al.*, 1996) are gaining popularity in countries possessing the required capital equipment and infrastructure. However, cheap, simple and easy to use detection methods for detecting protozoons in water and faecal samples are still necessary. An economic system for detecting *Cryptosporidium* oocysts and *Giardia* cysts in water, using the slide immunoenzymatic assay (SIA), was developed and is described in this chapter.

Slide immunomagnetic separation (SIA) has proved useful for various antigen-antibody systems such as immunologic studies of methanogenic bacteria and hybridoma technology (Conway de Macario *et al.*, 1986). It is based on the principles of the enzyme-linked immunosorbent assay (ELISA) and is carried out on a reaction area of a glass slide delimited by an epoxy-Teflon coating rather than conventional microtitre plates. This geometry allows rapid contact of reagents in the drop with the reagent anchored on the circle and enhances the specificity and sensitivity of SIA (Conway de Macario *et al.*, 1986). Other potential advantages of SIA attributable to its geometry are that it requires small volumes of sample ($\leq$ 10 $\mu$l) and reagent and allows microscopic examination for further confirmation (Conway de Macario *et al.*, 1986). The potential also exists for the development of a multiple solid-phase test (prefixed dry ready-for-use reagents) or a kit combining liquid and solid phases, for laboratory and field use (Conway de Macario *et al.*, 1987).

## 3.1 Materials and methods

The basic procedure from which all SIA variations derive involves the steps presented in Figure 3.1. A series of preliminary experiments was done to calibrate the system for the detection of *Cryptosporidium* oocysts and *Giardia* cysts, the technical details and variations of which are described where applicable. The number of oocysts and cysts present in the dilutions used was therefore determined once optimisation of the system was complete.

Apply oocyst / cyst suspension to reaction circle on SIA slide and air dry

↓

Heat-acetone fix

↓

Apply 10μl primary antibody (mouse anti-*Cryptosporidium* / anti-*Giardia*)

↓

Incubate (30 minutes; room temperature)

↓

Wash and air dry

↓

Apply 10μl secondary antibody (sheep anti-mouse)

↓

Incubate (30 minutes; room temperature)

↓

Wash and air dry

↓

Apply 10μl OPD substrate

↓

Read by direct visual observation or by spectrophotometry

**Figure 3.1. Flow diagram depicting the steps involved in SIA**

### 3.1.1 Calibration of the SIA system

The purified *Cryptosporidium* oocyst (Human AZ-1 strain, produced in mice and purified from faeces by sodium chloride centrifugation gradients, PRL DyNAgenics, Arizona, USA) and *Giardia* cyst suspensions (CH-3 strain, produced in Mongolian gerbils and purified from faeces by zinc sulphate centrifugation gradients, PRL DyNAgenics, Arizona, USA)) were homogenised by inverting the respective tube several times. 5 μl volumes of 1:5 and 1:25 dilutions of the oocyst suspension made in PBS (pH7.2) and 1:5 and 1:10 dilutions of the cyst suspension were applied in triplicate to reaction circles delimited on epoxy-Teflon coated SIA slides (Cel-Line Inc., New Jersey, USA) respectively and air dried. The positive control was 10 μl of each dilution applied in triplicate to the reaction circles and air dried. The oocysts / cysts were heat-acetone fixed onto the slide by passing the slide through a flame three times, allowing to cool and repeating the flaming process. The protozoa were further fixed by the addition of a drop (20 μl) of acetone. A 1:5 dilution of mouse anti-*Cryptosporidium* antibody (Waterborne, Inc.,Louisiana, USA) made in CM+(complete medium which is Dulbecco's modified Eagle medium; Sigma, Montana, USA) and undiluted mouse anti-*Giardia* antibody (Waterborne, Inc., Louisiana, USA) were applied to the respective reaction circles (10 μl). The negative control was 10 μl CM+ (used in place of the primary antibody). The respective slides were incubated in a humidified chamber at room temperature for 30 minutes.

Following incubation, the circles were washed gently with distilled water. A 1:50 dilution of secondary peroxidase labelled sheep anti-mouse antibody (Sigma, Montana, USA) was applied to the reaction circles on the slides. The slides were incubated for 30 minutes at room temperature in a humid chamber then washed with distilled water and air dried. The o-phenylenediamine (OPD; 1 mg OPD diluted in 1 ml of 0.1M citric acid buffer (pH 4.5) with 1 μl of 30% hydrogen peroxide added just prior to use) substrate solution (10 μl) was added to each reaction circle being tested and the slides were returned to the humid chamber. The slides were read by direct visual observation of a yellow colour (pale, mid or dark)(positive) and readings were taken at intervals between 5-45 minutes using a vertical beam spectrophotometer (OD$_{450nm}$) (Dynatech Laboratories Inc., Virginia, USA) and compared with the negative control.

As the use of concentrated primary antibody is expensive, the following experiments were performed using a 1:50 dilution of anti-*Cryptosporidium* antibody and a 1:5 dilution of anti-*Giardia* antibody and the procedure followed as described in Figure 3.1. Ten microlitres of a 1:10, 1:25 or 1:50 dilution of secondary peroxidase labelled sheep anti-mouse antibody was applied to the reaction circles on the slides to determine the optimum secondary antibody concentration which produced a visually detectable colour reaction.

### 3.1.2  Number of oocysts and cysts giving positive colour reactions with SIA

Microscopic quantification of oocysts or cysts present in samples giving a positive colour reaction after SIA was carried out in the following manner:

Five microlitres of a 1:5 dilution of purified *Cryptosporidium* or *Giardia* suspension was applied in triplicate to glass slide wells as higher dilutions did not produce visually detectable colour reactions. After air drying, 10 μl of primary antisera (mouse anti-*Cryptosporidium* or mouse anti-*Giardia* antibodies) were applied to the respective reaction circles and incubated for 30 minutes at room temperature in a humidified chamber. Following incubation the slide was washed and air dried and a 1:1000 dilution (made in CM+) of fluorescein isothiocyanate (FITC) labelled goat anti-mouse antibody (Sigma, Montana, USA) was applied (10 μl). The slide was incubated for 30 minutes at room temperature, washed and air dried. The sample was viewed using oil immersion with phase contrast, differential interference contrast (DIC) and fluorescence (450-490 nm) microscopy on a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany). The oocysts and cysts were identified as described in 3.3.

### 3.1.3  Multiple phase SIA

#### 3.1.3.1  Preparation of slides for multiple liquid and solid-phase SIA.

*Antigen slide.* Purified *Cryptosporidium* oocyst and *Giardia* cyst suspensions (PRL DyNAgenics, Arizona, USA) were homogenised by inverting the respective tubes several times. Five microlitre volumes of 1:5 and 1:25 dilutions (made in PBS (pH7.2) of the oocyst suspension and 1:5 and 1:10 dilutions of the cyst suspension were applied in triplicate to reaction circles delimited on epoxy-Teflon coated SIA slides (Cel-Line Inc., New Jersey, USA) respectively and air dried. The positive control was 10 μl of each dilution applied in triplicate to the reaction circles and air dried. The oocysts / cysts were heat-acetone fixed onto the slide by passing the slide through a flame three times, allowing to cool and repeating the flaming process.

The protists were further fixed by the addition of a drop (20 μl) of acetone. These slides were made immediately prior to conducting the experiments as the sample of interest would always be in the liquid phase.

*Primary antibody slide.* Ten microlitres of a 1:50 dilution of mouse anti-*Cryptosporidium* antibody (Waterborne, Inc., Louisiana, USA) made in CM+ and a 1:5 dilution of mouse anti-*Giardia* antibody (Waterborne, Inc., Louisiana, USA) were applied to the respective reaction circles and allowed to air dry. The negative control was 10 μl CM+ (used in place of the primary antibody). The slides were stored in a slide-box at 4°C for future use.

*Secondary antibody slide.* Ten microlitres of a 1:50 dilution of peroxidase labelled sheep anti-mouse antibody (Sigma, Montana, USA) was anchored onto each circle of SIA slides by air drying. The slides were stored as described above for the primary antibody slides.

*Substrate slide.* Ten microlitres of OPD solution were anchored onto each circle of SIA slides precoated with Sigmacote (Sigma, Montana, USA) and stored as described above for the primary antibody slides.

### 3.1.3.2 Basic procedure for multiple phase SIA.

The module, made in-house, for multiple phase SIA is composed of two slide frames joined by a hinge along one of their longer sides (Conway de Macario *et al.*, 1987) (Figure 3.2.).
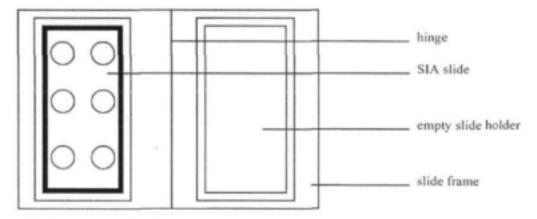


**Figure 3.2 Diagram of the basic multiple phase slide module**

The hinged frames were opened and positioned flat on the bench top. The antigen slide was inserted in the left hand side frame with the circles up. Likewise, the primary antibody slide was inserted in the right-hand side frame. Ten microlitres of distilled water was placed onto each circle of the antigen slide. The frame was closed so that the antibody slide lay on top of the antigen slide with matching circles in both slides facing each other so that the contents mix. The closed module was incubated at room temperature for five minutes. Following the incubation, the module was opened and the antibody slide removed and discarded. The solid phase secondary antibody slide was placed in the empty frame and the module closed or 10 μl of a 1:50 dilution of labelled secondary antibody (liquid phase) was applied to each reaction circle of the antigen slide and incubated at room temperature for a further five minutes.

After the second incubation the antigen slide was removed and the circles washed with five drops of distilled water. The slide was air dried and repositioned in the frame. The substrate was added in either the solid phase or liquid phase as follows:

1. The solid phase substrate slide was placed in the module and 10 µl drops of distilled water containing 0.02 % hydrogen peroxide (30%) were placed on each reaction circle of the antigen slide and substrate slide. The module was closed and the reaction read by direct visual observation of colour (yellow) and on the vertical beam spectrophotometer (450nm) (Dynatech Laboratories Inc., Virginia, USA)) at intervals (5-45 minutes).

2. The liquid phase of the substrate was applied as follows: OPD solution (10 µl) was added to each reaction circle being tested and the slides were returned to the humid chamber and read spectrophotometrically as stated above.

### 3.1.3.3 Multiple phase SIA development

The following combinations of liquid and solid phase solutions were evaluated for SIA use as described above:

a) A combination of the liquid phase antigen slides, solid phase primary and secondary antibody slides and solid phase substrate slides. All solid phase slides were used after one week storage at 4°C. The experiment was repeated two weeks later.

b) Liquid phase antigen slides were combined with solid phase primary antibody slides, liquid phase secondary antibody slides which were made up immediately prior to this experiment and solid phase substrate slides. As a pale yellow colour resulted from the positive control in the above experiment, fresh substrate slides were made up and stored at 4°C. Thereafter, experiments were always performed using the fresh substrate slides.

c) Multiple phase SIA was performed using liquid phase antigen slides, solid phase primary and secondary antibody slides and liquid phase substrate slides and the resulting colour determined by direct visual observation and spectrophotometry.

### 3.1.4 Costing analysis

A costing analysis was done to establish SIA's applicability for use in developing countries. The cost of equipment, consumables, reagent and technician time required to perform one SIA analysis for the detection of *Cryptosporidium* or *Giardia* was used in the calculation.

## 3.2 Results and discussion

### 3.2.1 Calibration of the SIA system

The optimum fixation method was determined to be a heat-acetone combination, which allowed for the fixation of the most protozoons with uniform distribution.

The 1:5 dilution of primary antibody against *Cryptosporidium* produced a dark yellow colour (Maximum absorbance: 0.46) with the 1:5 dilution of *Cryptosporidium* antigen and a lighter

yellow colour (maximum absorbance: 0.36) with the 1:25 dilution of antigen when used in combination with the 1:50 dilution of labelled secondary antibody.

The undiluted primary antibody against *Giardia* produced a much lighter yellow colour with the 1:5 (maximum absorbance: 0.42) and 1:10 dilutions (Maximum absorbance: 0.43) of *Giardia* antigen when used in combination with the 1:50 dilution of labelled secondary antibody. Spectrophotometric readings for both dilutions of both antigens were above the positive and negative controls.

The more dilute anti-*Cryptosporidium* (1:50 dilution) produced a pale yellow colour distinguishable from the background when reacted with the 1:5 dilution of *Cryptosporidium* antigen and 1:10 dilution of labelled secondary antibody. Maximum absorbance occurred after 30 minutes incubation (0.28) with the 1:5 dilution of *Cryptosporidium*. No colour formed when the same dilutions of primary and secondary antibodies reacted with the 1:25 dilution of *Cryptosporidium* (Absorbance range: 0.11 - 0.15).

The 1:5 dilution of antibody against *Giardia* produced a pale yellow colour (Maximum absorbance : 0.20) when reacted with 1:5 dilution of *Giardia* and 1:10 dilution of labelled secondary antibody while no colour resulted with the 1:10 dilution of *Giardia*.

A greater dilution of labelled secondary antibody (1:25 dilution) resulted in a pale yellow colour (Absorbance range: 0.22 - 0.24) being produced with the 1:5 dilution of *Cryptosporidium* and no colour being produced with the 1:25 dilution of *Cryptosporidium* when combined with a 1:50 dilution of primary antibody.

The 1:25 dilution of labelled antibody produced a pale yellow colour (Maximum absorbance: 0.25) with the 1:5 dilution of *Giardia* antigen whilst the 1:10 dilution of antigen was colourless (Maximum absorbance: 0.22) when combined with a 1:5 dilution of primary antibody.

The 1:5 dilution of *Cryptosporidium* was pale yellow in colour when reacted with 1:50 dilutions of primary and secondary labelled antibody which was most intense after 30 minutes incubation. The range of absorbance readings with time (0.27 - 0.55) were above the background readings (0.18 - 0.25) (Figure 3.3.).
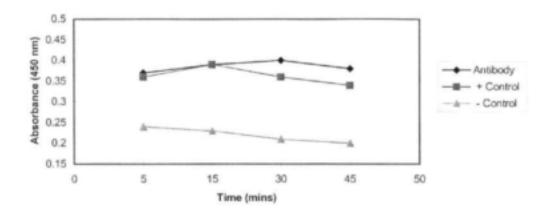
Figure 3.3. Absorbance readings using 5 μl of a 1:5 dilution of *Cryptosporidium* combined with a 1:50 dilution of primary and secondary antibody between 5-45 minutes

The 5 μl of a 1:25 dilution of *Cryptosporidium* did not produce a colour, which was significantly distinguishable from the negative control when reacted with the primary and secondary antibody although the absorbance readings ranged from 0.23-0.33 and were above the background readings (0.19 - 0.26) (Figure 3.4.).
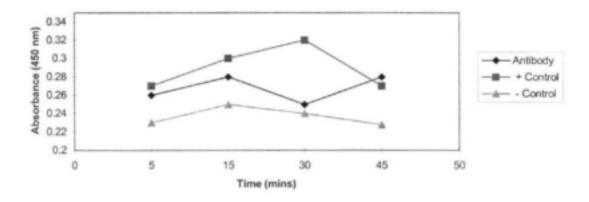


Figure 3.4 Absorbance readings using 5 μl of a 1:25 dilution of *Cryptosporidium* combined with a 1:50 dilution of primary and secondary antibody

The 5 μl volume of both *Giardia* dilutions produced a range of absorbance readings above the negative controls (1:5 dilution: 0.28 - 0.53; 1:10: 0.20 - 0.44) but direct visual observation of the 1:5 dilution was a distinct pale yellow colour while the 1:10 dilution could not be visually distinguished from the negative control despite the absorbance readings being above the background (Figure 3.5. and 3.6. respectively).

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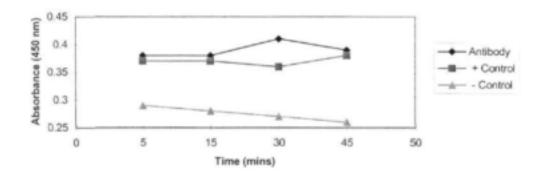
Figure 3.5   Absorbance readings using 5 µl of a 1:5 dilution of *Giardia* combined with a
1:5 dilution of primary antibody and a 1:50 dilution of secondary antibody
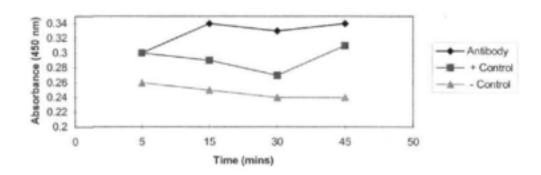


Figure 3.6   Absorbance readings using 5 µl of a 1:10 dilution of *Giardia* combined with a
1:5 dilution of primary antibody and a 1:50 dilution of secondary antibody

### 3.2.2   *Number of oocysts and cysts giving positive colour reactions with SIA*

From the triplicate samples examined, the number of (oo)cysts per five microlitres that
produced a visible colour was determined to average 154 and 74 respectively. A lower
minimum detection level would be possible by visual observation of colour and by
spectrophotometry using a more concentrated primary antibody against *Cryptosporidium* (1:5
dilution) combined with the 1:50 dilution of labelled secondary antibody. Similarly, undiluted
primary antibody against *Giardia* would allow fewer cysts to be detected by visual observation of
colour and spectrophotometrically when combined with the 1:50 dilution of labelled secondary
antibody. These systems are, however, less cost effective as they require 10 and five times
more primary antibody respectively. These were not quantified, but from extrapolation the
systems would be more sensitive and allow for the detection of 30 oocysts and 37 cysts
respectively.

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### 3.2.3 Multiple phase SIA development

a) A yellow colour was observed for both dilutions of *Cryptosporidium* and *Giardia* in liquid phase respectively when combined with the solid phase primary and secondary antibody and solid phase substrate.

The *Cryptosporidium* absorbance readings (0.28 - 1.80) were above the background (0.28 - 1.10) (Figure 3.7.) The *Giardia* absorbance readings (0.30 - 0.78) were also above background (0.15 - 0.34)(Figure 3.8.). When the experiment was repeated two weeks later with the same reagents, no colour was produced and the absorbance readings were similar to the negative control.
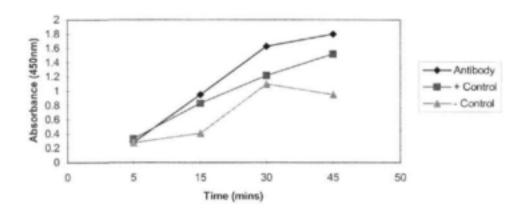


**Figure 3.7** Absorbance readings of a 1:5 dilution of *Cryptosporidium* in liquid phase when used in combination with solid phase primary and secondary antisera and solid phase substrate
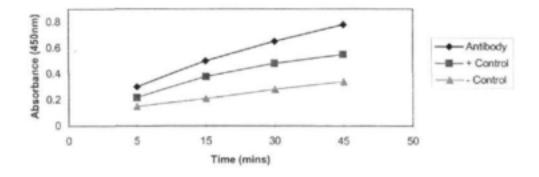


**Figure 3.8** Absorbance readings of a 1:5 dilution of *Giardia* in liquid phase when used in combination with solid phase primary and secondary antisera and solid phase substrate

b) The use of the secondary antibody in the liquid phase produced a colour reaction which could not be distinguished from the negative control. The use of the freshly prepared substrate produced a yellow colour reaction which could be seen by direct visual observation of the 1:5 dilution of *Cryptosporidium* and the 1:5 dilution of *Giardia*.

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The *Cryptosporidium* absorbance readings (0.10 - 0.14) were above the background (0.09 - 0.10)(Figure 3.9.) as were the *Giardia* absorbance readings (0.12 - 0.16)(Background: 0.11 - 0.16)(Figure 3.10.). The 1:25 dilution of *Cryptosporidium* and the 1:10 dilution of *Giardia* did not produce a colour that could be distinguished from the negative control.

c) The use of liquid phase substrate increased the absorbance and produced a yellow colour with the 1:5 and 1:25 dilution of *Cryptosporidium* (Maximum absorbance: 0.30) and the 1:5 and 1:10 dilution of *Giardia* which increased in intensity with time and was most intense from 15 to 30 minutes (Maximum absorbance: 0.24)(Figures 3.11. and 3.12.).
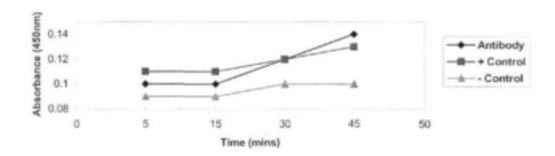


**Figure 3.9**   **Absorbance readings of a 1:5 dilution of *Cryptosporidium* in liquid phase when used in combination with solid phase primary antisera, liquid phase secondary antisera and solid phase substrate**

The positive control (10 µl) absorbance readings may be lower than that of the antisera reactions (5 µl) due to the unavailablity of antigenic sites as a result of clumping of the oocysts / cysts in the positive control (Figures 3.7., 3.8. and 3.11).
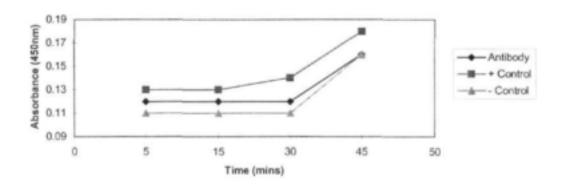


**Figure 3.10**   **Absorbance readings of a 1:5 dilution of *Giardia* in liquid phase when used in combination with solid phase primary antisera, liquid phase secondary antisera and solid phase substrate**
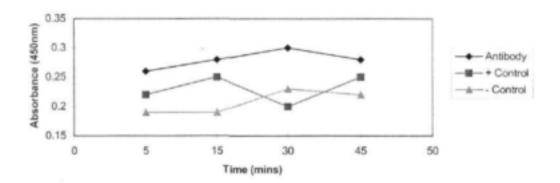
**Figure 3.11.** Absorbance readings of a 1:5 dilution of *Cryptosporidium* in liquid phase when used in combination with solid phase primary and secondary antisera and liquid phase substrate
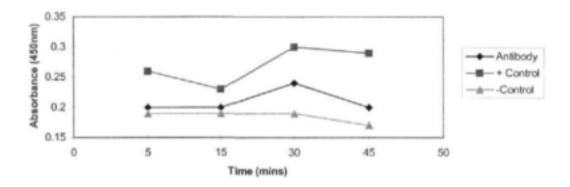


**Figure 3.12** Absorbance readings using a 1:5 dilution of *Giardia* in liquid phase when used in combination with solid phase primary and secondary antisera and liquid phase substrate

The basic SIA procedure can be further enhanced and optimised in many ways to increase the sensitivity:

(1) Detection of oocysts and cysts could be enhanced by applying small volumes (5 μl / 10 μl ) of the concentrated sample, allowing to air dry and repeating this procedure until the entire concentrated sample has been air dried on the slide reaction circle. The SIA procedure can then be used and will react with the protozoons present. However the increase in time for each sample is a disadvantage.

(2) A colour chart correlating colour intensity with oocyst / cyst concentration could be developed as a guide to the direct visual quantification of oocysts / cysts in samples. As spectrophotometric readings are able to distinguish the sample from the background (negative control) at lower concentrations of antigen sample, a cheap and easy to construct spectrophotometer could be used in place of expensive capital equipment (Smith *et al.*, 1991).

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(3) Incubation times for the different antibodies can be shortened to 15 minutes or less when the antisera are strong. This is time saving especially when multiple tests must be performed in one session.

As spectrophotometric readings are able to distinguish the sample from the background (negative control) at lower concentrations of antigen sample, a cheap and easy to construct spectrophotometer could be used in place of expensive capital equipment (Smith *et al.*, 1991).

This preliminary work showed that SIA is a sensitive technique for detecting *Cryptosporidium* oocysts and *Giardia* cysts. Results were produced in triplicate and the reproducibility of repeat samples was good. A practical advantage of SIA attributable to its geometry is that it requires only 10 µl or less of sample and reagents which saves sample and reduces costs (Conway de Macario *et al.*, 1986). SIA proved to be a cheap means of detecting *Cryptosporidium* and *Giardia* in water concentrates (R 30-00 per sample; APPENDIX 1). Other techniques such as IFA use FITC labelled antibodies which cost between R 420-00 and 85-00 per sample depending the volume of sample required for staining.

## 3.3 Conclusions

- A 1:50 dilution of primary antibody against *Cryptosporidium* and a 1:5 dilution of anti-*Giardia* antibody were required to produce a colour reaction that could be seen by the naked eye and would be cheaper than fluorogenic labelled antibodies. A 1:50 dilution of peroxidase labelled secondary antibody was used in both systems. These dilutions are dependent on reagent quality and could be increased with improved purity and specificity of primary or secondary antibody .
- Using a more cost effective system (higher dilution) the minimum number of oocysts and of cysts present in the samples giving a visibly detectable yellow reaction was 154 and 74 per 5 µl respectively. Fewer oocysts and cysts (30 and 37 respectively) produced a colour reaction that was visible to the naked eye by using an increased concentration of primary antibody against *Cryptosporidium* and *Giardia*. A greater increase in sensitivity may also be achieved using a spectrophotometer although this was not quantified.
- The SIA detection method is, as are other detection methods, dependent on the ability of the concentration technique to recover the oocysts and cysts present in the sample. Therefore a complementary continuous immunobead system was developed.
- SIA is a simple tool, which is less time-consuming and requires less capital expenditure than other methods. It also only requires small volumes of reagent which further reduces costs and can be performed at small sparsely equipped laboratories or in the field. In addition a simple and relatively inexpensive vertical-beam spectrophotometer could be constructed from locally available parts and operated either from a 12 volt battery or with available AC current (Smith *et al.*, 1991).
- The SIA results were reproducible and the potential exists for the development of a solid-phase system for storage of dry ready-for-use reagents for laboratory and field use. Versatility is a salient feature of SIA (Conway de Macario *et al.*, 1986) and slide design and distribution of circles on the slide surface can be varied according to the needs of the SIA kit. Further refinement will enhance the sensitivity of the SIA for oocyst and cyst detection and lead to the development of the optimum multi-phase combinations.

# 4    DEVELOPMENT OF A CONTINUOUS CAPTURE SYSTEM

The detection of *Cryptosporidium* and *Giardia* in concentrated water samples is hampered by extraneous debris, which can mask the oocysts and cysts (Smith *et al.*, 1995). Immunomagnetic separation and density gradient centrifugation have been used to alleviate this problem. However, researchers found that oocysts and cysts were lost during the sucrose gradient flotation step and recommend that this process be avoided when processing treated water samples (Nieminski *et al.*, 1995).

Immunomagnetic separation (IMS) or antibody-magnetite, using small paramagnetic beads coated with antibodies against surface antigens of cells, has been shown to be efficient in isolating prokaryotic organisms such as bacteria, as well as viruses from different media including water (Rossomando *et al.*, 1995). IMS allowed high recoveries for *Cryptosporidium*: 49 to 67% from raw water and 87 to 97.4% from turbid waters (Stanfield *et al.*, 1999). Previous studies have used the beads in "batch" mode (Bifulco and Schaefer, 1993). However, in contrast to other methods such as filtration or flocculation, which have the potential to process large volumes (>10 litres), the use of immunobeads in the batch mode is usually limited to smaller volumes (10 m$\ell$). However, even larger volumes can be processed using a continuous flow system.

As the sensitivity of the entire analytical method is limited by the concentration procedure, it would be beneficial to improve this step. The direct capture of (oo)cysts from flowing water would decrease the time taken to analyse for these protozoons and may enhance recovery.

## 4.1    Materials and methods

### 4.1.1    Quantification of oocysts and cysts for use in seeding experiments

Purified *Cryptosporidium* oocysts and *Giardia* cysts for use in seeding experiments were quantified as follows:

#### 4.1.1.1    Slide enumeration

A 1:5 working dilution of stock *Cryptosporidium* oocysts (Human AZ-1 strain, produced in mice and purified from faeces by sodium chloride centrifugation gradients, PRL DyNAgenics, Arizona, USA) or a 1:10 dilution of *Giardia* cysts (CH-3 strain, produced in Mongolian gerbils and purified from faeces by zinc sulphate centrifugation gradients, PRL DyNAgenics, Arizona, USA) were prepared in phosphate buffered saline (PBS; pH 7.4) and five microlitres of each suspension was applied to six wells each of a microscope slide. (Oo)cysts were stained and enumerated as described in 4.1.2.

#### 4.1.1.2    Flow cytometric enumeration

A FACSCalibur flow cytometer (Becton Dickinson, Belgium) was used as follows:
Purified suspensions of unstained *Cryptosporidium* oocysts and *Giardia* cysts (PRL DyNAgenics, Arizona, USA) were diluted in milli-Q water to give approximately $10^5$ m$\ell^{-1}$. A dot plot using forward scatter (FSC) and side scatter (SSC) was created and each suspension was run. The detector levels were adjusted to place the oocyst and cyst populations on scale until a well defined region for the oocysts and cysts respectively were obtained (Table 1).

**Table 4.1 Instrument settings for sorting of unstained *Cryptosporidium* oocysts and *Giardia* cysts for seeding experiments**

| Detector | *Cryptosporidium* | | | *Giardia* | | |
|---|---|---|---|---|---|---|
| | Voltage | Amp Gain | Mode | Voltage | Amp Gain | Mode |
| FSC | E00 | 2.00 | Lin | E-1 | n/a | Log |
| SSC | 400 | n/a | Log | 300 | n/a | Log |

n/a = not applicable

A threshold parameter of 54 or 525 was placed on the FSC detector for *Cryptosporidium* and *Giardia* respectively. The threshold was kept below 100 to reduce cells being aborted and a sort gate was drawn around the well defined region. The flow cytometer was requested to sort a particular number of oocysts or cysts in single cell mode. The acquired (oo)cysts were collected in 50 m*l* plastic tubes and stored at 0-10°C until use.

Sort efficiency was assessed by sorting 100 (oo)cysts onto 0.8 μm cellulose acetate membranes and enumerating the (oo)cysts as described in 4.1.2. This was repeated five times each.

### 4.1.2 Staining and enumeration of Cryptosporidium *oocysts and* Giardia *cysts*

#### 4.1.2.1 Staining in suspension

(Oo)cysts were stained in suspension by the addition of an equal volume of fluorescein isothiocyanate (FITC) labelled monoclonal antibodies against *Cryptosporidium* and *Giardia* (Crypto/Giardia–Cel IF, Cellabs, New South Wales, Australia) and incubated at 37°C for 30 minutes according to the manufacturer's instructions (APPENDIX 2). The entire stained sample was applied to microscope slide wells in 20 μl aliquots or filtered onto a 1.2 μm cellulose acetate membrane.

#### 4.1.2.2 Staining on a membrane

(Oo)cysts were stained by placing the membrane on a drop (20 μl) of FITC labelled antibodies (Crypto/Giardia–Cel IF, Cellabs, New South Wales, Australia) and incubating in a humidified chamber at 37°C for one hour.

#### 4.1.2.3 Staining on a slide well

The sample of interest was applied to a microscope slide well (20 - 50 μl), air dried and fixed with 20 μl of methanol. (Oo)cysts were stained by the addition of 25 μl of fluorescein isothiocyanate (FITC) labelled monoclonal antibodies against *Cryptosporidium* and *Giardia* (Crypto/Giardia–Cel IF, Cellabs, New South Wales, Australia) to the well and incubated at 37°C for 30 minutes according to the manufacturer's instructions (APPENDIX 2). After incubation the well was gently washed with PBS (pH 7.4) and allowed to air dry.

#### 4.1.2.4 Enumeration of stained *Cryptosporidium* oocysts and *Giardia* cysts

A Zeiss Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany) fitted with differential interference contrast (DIC) and selected excitation of 450 - 490 nm and barrier filter of 520 nm was used with X400 magnification to examine the stained samples.

Each well or membrane was systematically scanned, the (oo)cysts enumerated and identified using the following criteria: (a) distinct apple green fluorescence of the (oo)cyst wall; (b) shape; (c) size; (d) the presence of internal structure under differential interference contrast (DIC)(LeChevallier et al., 1995).The average number of (oo)cysts and the standard deviation was calculated.

### 4.1.3 Comparison of commonly used concentration techniques

The following concentration protocols were evaluated for their ability to recover Cryptosporidium oocysts and Giardia cysts from seeded potable water samples. After concentration each pellet was stained and (oo)cysts were enumerated as described in 4.1.2.

#### 4.1.3.1 Cartridge filtration

Three 100- litre seeded potable water samples ($10^4$ oocysts and $10^5$ cysts) were filtered through wound cartridge filters at a flow rate of three litres per minute (Badenoch et al., 1989) (APPENDIX 3 ). The concentrated samples were stained on a slide well and viewed using epifluorescence microscopy (4.1.2.3; 4.1.2.4).

#### 4.1.3.2 Membrane dissolution

Three 10-litre seeded potable water samples ($10^4$ oocysts and $10^5$ cysts) were concentrated by membrane dissolution (Aldom and Chagla, 1995) using 1.2 $\mu$m pore

sized cellulose acetate membranes. (APPENDIX 4). The concentrated sample was stained on a slide well and viewed using epifluorescence microscopy (4.1.2.3; 4.1.2.4)

#### 4.1.3.3 Calcium carbonate flocculation

Three 10-litre seeded potable water samples ($10^4$ oocysts and $10^5$ cysts) were concentrated by calcium carbonate flocculation (Vesey et al., 1993)(APPENDIX 5). The concentrated sample was stained on a slide well and viewed using epifluorescence microscopy (4.1.2.3; 4.1.2.4)

### 4.1.4 Testing of Dynal protocol for coating Dynabeads with Crypto or Giardia IgM

Due to the cost implications of purchasing commercially available anti-Cryptosporidium or anti-Giardia Dynabeads, uncoated paramagnetic beads (Dynabeads)(Dynal A.S., Oslo, Norway) and tosylactivated Dynabeads (Dynal A.S., Oslo, Norway) were coated with monoclonal antibodies against Cryptosporidium or Giardia (Waterborne, Inc., Louisiana, USA). These "homemade" Dynabeads were compared with commercially available coated Dynabeads for their ability to isolate oocysts or cysts from seeded water samples.

#### 4.1.4.1 Coating of Dynabeads or tosylactivated Dynabeads with Cryptosporidium IgM or Giardia IgM

**Prewash of commercially available uncoated beads**

Dynabeads M-450 or tosylactivated M-450 (Dynal A.S., Oslo, Norway) were resuspended in the vial by gently vortexing and shaking for one minute. Five hundred microlitres of Dynabeads M-450 /tosylactivated M-450 was transferred to an Eppendorff tube that was placed on the Magnetic Particle Concentrator (MPC)(Dynal A.S., Oslo, Norway) for two minutes. After the fluid was pipetted off, the tube was removed from the MPC and resuspended in an excess volume of washing buffer (PBS pH 7.4).

The tube was placed on the MPC for two minutes after which the fluid was pipetted off. The washed Dynabeads were resuspended in one millilitre of washing buffer until use.

## Coating of commercially available uncoated beads

The washed Dynabeads were resuspended thoroughly in 0.1 M phosphate buffer pH 7.4 by vortexing. Pure antibodies (50 µg) (1 mg ml⁻¹ of *Cryptosporidium* IgM or *Giardia* IgM; Waterborne, Inc.,Louisiana, USA) were added per $10^7$ Dynabeads and vortexed for two minutes. The beads and antibody were incubated for 10 minutes at 37°C with gentle tilt rotation using a blood rotator (Polychem Supplies cc., Durban, South Africa). After the incubation period, the blocking protein BSA (0.5%) was added to provide optimal orientation of the antibodies. The tube was placed on the rotator and incubated for 24 hours at 37°C. After incubation, the tube was placed in the MPC for three minutes and the supernatant was removed. The coated Dynabeads were washed three times in 0.1% PBS/BSA pH 7.4 for five minutes at 4°C and stored in 0.1% PBS/BSA until use.

### 4.1.4.2 Recovery of *Cryptosporidium* and *Giardia* from seeded potable water samples using "homemade and commercially available Dynabeads

#### "Homemade" Dynabeads / tosylactivated Dynabeads

One hundred microlitres of "homemade" uncoated beads or tosylactivated Dynabeads were resuspended and added to a 10 mℓ tube containing a known number of (oo)cysts seeded into potable water. The 10 mℓ potable water solution containing the oocysts and cysts was homogenised by carefully inverting the tube. The tube was rotated on a blood rotator for an hour at room temperature. Thereafter the tube was placed on the MPC and gently rocked. The supernatant was removed and PBS was added to the tube. After mixing, the liquid was transferred to an Eppendorff tube and placed in the MPC. After a gentle rock/roll action for one minute, the beads formed a clear "dot" at the back of the tube. The supernatant was removed and the bead/(oo)cyst complex was dissociated by the addition of 0.1M HCl.  All liquid remaining after dissociation was transferred to slide wells containing 1M NaOH, stained and quantified as described in 4.1.2.

#### Commercially coated Dynabeads

One hundred microlitres of commercially available anti-*Cryptosporidium* and anti-*Giardia* Dynabeads (GC Combo, Dynal A.S., Oslo, Norway) were resuspended and added to a 10 mℓ tube containing a known number (oo)cysts seeded into potable water. The samples were treated as described in the Dynal protocol for separation of oocysts and cysts using GC Combo Dynabeads (APPENDIX 6). All liquid remaining after dissociation was transferred to slide wells containing 1M NaOH, stained and quantified as described in 5.1.2.

### 4.1.4.3 Cost analysis

A cost analysis for the preparation of ordinary and tosylactivated Dynabeads was undertaken and compared with the cost of purchasing commercially available anti-*Cryptosporidium* and anti-*Giardia* Dynabeads (Dynal A.S., Oslo, Norway).

### *4.1.5 Comparison of* Cryptosporidium *oocysts from seeded water samples following various concentration techniques and "batch" immunomagnetic separation*

"Batch" immunomagnetic separation requires pre-concentration by a method of choice. In order to determine the efficiency of "batch" separation, the following experiments were performed with commonly used concentration techniques and commercially available anti-*Cryptosporidium* Dynabeads.

### 4.1.5.1 Seeded water sample concentration by membrane dissolution, cartridge filtration or calcium carbonate flocculation

Three 10-litre distilled water samples for each of three concentration methods to be evaluated were inoculated with five microlitres of 1:5 dilution of purified *Cryptosporidium* oocysts (PRL DyNAgenics, Arizona, USA) and homogenised by continuous stirring. Each of the three seeded water samples was concentrated by membrane dissolution (Aldom and Chagla, 1995)(APPENDIX 4) or cartridge filtration (Badenoch *et al.*, 1989)(APPENDIX 3) performed without the sucrose flotation procedure or calcium carbonate flocculation (Vesey *et al.*, 1993)(APPENDIX 5). Once concentration was complete the samples were stored at 0-10°C until isolation using Dynabeads anti-*Cryptosporidium* (Dynal A.S., Oslo, Norway).

The positive control was a 10 m*l* distilled water sample was seeded with five microlitres of the working dilution (1:5) of purified oocysts quantified in 4.1.1 and subjected to oocyst capture by immunomagnetic separation.

### 4.1.5.2 Concentrated water sample clarification by immunomagnetic separation

The concentrated water samples (4.1.5.1) were equilibrated to room temperature and the pellets re-suspended in milli-Q. The samples were centrifuged at 1050 *g* (10 min) until the final pellet was 0.5 m*l* per 10 m*l* of sample. Oocyst capture from concentrated water samples by paramagnetic beads coated with antibody against *Cryptosporidium* was performed as stated in the manufacturer's instructions using a Magnetic (neodymium iron boron) Particle Concentrator (APPENDIX 6).

### 4.1.5.3 Detection of oocysts from seeded water samples following immunomagnetic separation

The solution left after dissociation of the bead / oocyst complex, by the addition of hydrochloric acid (HCl)(APPENDIX 8), was transferred to slide wells containing 1M NaOH, stained and quantified as described in 4.1.2.

## 4.1.6 Continuous immunomagnetic capture

### 4.1.6.1 Construction of a laboratory-scale continuous immunomagnetic capture system

A prototype flow-through system, which allows water samples to circulate with immunomagnetic beads labelled specifically against *Cryptosporidium* or *Giardia* was designed and constructed by the workshop at the University of Natal, Pietermaritzburg (Figures 4.1 and 4.2). The system comprised a 1-litre glass reservoir with inlet (A) for the addition of water or paramagnetic beads, a peristaltic pump and magnetic housing. All components of the apparatus were connected with plastic tubing (5 mm).

The magnetic housing comprised a steel armature in which two neodymium iron boron magnets were seated. A Perspex wedge separated the magnets from the bottom of the steel armature and an eight millimetre gap separated the North and South poles of the magnets. An eight millimetre copper tube was made to fit in the gap. This tube was flattened in the centre to allow increased magnetic flux between the magnetic pole pieces, which could be inserted or removed as required for capture or release of the anti-*Cryptosporidium* or anti-*Giardia* labelled paramagnetic beads.
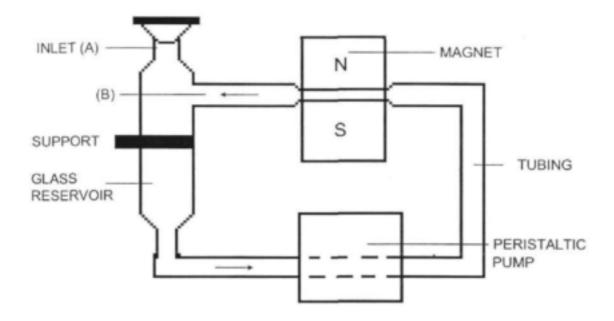
Figure 4.1  Laboratory-scale continuous immunomagnetic capture system
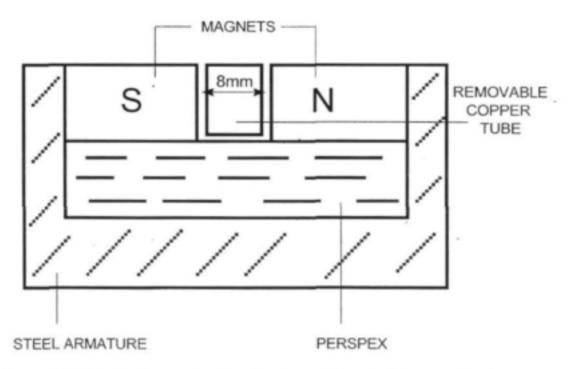


Figure 4.2  Housing for neodymium iron boron magnets (cross-section)

Distilled water samples (500 m$\ell$) were seeded with a known number of *Cryptosporidium* oocysts and *Giardia* cysts (PRL DyNAgenics, Arizona, USA) and homogenised. The sample was poured into the glass reservoir at the inlet (A) and the peristaltic pump switched on to allow the water to pre-equilibrate for 15 minutes at a flow rate of one litre per minute. Thereafter commercial anti-*Cryptosporidium* and anti-*Giardia* beads (GC Combo Dynal A.S., Oslo, Norway) were added through the inlet and the pump was switched on for a further five minutes to allow the beads to mix with the water sample and attach to the (oo)cysts.

The copper tube was placed between the neodymium iron boron magnets and the peristaltic pump was switched on for 30 minutes. The tube connected to (B) was removed and placed in a beaker and the pump switched on until all the water in the tubes was pumped to waste. Once the system was free of water, the tubing was carefully detached from the copper tube and an Eppendorff tube was fitted to one end of the tube. The copper tube was carefully removed, by keeping horizontal, from the magnet. The copper tube was carefully removed, by keeping horizontal, from the magnet. The copper tube was held vertical and washed with two millilitres of distilled water thereby capturing the bead / (oo)cyst complex in the Eppendorff tube.

The Eppendorff tube was placed in the Magnetic Particle Concentrator (MPC) (Dynal A.S., Oslo, Norway) and rocked gently through 180º for one minute during which the beads and (oo)cysts formed a clear dot on the back of the tube. The bead / (oo)cyst complex was dissociated according to the manufacturer's instructions (Dynal A.S., Oslo, Norway; APPENDIX 8) and the (oo)cysts were detected as described in 4.1.2. The isolated beads were also examined using bright field microscopy and DIC so that any (oo)cysts that may have remained bead-bound could be detected.

### 4.1.7 Capture of (oo)cysts using coated polypropylene beads

#### 4.1.7.1 Coating of polypropylene beads with anti-*Cryptosporidium* and anti-*Giardia* antibody

Polypropylene beads (16 mm) were incubated in 250 $\mu\ell$ of anti-*Cryptosporidium* antibody (1:50 or 1:10 dilution of 1 mg $ml^{-1}$) or anti-*Giardia* antibody (1:5 or undiluted 1 mg $ml^{-1}$) (Waterborne, Inc., Louisiana, USA). The tubes were placed on a blood rotator (Polychem Supplies cc., Durban, South Africa) with gentle tilt rotation for 10 minutes at 37ºC. One millilitres of BSA (0.5%) was added to block the reaction and vortexed. The tubes were replaced in the rotator and incubated as at 37ºC for 24 hours. After incubation the beads were washed with PBS containing 0.1% Tween 20. This step was repeated. Beads were stored in one millilitre of 0.1% PBS/BSA until use.

#### 4.1.7.2 Batch capture of oocysts or cysts from seeded potable water samples using coated polypropylene beads

A 15 m$\ell$ centrifuge tube containing potable water was seeded with a known number of *Cryptosporidium / Giardia* (oo)cysts as determined in 4.1.1. In order to determine the optimal concentration of antibody required to maximise (oo)cyst recovery, a polypropylene bead, coated with each antibody dilution specific to the protist of interest, was placed in the tube and allowed to mix in a blood rotator (Polychem Supplies cc., Durban, South Africa) for 30 minutes at room temperature. The supernatant was collected in a clean centrifuge tube for further analysis to quantify any (oo)cysts which had not been captured. The bead was removed and placed in a clean centrifuge tube to which 50 $\mu$l of 0.1M HCl was added. The tube was vortexed for five seconds. The tube was allowed to stand in a vertical position for five minutes at room temperature after which it was further vortexed for five seconds. All the liquid from the tube was transferred onto slide wells which contained five microlitres of 1M NaOH. The slide wells were air dried, stained and enumerated as described in 4.1.2.

#### 4.1.7.3 Continuous capture of oocysts or cysts from seeded potable water samples using coated polypropylene beads

A filter-housing unit was filled with the optimal dilution of anti-*Cryptosporidium* and anti-*Giardia* coated (undiluted) polypropylene beads (7 anti-*Cryptosporidium* beads; 7 anti-*Giardia* beads). The inflow pipe to the filter housing unit was connected via a pump to a bucket containing the potable water sample seeded with a known quantity of (oo)cysts (4.1.1). The pump was battery operated, allowing a flow rate of 10 $\ell$ min$^{-1}$ (slower flow rates could not be achieved due to the nature of the pump). The outflow pipe allowed the water exiting the filter housing unit to be collected in a separate bucket for further analysis to determine the number of non-recovered (oo)cysts.

Once the seeded water sample had passed through the filter housing, the polypropylene beads were collected and subjected to hydrochloric acid to dissociate the bead / (oo)cyst complex (5.1.6.2). (Oo)cysts were stained on slide wells and enumerated as described in 4.1.2.

The water collected after filtration was concentrated by calcium carbonate flocculation (APPENDIX 5) and the concentrated pellet was stained and evaluated for the presence of (oo)cysts (4.1.2).

### 4.2 Results and discussion

### 4.2.1 Quantification of Cryptosporidium oocysts for use in seeding experiments

#### 4.2.1.1 Slide enumeration

The following results were obtained from the counting of *Cryptosporidium* oocysts or *Giardia* cysts in each microscope well using FITC labelled antibodies (Table 4.2). The average number of oocysts per 5 µl of the 1:5 dilution was determined to be 80 oocysts with a standard deviation of 9.94. The average number of cysts per 5 µl of the 1:10 dilution was determined to be 59.20 with a standard deviation of 13.44.

**Table 4.2 Number of (oo)cysts counted in each of six microscope wells**

| Replicate number | Number of *Cryptosporidium* oocysts | Number of *Giardia* cysts |
|---|---|---|
| 1 | 90 | 42 |
| 2 | 64 | 53 |
| 3 | 83 | 57 |
| 4 | 90 | 65 |
| 5 | 75 | 56 |
| 6 | 78 | 82 |

### 4.2.1.2 Flow cytometric enumeration

The average number of *Cryptosporidium* oocysts sorted by flow cytometry was 69.8 with a standard deviation of 6.61. The average number of *Giardia* cysts was 64.4 with a standard deviation of 12.9 (Table 4.3).

**Table 4.3 Number of purified (oo)cysts sorted by flow cytometry**

| Replicate number | Number of *Cryptosporidium* oocysts | Number of *Giardia* cysts |
|---|---|---|
| 1 | 71 | 73 |
| 2 | 62 | 42 |
| 3 | 64 | 72 |
| 4 | 75 | 65 |
| 5 | 77 | 70 |

Variances in the sorting efficiency are probably due to impurities (such as yeast cells) being present in the sample.

### 4.2.2 Concentration by membrane dissolution, cartridge filtration or calcium carbonate flocculation

Wound cartridge filtration and membrane dissolution had poor recoveries of *Cryptosporidium* oocysts and *Giardia* cysts from seeded potable water samples (Table 4.4 and 4.5).

**Table 4.4 Recovery of *Cryptosporidium* oocysts and *Giardia* cysts using wound cartridge filtration**

| Replicate number | No. of oocysts recovered | % oocysts recovered | No. of cysts recovered | % cysts recovered |
|---|---|---|---|---|
| 1 | 4 500 | 4.5 | 1252 | 11.8 |
| 2 | 3 900 | 3.9 | 1039 | 9.8 |
| 3 | 15 100 | 15.1 | 2364 | 22.3 |

The recovery of *Cryptosporidium* oocysts and *Giardia* cysts using wound cartridge filtration was poor (Table 4.4). The average recovery of oocysts was 7.8% whilst an average of 14.6 cysts were recovered. The higher recovery of *Giardia* cysts using wound cartridge filtration may be due to the larger size of the cysts, which are more likely to be retained in the filter cartridge than *Cryptosporidium* oocysts. It has been reported that between five and 30% of oocysts may pass through the cartridge filter (Vesey and Slade, 1991). Musial *et al* (1987) demonstrated that the seed size affects the recovery when cartridge filters and the lower the concentration of oocysts and cysts present, the less likely they are to be retained by the filter. Large variations in recovery efficiencies using this method have been reported between laboratories and even within a single laboratory (Clancy *et al.*, 1994; LeChevallier *et al.*, 1991). Recoveries of *Cryptosporidium* oocysts from potable water range from 70.0 to 80.0% (Rose *et al.*, 1986; LeChevallier *et al.*, 1991) to 1.0% or less (Colburn, 1989; Vesey and Slade, 1991) while an average recovery efficiency of 9.0 - 68.6% has been achieved for *Giardia* cysts (LeChevallier *et al.*, 1991; Clancy *et al.*, 1994).

Recoveries from turbid waters using this method was often less than 5% (Vesey *et al.*, 1993). Losses have been reported to occur throughout the procedure with large numbers passing through the filter or adhering to the filter material and not being recovered (Vesey and Slade, 1991).

**Table 4.5   Recovery of *Cryptosporidium* oocysts and *Giardia* cysts using membrane dissolution**

| Replicate number | No. of oocysts recovered | % oocysts recovered | No. of cysts recovered | % cysts recovered |
|---|---|---|---|---|
| 1 | 5 000 | 5 | 3 000 | 28.3 |
| 2 | 6 500 | 6.5 | 4 500 | 42.5 |
| 3 | 0 | 0 | 0 | 0 |

The membrane dissolution method was developed for application to potable waters and has been reported to have a 70.5% recovery of oocysts (Aldom and Chagla, 1995). However, our findings indicate a low average recovery of oocysts (3.8%) and cysts (23.6%)(Table 4.5). It is suspected that the (oo)cysts may have been lost during aspiration of the supernatant or the high centrifugation speed may have resulted in compaction of the particles from the water, onto and around the (oo)cysts. The lack of recovery experienced during one replicate, may have been due to the inability of the membrane to dissolve adequately. Recoveries were low for the membrane dissolution technique as (oo)cysts may have remained embedded in debris which did not dissolve in the acetone. Poor recoveries of oocysts and cysts using this method have also been experienced by other researchers (Clancy *et al.*, 1997).

Calcium carbonate flocculation had the highest average recovery of oocysts (66.5%) and cysts (70.0%) (Table 4.6).

**Table 4.6   Recovery of *Cryptosporidium* oocysts and *Giardia* cysts using calcium carbonate flocculation**

| Replicate number | No. of oocysts recovered | % oocysts recovered | No. of cysts recovered | % cysts recovered |
|---|---|---|---|---|
| 1 | 66 000 | 66.0 | 8 173 | 77.1 |
| 2 | 65 300 | 65.3 | 7229 | 68.2 |
| 3 | 68 200 | 68.2 | 6858 | 64.1 |

The calcium-carbonate flocculation technique was inexpensive and simple to use. A number of samples could be flocculated simultaneously although this step was time consuming, taking a minimum of four hours.

### 4.2.3   Recovery using "homemade" and commercially available Dynabeads

**"Homemade" Dynabeads**

Recovery of oocysts and cysts from seeded potable water samples using "homemade" uncoated Dynabeads M-450 were as follows:

**Table 4.7** Recovery of *Cryptosporidium* oocysts and *Giardia* cysts following IMS using "homemade" uncoated Dynabeads M-450

| *Cryptosporidium* | | | *Giardia* | | |
|---|---|---|---|---|---|
| No. oocysts seeded | No. oocysts recovered | Percentage oocysts (%) | No. cysts seeded | No. cysts recovered | Percentage cysts (%) |
| 1000 | 720 | 72.0 | 1000 | 729 | 72.9 |
| 1000 | 754 | 75.4 | 1000 | 717 | 71.7 |
| 966 | 712 | 73.7 | 890 | 634 | 71.2 |

**"Homemade" tosylactivated Dynabeads M-450**

Experiments using tosylactivated Dynabeads M-450, which had been coated with monoclonal antibodies against *Cryptosporidium* and *Giardia*, were found to have a good recovery of oocysts and cysts (Table 4.8).

**Table 4.8** Recovery of *Cryptosporidium* oocysts and *Giardia* cysts using "homemade" tosylactivated Dynabeads M-450

| *Cryptosporidium* | | | *Giardia* | | |
|---|---|---|---|---|---|
| No. oocysts seeded | No. oocysts recovered | Percentage oocysts (%) | No. cysts seeded | No. cysts recovered | Percentage cysts (%) |
| 750 | 645 | 86 | 750 | 580 | 77.3 |
| 1000 | 955 | 95.5 | 1000 | 801 | 80.1 |
| 1000 | 940 | 94.0 | 1000 | 784 | 78.4 |

**Commercially coated Dynabeads**

Initial recovery of oocysts and cysts using the commercially available Dynabeads was poor although subsequent recoveries were high, although this was explained later (Table 4.9).

**Table 4.9** Recovery of *Cryptosporidium* oocysts and *Giardia* cysts using commercially coated Dynabeads

| *Cryptosporidium* | | | *Giardia* | | |
|---|---|---|---|---|---|
| No. oocysts seeded | No. oocysts recovered | Percentage oocysts (%) | No. cysts seeded | No. cysts recovered | Percentage cysts (%) |
| 1364 | 41 | 2.99 | 1094 | 84 | 7.68 |
| 966 | 712 | 73.7 | 890 | 634 | 71.2 |
| 1000 | 852 | 85.2 | 1000 | 875 | 87.5 |
| 1000 | 887 | 88.7 | 1000 | 824 | 82.4 |
| 1000 | 670 | 67 | 1000 | 867 | 86.7 |

Microscopy of the beads from the poor recoveries revealed that a number of oocysts and cysts remained attached following dissociation, thereby resulting in poor recovery (2.99% and 7.68% respectively).

Evaluation of the "homemade" Dynabeads has indicated that tosylactivated Dynabeads coated with monoclonal antibodies against *Cryptosporidium* or *Giardia* had better separation and recovery of oocysts (95.5%) and cysts (80.1%) (Table 4.8) from seeded potable water samples than the uncoated Dynabeads (Table 4.7). This may be due to better binding of the antibody due to the bead surface being activated by p-toluene sulfonyl chloride. It has been shown that the efficiency of antibody-antigen binding is increased when a "spacer" is used. This prevents steric hindrance between the bead and the target surface and may ensure optimal orientation of the primary antibody. The "homemade" tosylactivated Dynabeads compared favourably with the recovery of oocysts (78.7%) and cysts (82.0%) using commercially available Dynabeads.

The initial poor recovery obtained when using the commercially available Dynabeads may have been due to the poor dissociation as oocysts and cysts were still attached to the beads. Dissociation of the bead / (oo)cyst complex was improved by vortexing adequately to ensure the acid was well dispersed (Table 4.8).

### 4.2.4 Cost analysis
The cost of commercially available anti-Cryptosporidium and anti-Giardia Dynabeads is R 364.80 per sample whilst "homemade" beads cost R 369.57 and "homemade" tosylactivated beads cost R 413.32 to produce (APPENDIX 7)

### 4.2.5 Recovery of oocysts from seeded water samples following various concentration techniques and "batch" immuno-magnetic separation
The recovery of oocysts from the positive control water samples following separation by paramagnetic beads was fairly high (75.0%). The oocysts stained with 2-3+ intensity and excessive red staining of the background occurred. The recovery of oocysts following membrane dissolution (45.4%) and cartridge filtration (26.7%) was poor (Table 4.10) whilst recovery of oocysts following calcium carbonate flocculation was higher (57.7%).

Table 4.10 Percentage recovery from seeded water samples following various concentration techniques and immuno-magnetic separation (IMS)

| Percentage recovery of oocysts using different concentration methods | | | |
|---|---|---|---|
| Membrane dissolution | Cartridge filtration | Calcium carbonate flocculation | Positive control |
| 51 (41/80) | 25 (20/80) | 54 (43/80) | 75 (60/80) |
| 49 (39/80) | 16 (13/80) | 61 (49/80) | |
| 36 (29/80) | 23 (18/80) | 58 (46/80) | |
| Ave. 45.4 | Ave. 26.7 | Ave. 57.7 | |

The recovery of oocysts from the distilled water samples (75%)(positive control) in the "batch" mode was satisfactory although the manufacturer's state that recovery can be as high as 90%. The use of rounded polypropylene tubes for the isolation technique instead of flat sided glass

tube may have reduced the recovery. The aspiration of the supernatant from the Eppendorff tube may have resulted in loss of the oocyst / bead complex although this was performed carefully with a micro-pipette.

The red staining background during fluorescence microscopy is probably due to the Evan's blue dye used as a counter stain with the fluorescein isothiocyanate (FITC) labelled antibodies used to detect the oocysts. Dynal recommends that immunofluorescence assays with high concentrations of this dye should not be used.

The poor recovery following different concentration methods combined with IMS is probably due to losses occurring primarily during the concentration procedures. The use of IMS did, however, clarify the sample making microscopy easier and less time-consuming.

### 4.2.6 Continuous immuno-magnetic capture

The addition of the commercially available anti-*Cryptosporidium* and anti-*Giardia* beads to the fluid contained in the reservoir was clearly visible as a brown streak. Once the peristaltic pump was switched on, the beads were distributed in the fluid and were not visible.

The beads were easily captured by the neodymium iron boron magnet. Following the magnetic capture of the beads, they were easily eluted from the copper tube by removal of the tube from the magnets. Again the beads were visible as a brown mass in the Eppendorff tube.

Recovery of (oo)cysts from flowing water was poor (Table 4.11) as few (oo)cysts were detected when viewed by epifluorescence microscopy following dissociation of the bead / oocyst complex. The beads, however, were clearly visible under DIC microscopy without any oocysts attached.

Table 4.11 Recovery of *Cryptosporidium* oocysts and *Giardia* cysts following continuous immuno-magnetic capture

| Cryptosporidium | | | Giardia | | |
|---|---|---|---|---|---|
| No. oocysts seeded | No. oocysts recovered | Percentage oocysts (%) | No. cysts seeded | No. cysts recovered | Percentage cysts (%) |
| 1250 | 102 | 8.16 | 1375 | 78 | 5.7 |
| 1000 | 110 | 11.0 | 1000 | 97 | 9.7 |
| 1000 | 62 | 6.2 | 1000 | 143 | 14.3 |

Due to the large size of the oocysts and cysts compared to the beads (4.5 μm), a number of beads would be required to adhere to one oocyst or cyst to enable capture. Rossomando *et al.* (1995) have shown that maximum recovery occurred when the protozoons interacted with clustered immunobeads.

Although the continuous immuno-magnetic capture of (oo)cysts was poor when the commercially available Dynabeads were mixed with water samples (Table 4.11), it did indicate

that the antigen-antibody binding affinities were of sufficient strength to allow recovery from flowing water. The poor recovery may be due to the lack of contact with the coated beads due to the low flow rate of one litre per minute or due to insufficient bead numbers. Poor recovery may also be due to too few beads in the system as pathogen recovery increases with the increase in bead number, with the maximum (100%; 50) number of oocysts recovered with 10 million beads per 500ml (Rossomando et al., 1995).

### 4.2.7 Capture of (oo)cysts using coated polypropylene beads

#### 4.2.7.1 Batch capture of oocysts or cysts from seeded potable water samples using coated polypropylene beads

Initial results were disappointing as Cryptosporidium oocysts and Giardia cysts were not recovered from the polypropylene beads coated specifically against Cryptosporidium or Giardia. However, (oo)cysts were found to still be attached to the beads following dissolution of the (oo)cyst / bead complex (Table 4.12 and 4.13). Thereafter the volume of hydrochloric acid used in the dissolution step was increased which assisted in breaking the (oo)cyst / bead bond enabling more (oo)cysts to be detected in subsequent experiments (Table 4.12 and 4.13).

Table 4.12 Number of oocysts and cysts recovered following batch capture using polypropylene beads coated with anti-Cryptosporidium (1:50 dilution) and anti-Giardia antibody (1:5 dilution)

| Cryptosporidium | | Giardia | |
|---|---|---|---|
| No. recovered oocysts | No. non-recovered oocysts | No. recovered cysts | No. non-recovered cysts |
| 0 | 55 | 0 | 29 |
| 18 | n/d | 39 | n/d |
| 22 | n/d | 35 | n/d |

n/d = not determined

A low percentage of oocysts were recovered (0-31.5%)(std. dev. 11.7) whilst recovery of cysts ranged from 0-60.6% (std. dev. 21.5)(Table 4.12).

Table 4.13 Number of oocysts and cysts recovered following batch capture using polypropylene beads coated with anti-Cryptosporidium (1:10 dilution) and anti-Giardia antibody (undiluted)

| Cryptosporidium | | Giardia | |
|---|---|---|---|
| No. recovered oocysts | No. non-recovered oocysts | No. recovered cysts | No. non-recovered cysts |
| 63 | n/d | 32 | 0 |
| 24 | 0 | 54 | n/d |
| 10 | 0 | 18 | 0 |

n/d = not determined

Polypropylene beads coated with higher concentrations of antibody had better recoveries of (oo)cysts although the range of recovery was wide. Cryptosporidium oocyst recovery ranged from 14.3 - 90.3% (std. dev. 27.5) whilst Giardia cyst recovery ranged from 28.0 - 83.6% (std.

dev. 18.1)(Table 4.13). As (oo)cysts were not detected in the supernatant collected after the beads had been incubated with the seeded water samples and removed for further analysis, it may indicate that (oo)cysts remained attached to the beads although this could not be determined microscopically due to the size of the beads.

#### 4.2.7.2 Continuous capture of oocysts or cysts from seeded potable water samples using coated polypropylene beads

Table 4.14  Number of *Cryptosporidium* oocysts recovered following continuous capture using polypropylene beads coated with anti-*Cryptosporidium* (1:10 dilution)

| No. recovered oocysts | No. non-recovered oocysts |
|---|---|
| 0 | 33 |
| 15 | 26 |
| 19 | 30 |

Table 4.15  Number of *Giardia* cysts recovered following continuous capture using polypropylene beads coated with anti-*Giardia* antibody (undiluted)

| No. recovered cysts | No. non-recovered cysts |
|---|---|
| 0 | 13 |
| 27 | 21 |
| 14 | 29 |

Following continuous capture from flowing water, up to 27.2% of oocysts were recovered (std. dev. 14.3) and up to 41.9% of cysts were recovered (std. dev. 21.0).

(Oo)cyst attachment to the coated polypropylene beads may not have occurred due to the low number of beads (7 anti-*Cryptosporidium* beads; 7 anti-*Giardia* beads) contained within the filter housing. The low number of recovered and non-recovered (oo)cysts which were detected may be due to attachment of (oo)cysts to the wall of the bucket containing the seeded potable water sample or as a result of insufficient rinsing of the bucket in which the water was collected following filtration. In addition, inadequate mixing of the inflow to the filter housing may have resulted in (oo)cysts by-passing the beads.

### 4.3  Conclusions

- Calcium carbonate flocculation had the highest average recovery of oocysts (66.5%) and cysts (70.0%) from seeded water samples in comparison to membrane dissolution and cartridge filtration.
- "Homemade" tosylactivated Dynabeads coated with monoclonal antibodies against *Cryptosporidium* or *Giardia* had better separation and recovery of oocysts (95.5%) and cysts (80.1%) from seeded batch potable water samples than "homemade" ordinary Dynabeads.
- Although the "homemade" coated tosylactivated Dynabeads had good recoveries of (oo)cysts, they cost slightly more (R 413.32) than the " homemade" ordinary Dynabeads (R

369.57) or commercially available anti-*Cryptosporidium* and anti-*Giardia* Dynabeads (R 364.80), which gave similar recoveries.

- Recovery of (oo)cysts from seeded batch potable water samples using commercially available Dynabeads was comparable to the "homemade" tosylactivated beads (Oocysts: 78.7%; cysts: 82.0%).
- Although "batch" immunomagnetic separation had a good recovery of oocysts from seeded distilled water samples, recovery following concentration of large volumes of water dropped dramatically.
- Recovery of *Cryptosporidium* oocysts by IMS following concentration by flocculation was higher (57.7%) than recovery following membrane dissolution (45.4%) and cartridge filtration (26.7%).
- The use of IMS for separation of oocysts from the background debris present in the water concentrate made microscopy easier and less time-consuming.
- Although the continuous immunomagnetic capture of (oo)cysts was poor (oocysts: 8.5%; cysts: 9.9%) when the commercially available Dynabeads were mixed with flowing water samples, it did indicate that the antigen-antibody binding affinities were of sufficient strength to allow recovery from flowing water.
- "Homemade" polypropylene beads coated with a 1:10 dilution of anti-*Cryptosporidium* antibody and undiluted anti-*Giardia* antibody had fairly high average recoveries of oocysts (46.3%) and cysts (53.8%) from seeded batch potable water samples.
- Continuous capture of oocysts and cysts from seeded flowing potable water using coated polypropylene beads was reduced to an average of 16.2% and 21.2% respectively, although this was higher than that achieved with the commercially available Dynabeads.

# 5    FIELD TESTING CONTINUOUS CAPTURE SYSTEM AND MULTIPLE PHASE SIA

The concentration and detection of *Cryptosporidium* oocysts and *Giardia* cysts in water is laborious and time-consuming taking up to three days before a result is available. Whilst specialised cartridge filtration (Envirocheck or Genera) combined with IMS for clarification of water concentrates has become the standard technique for isolating *Cryptosporidium* and *Giardia* in the UK and USA, this method is expensive. In addition, these cartridges are limited to low turbidity water (< 5 NTU) unlike continuous capture. Ideally a technique which combines concentration and clarification of oocysts and cysts from water would be beneficial to the water industry.

This chapter describes the use of continuous capture of *Cryptosporidium* and *Giardia* from flowing environmental water using coated polypropylene beads.

## 5.1    Materials and methods

### 5.1.1    Continuous capture of oocysts from seeded environmental water

Three ten-litre environmental water samples having a turbidity of 10.1 NTU (determined using a Hach 50 turbidimeter (Hach, Colorado, USA) according to the method described in Standard Methods (1995)) were seeded with a known quantity of (oo)cysts as determined by flow cytometry (4.1.1). Each sample was pumped (9-11 $\ell$ min$^{-1}$) through the filter housing unit filled with 10 - 95 anti-*Cryptosporidium* beads as described in 4.1.7.3 (Plate 5.1). The coated polypropylene beads were contained in the "bead holder" (B) within the filter housing (A)(Plate 5.1). Further mixing of the water with the beads was achieved by gently rocking the filter house end-to-end. The bead bound (oo)cysts were collected after filtration and subjected to acid extraction (4.1.6.2), stained in suspension and enumerated (4.1.2). DAPI staining was used to confirm the identification of *Cryptosporidium* oocysts from the seeded environmental water samples (5.1.2).

The turbid water collected after filtration was concentrated by calcium carbonate flocculation (APPENDIX 5) and the concentrated pellet was stained in suspension, filtered onto a membrane and evaluated for the presence of oocysts (4.1.2)(5.1.2).

Three ten-litre environmental samples (10.1 NTU) were not seeded with oocysts or cysts, but were filtered through the filter housing containing the polypropylene immunobeads. After filtration the beads were treated with hydrochloric acid and the extract stained with FITC and DAPI to determine the ability of other environmental particles to attach to the polypropylene immunobeads.
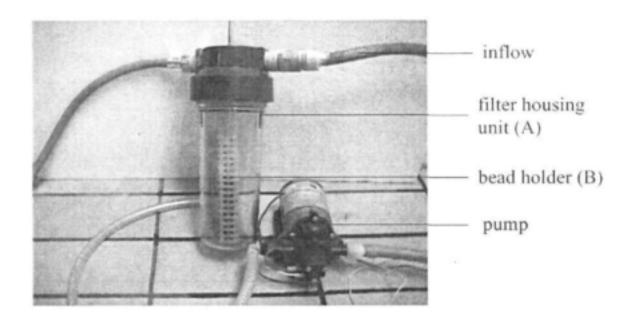
inflow

filter housing
unit (A)

bead holder (B)

pump

**Plate 5.1** Filter housing unit containing inner bead holder for polypropylene immuno beads

### 5.1.2 Confirmation of Cryptosporidium in environmental water samples

The membrane onto which the FITC-stained water concentrate had been filtered, was rinsed with PBS prior to the addition of 100 μl DAPI (1:5000 dilution of the 2 mg/ml stock solution). The filter was incubated at room temperature for five minutes after which it was rinsed with PBS and mounted onto a slide (APPENDIX 9). A Zeiss Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany) fitted with selected excitation of 330-385 nm and barrier filter of 420 nm was used with X400 magnification to examine the stained samples. Identity of the oocysts was confirmed by the presence of 3 - 4 distinct sky-blue staining nuclei.

### 5.1.3 Costing analysis of polyrop beads coated with anti-Crypto/Giardia antibodies

A costing analysis was undertaken to determine the cost of producing polypropylene immunobeads in-house (APPENDIX 10).

### 5.1.4 Detection of Cryptosporidium and Giardia in turbid water samples using SIA

Turbid water samples (0.1 NTU; 6 NTU; 60 NTU; 600 NTU; 4000 NTU) were simulated by mixing potable water with Loamy soil, from a greenhouse where Cryptosporidium and Giardia were unlikely to be present. Five hundred microlitres of each turbid water sample, and a potable water sample (0.1 NTU) were seeded with approximately $10^3$ oocysts and $10^3$ cysts and homogenised by vortexing. Five microlitres of the seeded turbid water were applied in triplicate to reaction circles on SIA slides and assayed, as shown in Figure 4.1., using optimised reagent concentrations as determined in 4.2.1. The negative control was 10 μl CM+ used in place of the antigen. The slides were read by direct visual observation of colour (pale, mid or dark)(positive) at intervals between 5, 15, 30 and 45 minutes. Absorbance readings were not performed as compatible equipment was not available at the time.

### 5.1.5 Evaluation of anti-Crypto and anti-Giardia monoclonal antibody specificity

*Escherichia coli* (*E.coli*) and coliform suspensions replaced the *Cryptosporidium* oocyst and *Giardia* cyst suspensions and SIA was carried out as shown in Figure 4.1.

### 5.1.6 Development of a simple photometer for semi-quantitative analysis by SIA

A simple spectrophotometer comprised of a low noise amplifier, light sensor diode (LSD), light emitting diode (LED) and a voltmeter for digital readout was manufactured for semi-quantitative analysis of *Cryptosporidium* and *Giardia* by SIA (Plate 5.2)(LabsFirst, Pietermaritzburg, South Africa). The system is based on a blue LED which emits light at an optimum of 470 nm. Light emitted from the diode travels through air and is picked up by the optical sensor. Therefore a substance, such as OPD, which absorbs light between 420 nm and 520 nm will absorb the blue light emitted. The system is powered by a 9V battery.

The higher the number of protozoons present in a concentrated sample, the darker the yellow colour produced by the addition of the substrate OPD and the greater the amount of blue light absorbed.

This simple spectrophotometer was calibrated using appropriately coloured cellophane (orange)(APPENDIX 11) and evaluated by seeding potable water concentrates with 1000, 100, 50 (oo)cysts in triplicate (as determined in 4.1.1). Each water concentrate (1 m$\ell$) was pipetted onto a slide well in 25 $\mu\ell$ aliquots and allowed to air dry. This was repeated until the entire concentrate was air dried on the same well. Multiple phase SIA (Chapter 4) was applied and the colour reactions and volt read-outs using the simple spectrophotometer were recorded at intervals (5-45 min.)(APPENDIX 11).



The colour reactions were slower to develop with the *Giardia* - seeded samples. No colour was visible until 30 minutes had passed. The potable and turbid samples were pale yellow in colour and the intensity of the colour changed to yellow after 30 minutes. The colour reactions were easily distinguishable from the negative controls which remained pale yellow.

### 5.2.3 Evaluation of anti-Crypto and anti-Giardia monoclonal antibody specificity

No colour reactions occurred with the *E. coli* or coliform suspensions indicating that the monoclonal antibodies against *Cryptosporidium* and *Giardia* (Waterborne, Inc., Louisiana, USA) are specific to *Cryptosporidium* or *Giardia* and do not cross-react with other micro-organisms found in faecally contaminated and environmental waters. However, other researchers have shown that anti-*Cryptosporidium* or anti-*Giardia* antibodies can cross react with some 24 algal

species such as *Navicula minima*, a diatom, and *Synechococcus elongatus*, a cyanobacterium (Rodgers *et al.*, 1995).

Stibbs (per comm.) found that the anti-*Cryptosporidium* monoclonal 2C9 also cross reacts extensively with other species of *Cryptosporidium* tested including *C. muris, C. baileyi, C. serpentis* and *C. parvum* of mice, cattle and man. The nature of the antigen against which this reacts is not yet known although it appears to be in the oocyst wall. The anti-*Giardia* monoclonals were tested by Waterborne Inc. and cross react with all *Giardia* species namely G. *muris* (weak but some binding occurred), dog *Giardia* and *G. simoni*. The monoclonal antibody binds the GSA 65 antigen on the cyst wall (Stibbs, pers comm.).

### 5.2.4 Development of a simple photometer for semi-quantitative analysis by SIA

Initial evaluation of the simple spectrophotometer was not conclusive owing to a lack of sensitivity. Although colour changes occurred from pale yellow to yellow, the voltage continuously registetrd 1.1 from the 5 to 45 minutes. This may indicate that this simple spectrophotometer was not sufficiently sensitive for lower voltage outputs.

## 5.3 Conclusions

- The highest recovery of *Cryptosporidium* oocysts from flowing environmental waters (11 $\ell$ min$^{-1}$), using 95 (10mm diam) polypropylene immunobeads, was 74.8%.
- Polypropylene immuno-beads cost R 33 per anti-*Cryptosporidium* coated bead and R 165 per anti-*Giardia* coated bead making this technique appropriate, but rather expensive.
- The polypropylene immunobead continuous capture system (PICCS) is fully portable and can be used for environmental water sampling in the field or for potable water sampling at water treatment plants.
- The anti-*Cryptosporidium* or anti-*Giardia* antibodies, used in SIA or PICCS, did not cross-react with *E. coli* or coliforms present in contaminated water.
- SIA was found to react successfully with *Cryptosporidium* or *Giardia* seeded turbid water samples. The colour reactions produced were easily distinguishable from the negative controls.
- ctrophotometer ne                     eds to be optimised and calibrated in order to register from a low (oo)cysts concentration (pale yellow colour) to a high (oo)cyst concentration (dark yellow colour).

# 6    GENERAL DISCUSSION AND CONCLUSIONS

The intestinal parasites *Cryptosporidium parvum* and *Giardia lamblia* cause gastroenteritis infection worldwide and their widespread occurrence indicates their adaptation to numerous environments (Smith *et al.*, 1995). Although most infections are probably transmitted person-to-person, waterborne transmission does occur and can result in waterborne outbreaks. This highlights the importance of environmental monitoring for both these parasites, however the concentration and detection techniques used are expensive and usually complex; cheaper, simple but efficient methods are required.

Detection of oocysts and cysts in water concentrates is tedious and requires expensive fluorescence microscopes fitted with suitable filters and halogen lamps. To avoid this requirement, a simple and economical method using the slide immunoenzymatic assay (SIA) (Conway de Macario *et al.*, 1986) which is based on the enzyme linked immunosorbent asay (ELISA), was adapted to detect *Cryptosporidium* and *Giardia*. This method proved to be suitable for detecting (oo)cysts in clear water by both direct visual observation and spectrophotometrically, as the resulting colour reactions were distinguishable from the negative controls. An initial study, using visual observation only, indicated that this system may well be suitable for use with turbid water samples too. Further refinement to enhance the sensitivity of SIA is required as this system is presently only able to detect 154 oocysts and 74 cysts in a concentrated water sample (e.g. 1 m*l* by repeat dying), by direct visual observation of colour (although 30 and 37 respectively were possible with more concentrated reagents). A colour chart correlating colour intensity with (oo)cyst concentration could be developed as a guide to the direct visual quantification of (oo)cysts in samples. Visual confirmation of identity using DIC is also possible as the (oo)cysts are mounted on microscope slides. A simple spectrophotometer suitable for the detection of colour over a range (pale, mid and dark yellow) is required for semi-quantitative analysis using SIA. In addition, lower numbers of (oo)cysts could be detected by the spectrophotometer due to its increased sensitivity.

Environmental monitoring for both these parasites is made problematic by their small size, relatively low concentration in most waters, inability to augment their numbers by *in vitro* culture and the difficulty experienced in identifying them amongst other particles and debris (Smith *et al.*, 1995). Evaluation of commonly used concentration methods, which are also required by SIA, indicated that the recovery of *Cryptosporidium* and *Giardia* from seeded potable water samples using cartridge filtration or membrane dissolution was poor (0 - 15.1%). Recovery of these organisms by calcium carbonate flocculation was, however, much higher (64.1 - 77.1%). Commercially available immunomagnetic separation (IMS) of the concentrates following cartridge filtration or membrane dissolution was found to increase the recovery of oocysts (16 - 51%) as microscopy was made easier and less-time-consuming. Recovery of oocysts following calcium carbonate flocculation combined with IMS, however, was not further improved (54 - 61%), possibly due to the interferences, or the use of rounded polypropylene tubes for the isolation technique instead of the recommended flat-sided tubes (as these were unavailable). This may also have reduced the number of oocysts recovered from the positive control sample (75%) as the manufacturer's state that the recovery can be as high as 90%.

Due to the cost of the commercially available anti-*Cryptosporidium* or anti-*Giardia* paramagnetic beads, uncoated Dynabeads and tosylactivated Dynabeads were coated in-house with monoclonal antibodies against *Cryptosporidium* or *Giardia*. Although the in-house coated tosylactivated beads were better in recovering (oo)cysts (77.3 - 95.5%) than the commercially coated beads (67 - 88.7%)(R 365 per sample), their cost was even more expensive (R 413 per sample) making it impractical for use in a routine water monitoring programme.

As currently available concentration procedures are time-consuming and subject to (oo)cyst losses, a neodymium iron boron magnet was developed for continuous capture of the commercially available beads following their addition to flowing water. Recovery of (oo)cysts from the flowing water was poor, as dissociation of the (oo)cyst / bead complex appeared to have occurred. In addition, inadequate bead numbers and low flow rates may have resulted in further losses.

Batch capture of (oo)cysts from seeded potable water samples using antibody coated polypropylene beads had a wide range of recovery (14.3 - 90.3%), possibly because (oo)cysts remained attached to the beads. This was improved by an additional application of hydrochloric acid to the (oo)cyst / bead complex.

The cost of coating polypropylene beads was R 33 for each anti-*Cryptosporidium* bead and R 165 per anti-*Giardia* bead. 95 polypropylene beads incorporated into a filter-housing unit were able to capture a maximum 74.8% of oocysts from flowing water (11 $\ell$ min$^{-1}$), although smaller numbers of beads were more cost effective. Due to their large size (10mm), fewer polypropylene beads would be required to capture high numbers of (oo)cysts present in water. The beads can be re-coated and centrifugation is not required to further concentrate the sample, thereby making them more cost-effective. The polypropylene immuno-bead continuous capture system (PICCS) would allow on-site concentration and clarification of potable or environmental water, thereby reducing the process time of *Cryptosporidium* and *Giardia* results. The (oo)cyst / bead complex could be collected in a sterile container for transport to a laboratory. Following dissociation of the bead (oo)cyst complex, the volume containing the (oo)cysts (approx. 5 m$\ell$) can be repeatedly pipetted onto a slide well and air dried. Thereafter the (oo)cysts can be detected by Slide Immuno-Assay or Immuno Fluorescent Assay.

All but the last objective as specified in the contract were completed, to a greater or lesser extent, as described above:

- Although the operating principles were demonstrated to perform, the processes generally still need to be optimised and refined.
- Owing to the complexity of developing the SIA and PICCS concentration and detection system for recovering (oo)cysts from water samples, it was not possible to evaluate faecal or dung specimens owing to time constraints. However, as (oo)cysts are usually present in much higher numbers in infected faeces and dung, SIA should be able to recover (oo)cysts from such samples.
- It was also not possible to field test the system as such, but some environmental samples were tested, however. Indeed the apparatus could work well in a simple laboratory, but a concentration step would be required eg. flocculation, which would

then also need a centrifuge, unless the PICCS system was additionally al fluorescence microscope a

- In its present un-optimised form the SIA technique could be used to detect and monitor oocysts and cysts at contamination sources where the protists are in high quantities. These could include run-off points from livestock farms, informal settlement areas or especially for monitoring sewage effluents.
- A comprehensive literature review has been provided of the commonly used concentration methods, together with a comparative study of these.

## 6.1 Recommendations for future research

- The whole SIA process needs to be further optimised and refined for greater sensitivity, but shows great potential.
- An SIA colour chart correlating colour intensity with (oo)cyst concentration could be developed as a guide to the direct visual quantification of (oo)cysts in samples
- The simple low cost spectrophotometer for reading of SIA slides needs to be optimised/developed.
- The PICCS needs to be optimised in terms of flow rates and size of polypropylene beads to maximises (oo)cysts recovery from flowing water.
- The SIA should be tested against (oo)cysts in faecal and dung samples, as there is great potential for this application.

## 7 TECHNOLOGY TRANSFER

The whole SIA process needs to be further optimised and refined for greater sensitivity, but shows great potential and therefore this report needs to be circulated to commercial development, research and educational establishments.

## 7.1 Publications

C. Jarmey-Swan, A. J. L. Macario, I. W. Bailey and A. R. Howgrave-Graham. 1998. Preparation of kits for the detection of *Cryptosporidium* and *Giardia* in water supplies of developing countries. In *WISA proceedings*, **2**, paper 2B-1.

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## 8 CAPACITY BUILDING

Ms Jarmey-Swan spent three months at the Wadsworth Centre, New York State Department of Health under the auspices of Dr. Macario. During this time the SIA techniques were mastered and applied to detecting *Cryptosporidium* and *Giardia* in water and used to train the staff below.

Four previously disadvantaged individuals were employed during the duration of this project namely Zola Msiska, Lungile Mthembu, Nosipho Gulwa and Melanie Naidoo. They were trained in concentration of protists in water, clarification of water sample using IMS, coating of Dynabeads and polypropylene beads and the use of SIA and IFA for the detection of (oo)cysts.

Ms. Mthembu has subsequently been employed as a permanent technician at Umgeni Water.
Ms. Gulwa is now employed as a microbiologist with Amatola Water in the Eastern Cape.
Ms. Msiska obtained a BSc and was employed by the Agricultrual Research Council
Ms. Naidoo obtained a BSc and was employed by the WITS Medical School
Ms. Jarmey-Swan obtained a MSc


## 9 ARCHIVING OF DATA

Data from this study is available from Umgeni Water Box 9 Pietermaritzburg 3200.

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# APPENDIX 1

## Slide immunoenzymatic (SIA) costing analysis

**EQUIPMENT**

| | | | |
|---|---|---|---|
| Simple spectrophotometer | | R 1- 54 | |
| Microp1672x1480Y | | R 0-18 | R 1-72 |

**CONSUMABLES**

| | | | |
|---|---|---|---|
| Lighter | R 0-03 | | |
| Microscope slides | | R 0-22 | R 0-25 |

**REAGENTS**

| | | | |
|---|---|---|---|
| Acetone | | R 0-0003 | |
| *Cryptosporidium* antibody | 1:50 | R 1-35 | |
| *Giardia* antibody | 1:5 | R 5-40 | |
| Complete media | | R 0-58 | |
| Secondary antibody | | R 0-07 | |
| o-phenylenediamine | | R 0-01 | |
| Citric acid | | R 1-77 | |
| Peroxide | | R 0-30 | R 9-48 |

**TECHNICIAN TIME**

| | | |
|---|---|---|
| Slide preparation | R 4-17 | |
| Sample addition | R 0-83 | |
| Wash, dry & incubation time | R 4-17 | |
| Addition of antibody | R 0-83 | |
| Wash, dry & incubation time | R 4-17 | |
| Addition of substrate | R 0-83 | R 15-00 |

| | | |
|---|---|---|
| **SHIPPING & HANDLING** | R 3-55 | R 3-55 |

| | | |
|---|---|---|
| | Total cost per sample | R 30-00 |

# APPENDIX 2

## Immuno fluorescence assay method (Cellabs)
### (Crypto/Giardia–Cel IF, Cellabs, New South Wales, Australia)

1. Place 20 µℓ of a concentrated water sample on a well of a glass microscope slide.

2. Allow the sample to air dry completely.

3. Apply a drop of acetone to the sample and air dry.

4. Place 25 µℓ of FITC labelled monoclonal antibody on the sample.

5. Incubate the slide in a humidified chamber at 37°C for 30 minutes.

6. Wash the slide gently with PBS (pH 7.4).

7. Drain the slide and allow to air dry.

8. Add a drop of mounting fluid, apply a coverslip and remove air bubbles.

9. Examine slide using epifluorescence microscopy.

# APPENDIX 3

## Cartridge filtration method (Badenoch et al., 1989)

1. Filter sample through the wound cartridge filter contained within the filter house at a flow rate of 3 $\ell$ min$^{-1}$.

2. Remove the wound cartridge filetr from the filter house and cut the fibres from the plastic core. Cut the filter into sections.

3. Place some of the filter fibres into a plastic bag and agitate mechanically using a stomacher (10 min).

4. Add 750 m$\ell$ 0.1% Tween 80 and continue agitating.

5. Repeat steps 3 and 4 until all the fibres have been washed clean.

6. Drain the washings into a clean beaker.

7. Centrifuge the washings at 1500$g$ for 10 minute. Carefully aspirate the supernatant and collect the pellets.

8. Concentrate the pooled pellets by centrifugation at 1500$g$ for 10 minutes.

9. Carefully aspirate the supernatant, leaving approximately 0.5 m$\ell$ of fluid above the pellets.

10. Immunoassay.

# APPENDIX 4

## Membrane dissolution (Aldom and Chagla, 1995)

1.  Filter a 10 $\ell$ water sample through a 1.2 mm cellulose acetate flat membrane placed on a filter manifold (Millipore; 142 mm diameter).

2.  Place the membrane in a polypropylene centrifuge tube.

3.  Add 200 m$\ell$ acetone and wait two minutes.

4.  Centrifuge at 650 $g$ (15 minutes).

5.  Resuspend in 50 m$\ell$ acetone and centrifuge (650 $g$; 15 minutes).

6.  Discard supernatant and resuspend in 50 m$\ell$ of 95% ethanol.

7.  Centrifuge at 650 $g$ (15 minutes) and discard supernatant.

8.  Resuspend in 50 m$\ell$ of 70% ethanol and centrifuge (650 $g$; 15 minutes).

9.  Resuspend in 10 m$\ell$ eluting fluid.

10. Immunoassay

## APPENDIX 5

### Calcium carbonate flocculation (Vesey *et al.*, 1993)

1.  Collect a 10 $\ell$ water sample and place sample in a flat-bottomed polypropylene bucket.

2.  Mix thoroughly and add 100 ml of 1 mol $\ell^{-1}$ calcium carbonate and 100 m$\ell$ of 1 mol $\ell^{-1}$ sodium bicarbonate.

3.  Mix and adjust pH to 10.

4.  Allow sample to flocculate for a minimum of four hours.

5.  Remove supernatant fluid by vacuum aspiration.

6.  Dissolve residue by adding 200 m$\ell$ of 10% sulphamic acid and shake vigorously for 15 seconds before decanting into centrifuge tubes.

7.  Rinse barrels with 200 m$\ell$ of 0.01% Tween 80 and shake.

8.  Decant and repeat with 100 m$\ell$ Tween 80.

9.  Centrifuge at 3 000 *g* for 10 minutes.

10. Remove supernatant leaving 40-50 ml.

11. Resuspend pellet and transfer to 50 m$\ell$ centrifuge tubes.

12. Centrifuge at 3 000 *g* and remove supernatant.

13. Rinse original centrifuge tube with Tween 80 and resuspend the pellet.

14. Centrifuge again and remove the supernatant leaving 2-5 m$\ell$.

15. Immunoassay.

# APPENDIX 6

## IMS procedure for using GC COMBO DYNABEADS (Dynal A.S., Oslo, Norway)

1X dilution Buffer A: For every 1ml buffer A required , take 100µl of 10X buffer A and dilute to 1ml of milli-Q

Sample preparation:

To the 10 ml sample add :     1ml of 10x SL buffer A
                              1ml of 10X SL buffer B

1.   Vortex the anti-*Cryptosporidium* Dynabeads for 10 seconds.
2.   Resuspend the beads by inverting the vial.
3.   Add 100 µl of the anti-*Cryptosporidium* Dynabeads to tube containing 10 ml sample and the SL buffers.
4.   Repeat the above 3 steps for the Dynabeads anti- *Giardia* vial.
5.   Rotate the 10 ml sample containing reagents at 15-20 RPM for 1hr at RT.
6.   Place tube on magnetic particle concentrator (MPC).
7.   Gently rock the tube end to end through 90° (tilting cap end and base end up and down) for two minutes (1 tilt /sec)
8.   Return tube to upright position and immediately decant supernatant to a suitable container.  NB: Do not remove tube from magnetic concentrator during this step.
9.   Remove the tube from the MPC, resuspend sample in 1ml 1X SL buffer. Mix gently (Do not vortex).
10.  Transfer all liquid to microcentrifuge tube and place tube on MPC. Gently rock for 1min through 90°. Dynabeads-organism complex should form a clear dot on back of tube.
11.  Aspirate supernatant immediately (Do not remove from MPC).

APPENDIX 7

## Cost analysis for the preparation of ordinary and tosylactivated Dynabeads

| EQUIPMENT | | SUB-TOTAL |
|---|---|---|
| Blood rotator | R 0-39 | |
| Micropipette | R 0-18 | |
| MPC | R 0.95 | R 1-52 |

| CONSUMABLES | | |
|---|---|---|
| Centrifuge tubes | R 1-51 | |
| Pipette tips | R 0-35 | |
| | R 306-25 | R 308-11 |
| Tosylactivated Dynabeads | R 350-00 | R 351-86 |

| REAGENTS | | |
|---|---|---|
| *Cryptosporidium* antibody | R 43-75 | |
| *Giardia* antibody | R 43-75 | |
| PBS/BSA | R 0-13 | |
| PBS | R 0-12 | |
| HCl | R 0-01 | R 43-89 |

| TECHNICIAN TIME | | |
|---|---|---|
| Preparation | R 12-50 | |
| | ———— | 12-50 |

| SHIPPING & HANDLING | R 3-55 | R 3-55 |
|---|---|---|

| Total costs for ordinary Dynabeads | R 369.57 |
|---|---|
| Total costs for tosylactivated Dynabeads | R 413.32 |

# APPENDIX 8

## Dissociation of Dynabeads-cysts/oocysts complex

1.  Remove magnetic strip from MPC.

2.  Add 100μl 0.1M HCl to micro-centrifuge tube, vortex for 10 seconds.

3.  Place tube in MPC without magnetic strip; allow to stand for five minutes in vertical position at room temperature (RT).

4.  Vortex for 10 seconds.

5.  Place in MPC, insert magnetic strip and allow to stand for 10 minutes.

6.  Remove supernatant into clean Eppendorff tube and add 100 μl HCl, allow to stand at RT for 5 min. (Do not disturb beads while transferring).

7.  Place in MPC, insert magnetic strip and allow to stand for 10 minutes.

8.  Label slide, add 5 μl 1M NaOH to sample well.

9.  Transfer all liquid from tube onto well containing NaOH.

10. Air-dry sample on slide.

## APPENDIX 9

## DAPI staining

1. Add 100 µl DAPI (1:5000 working dilution) to the membrane.

2. Incubate at RT for 5 min.

3. Rinse the membrane with PBS.

4. Mount membrane on a drop of mounting fluid.

5. Add a drop of mounting fluid on top of the membrane and cover with a coverslip.

6. View the stained membrane with X400 magnification using an epifluorescence microscope with selected excitation of 330-385 nm and barrier filter of 420 nm.

## Costing of polypropylene immunobeads

| | | SUB-TOTAL |
|---|---|---|
| **EQUIPMENT** | | |
| Blood rotator | R 0-39 | |
| Micropipette | R 0-18 | R 0-57 |
| | | |
| **CONSUMABLES** | | |
| Centrifuge tubes | R 1-51 | |
| Pipette tips | R 0-35 | |
| Polypropylene beads | R 0-20 | R 2-06 |
| | | |
| **REAGENTS** | | |
| *Cryptosporidium* antibody (1:10 dilution) | R 14-58 | |
| PBS/BSA | R 0-13 | |
| PBS | R 0-12 | |
| HCl | R 0-01 | R 14-84 |
| | | |
| *Giardia* antibody (undiluted) | R 145-83 | |
| PBS/BSA | R 0-13 | |
| PBS | R 0-12 | |
| HCl | R 0-01 | R 146-09 |
| | | |
| **TECHNICIAN TIME** | | |
| Preparation | R 12-50 | |
| | | R12-50 |
| | | |
| **SHIPPING & HANDLING** | R 3-55 | R 3-55 |
| | | |
| **TOTAL COST PER BEAD** | | |
| **Anti-*Cryptosporidium* bead** | | R 33-52 |
| **Anti-*Giardia* bead** | | R 164-77 |

# APPENDIX 11

## Operation of the simple spectrophotometer

There are three knobs on the spectrophotometer casing: coarse tuning with the fine tuning above ; and the baseline tuning alongside the coarse knob.

1.  Switch on voltmeter to 200 mV.

2.  Switch on spectrophotometer.

3.  Setting the baseline: switch to 200mV and set to 1:00 by turning the baseline knob anticlockwise. Close lid to block light.

4.  Set the upper base line for the highest concentration. Use the darkest colour cellophane slide (orange). Switch to 20V and set by turning the coarse knob to approx. 2.5.

5.  When reading a slide, the well must be centred and the sample well-mixed.

6.  If the reading is too high (goes to 1), change the voltage setting.

7.  If the reading is zero, move the voltage setting to get a better reading.

# APPENDIX 12

## CAPACITY BUILDING OUTCOMES EMANATING FROM THIS PROJECT 825

1    Achievement of capacity building objectives as stated in the contract documentation:

| Objectives put forward in original proposal | Extent to which objectives have been achieved | |
|---|---|---|
| No specific objectives were included in proposal. | | |
| Previously Disadvantaged students were trained each year and achieved BSc Hons and/or permanent employment | | |
| | **Qualification obtained** | **Permanent post** |
| 1997 M.Naidoo, | BSc. Hons. | Wits Medical School |
| 1998 Z Msiska | BSc. Hons. | Agric Research Council |
| 1998 L Mthembu | | Umgeni Water |
| 1999 N Gulwa | | Amatola Water |
| | | |
| | | |
| | | |
| | | |
| | | |

2    Formal qualifications emanating from this research of capacity building:

| Certificate | Previously Disadvantaged Persons | Total |
|---|---|---|
| **Diploma** | | |
| **Bachelors** (Honours) | M.Naidoo, Z Msiska | 2 |
| **Masters** | C. Jarmey-Swan | 1 |
| **Doctoral** | | |

# Other related WRC reports available:

**The evaluation of alternative disinfection processes for the removal of protozoan oocysts and cysts and other micro-organisms, in the treatment of final wastewater**

*FreeseSD; Bailey I; Nozaic D*

This project forms part of the programme on the detection and quantification of *Cryptosporidium* oocysts and *Giardia* cysts in water supplies.

*Cryptosporidium* and *Giardia* are self-limiting disease-causing pathogenic parasitic protozoa for which no medication is available. Well-trained and experienced technicians are required to conduct presently available and expensive test methods to detect these pathogens. Additional knowledge is needed on the presence, viability and removal of pathogens during wastewater treatment processes and evaluation of the contribution of sources to their occurrence in the aquatic environment to prevent possible pollution from these sources. These pathogenic cysts and also viruses are highly resistant to chlorine. It is therefore obvious that effective disinfection of wastewater effluents is also required.