DEVELOPMENT OF CONCENTRATION AND DETECTION TECHNIQUES FOR *CRYPTOSPORIDIUM* AND *GIARDIA* AND THE SIGNIFICANCE OF THESE PROTISTS IN KWAZULU- NATAL

by

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ABSTRACT

Cryptosporidium and *Giardia* are waterborne parasitic protozoons that have been associated with some diarrhoeal illness in most parts of the world. They are considered of major importance for drinking water safety as waterborne outbreaks still occur regularly in both developed and developing countries, sometimes with fatal consequences. To determine their incidence in the KwaZulu-Natal population, an epidemiological review of pathology laboratory data was compiled. Both protists were found to be endemic although their occurrence did not appear to correlate with climatic factors such as rainfall, season or year, possibly indicating that other factors such as personal hygiene, potable water supply, sanitation and education probably have a more significant impact here rather than waterborne transmission. The results, however, may not be representative of the entire population as detection techniques are not standardised and data were only collected from laboratories willing to supply in the Durban and Pietermaritzburg areas which represent under 10% of laboratories in KwaZulu-Natal that test for *Cryptosporidium* and *Giardia*. These laboratories, however, perform most of the testing from throughout KwaZulu-Natal and are situated in the metropolitan areas. In addition, poor recording of patient details made conciling of data difficult.

Evaluation of a calcium carbonate flocculation, membrane filtration and membrane dissolution technique for concentrating *Cryptosporidium* oocysts and *Giardia* cysts from water is necessary to accurately quantify (oo)cysts in water. Methods currently used result in varying recoveries and the best method had to be identified and enhanced for this study. Greater (oo)cyst losses occurred as turbidity increased irrespective of the method used. The calcium carbonate flocculation method proved to have the best recovery for all water types and is recommended for use in the regular routine monitoring of smaller volumes of water. Pre-filtration prior to flocculation had the potential to make microscopy easier although losses of (oo)cysts still occurred. Sucrose flotation following flocculation reduced recovery whilst pH adjustment to 6 ± 0.5 following flocculation improved recovery and is recommended with turbid water samples.

A cheap and simple detection method using the slide immunoenzymatic assay (SIA) which is based on the principles of enzyme linked immunosorbent assay (ELISA), was adapted to detect *Cryptosporidium* and *Giardia* in potable and turbid water concentrates.

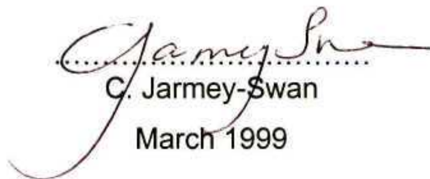
The results were reproducible and sensitivity was improved using a spectrophotometer. A multi-phase SIA system, using reagents in liquid and dry ready-to-use forms, was investigated and the potential exists for its further development. Unfortunately this method does not indicate the viability status of (oo)cysts therefore a novel method to do so was developed using fluorescein diacetate (FDA) and tetramethyl red (TMR) labelled anti-*Giardia* monoclonal antibodies. This combination stained viable cysts green internally with a red wall while non-viable cysts stained red only. While the use of FDA overestimates viability, any error would err in favour of safety, and could be complemented with fluorescein isothiocyanate / propidium iodide for confirmation of viability status.

As *Cryptosporidium* and *Giardia* were found to be present in the KwaZulu-Natal population, monitoring of water bodies, water supplies, wastewaters and sludges, using the enhanced flocculation procedure and immunofluorescence assay, was undertaken to determine their source. Oocysts and cysts were detected in dam, river and raw waters, treated effluent and sludge samples. No oocysts or cysts were detected in the treated water samples although this may be due to the inability of the method used to detect low numbers of (oo)cysts.

This research confirmed the occurrence of *Cryptosporidium* and *Giardia* in the KwaZulu-Natal population and water matrices. The optimum concentration method for use with water samples was established and further enhanced for use with turbid waters while a simpler and cheaper means of detecting (oo)cysts and a novel viability-detection stain for cysts were developed.

DECLARATION

I hereby certify that this research, unless specifically indicated in the text, is the result of my own investigations.



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CHAPTER 1

SIGNIFICANCE OF *CRYPTOSPORIDIUM* AND *GIARDIA* AND THEIR DETECTION IN ENVIRONMENTAL WATERS: LITERATURE REVIEW

1.1. INTRODUCTION

Since the mid-1970s, when both *Cryptosporidium parvum* and *Giardia lamblia* became recognised as important waterborne pathogenic protists of man, with the potential to cause diarrhoeal disease, considerable attention and resources have been directed at unravelling the epidemiology of these diseases and limiting the spread of the organisms (Smith *et al.*, 1995).

Both *Cryptosporidium* oocysts and *Giardia* cysts are currently considered of major importance for drinking water safety due to their relative robustness, low infectivity doses and the variable state of immunity elicited by the population at risk (Smith *et al.*, 1995). Human volunteer trials for *Cryptosporidium* infection suggested a minimum infective dose of ≤ 30.0 and a median dose of 132.0 oocysts (DuPont *et al.*, 1995). For *Giardia*, human volunteer studies indicate that the median infectious dose is between 25.0 and 100.0 cysts although as few as 10.0 cysts have initiated infection (Rendtorff, 1979).

The presence of *Cryptosporidium* in water challenges regulators to develop public policies that limit contamination of source waters, improve water treatment and protect public health (Rose *et al.*, 1997). 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 immunocompromised died (Lisle and Rose, 1995). In addition, this outbreak was estimated to cost the community millions of dollars (Lisle and Rose, 1995).

In the UK, a report, entitled "Parasites on tap land company in deep water" (Pearce, 1995), arose as government water inspectors planned to prosecute water utilities for failing to provide "wholesome water". Waterborne outbreaks of *Cryptosporidium* and *Giardia* pathogens may have resulted from faults in operational procedures or mechanical

and operational deficiencies at water treatment plants (Smith *et al.*, 1995). In addition oocysts are able to survive physical treatment and disinfection while cysts can survive water treatment processes, but are sensitive to some disinfectants used in water treatment (Smith *et al.*, 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).

Properly operated conventional treatment (coagulation, sedimentation and filtration) can remove 99.0% or more oocysts and cysts (Nieminski, 1994). In general, treatment of *Cryptosporidium* is more difficult than *Giardia*, possibly because of (1) its smaller size, (2) its lower sedimentation rate, and (3) its increased disinfection resistance (Rose *et al.*, 1997). For control of *Cryptosporidium* and *Giardia*, a combination of filtration and disinfection is required. The commonly used water treatment disinfectant, chlorine, has been shown to be ineffective against *Cryptosporidium* oocysts (Korich *et al.*,1990; Ransome *et al.*, 1993). Other disinfectants such as chlorine dioxide, hydrogen peroxide, ultraviolet irradiation, iodine and peroxone show some potential but in general terms the required dosage levels would render the water unpalatable or may exceed legislation (Ransome *et al.*, 1993). However, ozone has emerged as one of the most promising alternatives for inactivation of *Cryptosporidium* and *Giardia*, although disinfection by-products may form (Ozekin and Westerhoff, 1998).

Unlike conventional media amplification methods for detecting indicator organisms of significance to public health and the water industry, techniques for detecting *Cryptosporidium* or *Giardia* in water are composed of three stages namely sample collection, separation of oocysts and cysts from contaminating debris and detection and viability determination of the oocysts and cysts (Smith *et al.*, 1995).

Present methods used to concentrate water samples for the detection of oocysts and cysts are labour intensive, expensive and inefficient (Vesey *et al.*, 1993a). Furthermore

detection assays require skilled operators trained to identify oocysts and cysts amid an array of background particulate matter. Limitations in the methods have included: (1) poor recoveries from water (2) the possibility of inadequate antigen recognition (3) inability to assess the identification of the species or viability thereof (Rose *et al.*, 1989).

In this chapter, an introduction to these pathogenic protozoa and methods for their concentration and detection in water and the assessment of their viability are described. An overview of the occurrence of these protozoa and sources of contamination will also be discussed.

1.2. MORPHOLOGY, LIFE CYCLE, TRANSMISSION AND SIGNIFICANCE OF *CRYPTOSPORIDIUM* AND *GIARDIA*

Cryptosporidium and *Giardia* are cosmopolitan parasitic protozoa. The genus *Cryptosporidium* is taxonomically described as a coccidian protozoan from the phylum Apicomplexa, order Eucoccidiida and family Cryptosporidiae (Despommier and Karapelou, 1987). Several species of *Cryptosporidium* have been isolated and characterised. Carriers have included chickens, cats, turkeys, house mice, fish, corn and rat snakes, guinea pigs, amphibia, reptiles, quail and ostriches (Fayer *et al.*, 1997). *C. parvum* appears to be infectious for 79 species of mammals including humans (O' Donoghue, 1995). *Cryptosporidium* oocysts are spherical in shape, 4 μm in diameter with the following internal morphological structures: one to four crescent shaped sporozoites (motile infective stage) and a granular residual body (contains ribosomes, endoplasmic reticulum and micronemes) may be present (Plate 1.1) (Standard Methods, 1995).

The flagellate *Giardia* belongs to the phylum Sarcomastigophora, order Diplomonadida and family Hexamitidae (Despommier and Karapelou, 1987). Species of *Giardia* from the small intestine of man, dogs, cats, cattle, rabbits, amphibia and reptiles have been described (Kreier and Baker, 1987). All species are transmitted by means of cysts passed out in faeces and *G. canis* and *G. lamblia* are known to be pathogenic, the latter inhabiting the small intestine of man, monkeys and pigs worldwide; beavers may also serve as a reservoir for human infection (Kreier and Baker, 1987). *Giardia* cysts are oval in shape, 8-18 μm in length and 5-15 μm in width and have the following internal

morphological structures: axoneme (a bundle of fibres at the centre of a flagellum), two to four nuclei and median bodies (claw-hammer shaped paired organelles found in the posterior half of the cyst) (Plates 1.2. and 1.3.) (Standard Methods, 1995).

Although the primary route of transmission of oocysts and cysts is faecal-oral, drinking or recreational waters serve as vehicles for transmission following faecal contamination and several outbreaks have been reported from both sources (Craun, 1988; Solo-Gabriele and Neumeister, 1996). Foodborne transmission has also been recorded for cryptosporidiosis (Fayer and Ungar, 1986; Casemore, 1990) whilst giardiasis has been reported to have been sexually transmitted (Phillips *et al.*, 1981).

The life cycle of *C. parvum* is complex, involving both asexual and sexual reproductive cycles resulting in the production of large numbers of oocysts which are released in the gut lumen (Figure 1.1). In contrast, the life cycle of *G. lamblia* is simple in which the reproductive trophozoite encysts, resulting in cyst production (Figure 1.2.) (Table 1.1.) (Smith *et al.*, 1995).

Table 1.1. Comparison of life cycles of *Cryptosporidium* and *Giardia* (Smith *et al.*, 1995).

Cryptosporidium	*Giardia*
1. Ingestion of oocyst by susceptible host.	Ingestion of cyst by susceptible host.
2. Excystation in intestine; sporozoites (4/oocyst) infect cells on surface of intestine.	Excystation in intestine; trophozoites (2/ cyst) colonise the outer surfaces of the intestine.
3. Asexual multiplication of the parasite resulting in the production of large numbers of organisms.	Asexual multiplication of the parasites resulting in the production of large numbers of organisms.
4. Sexual cycle initiated resulting in the development and excretion of the oocyst.	No sexual cycle occurs. Encystation of asexually produced trophozoites resulting in the formation of the cyst.
5. Excretion of the environmentally resistant oocyst.	Excretion of the environmentally resistant cyst.

1.3. SIGNIFICANCE OF *CRYPTOSPORIDIUM* AND *GIARDIA* IN HUMAN POPULATIONS

Clinical symptoms of *Cryptosporidium* and *Giardia* infections include a flu-like illness, diarrhoea, malaise, abdominal pain, anorexia, nausea, flatulence, malabsorption of nutrients, vomiting, mild fever and weight loss (Fayer and Ungar, 1986). In most immunocompetent individuals both are self-limiting whilst in some immunocompromised individuals, such as those with Acquired Immune Deficiency Syndrome (AIDS), cryptosporidiosis can be life-threatening, causing profuse diarrhoea with severe dehydration, malabsorption of nutrients and wasting, with spread to other organs (Crawford and Vermund, 1988).

Whilst several drugs, including nitroimidazole compounds, are available for treating giardiasis (Smith *et al.*, 1995), there is presently no consistently efficacious therapy for cryptosporidiosis in humans (Blagburn and Soave, 1997).

It is difficult to determine incidence rates or prevalence relating to *Cryptosporidium* or *Giardia* as population surveys are often based on diagnostic specimens received by laboratories with which age and gender data are often not recorded (Casemore *et al.*, 1997). In addition, few surveys are adequately controlled and the resulting data may be biased towards children as medical intervention is more likely to result from gastrointestinal symptoms appearing in younger patients (Casemore, 1990).

Prevalence rates in the developed world have ranged from 0.6% to 20.0%, while prevalence rates in developing countries have ranged from three percent to 30.0% (Casemore, 1990; Ungar, 1990).

Few studies have been undertaken in South Africa to determine the incidence or prevalence of cryptosporidiosis or giardiasis in the population. Published reports have, however, highlighted the risk to young children (Schutte *et al.*, 1981; Berkowitz *et al.*, 1988; Walters *et al.*, 1988; Moodley *et al.*, 1991a).

Seasonal and temporal trends of human infections have been observed in a number of investigations. Those trends often coincide with periods of maximum rainfall that may reflect seasonal agricultural practices and environmental and zoonotic transmission (Moodley *et al.*, 1991a).

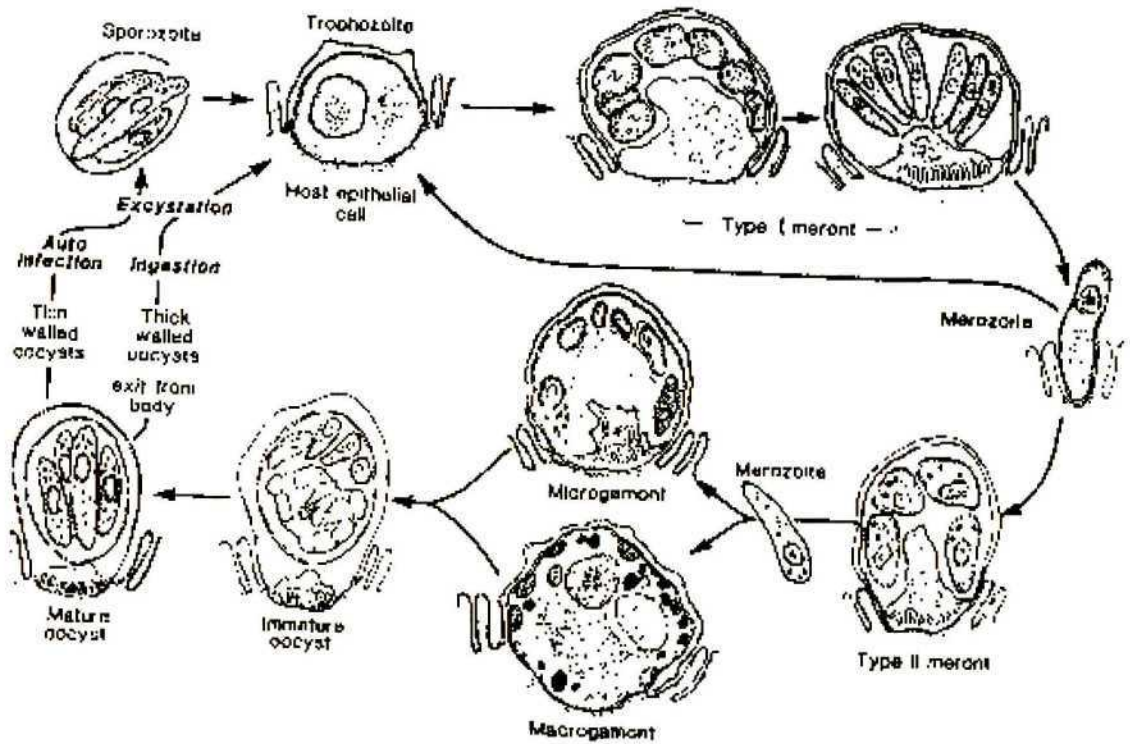


Figure 1.1. Diagram of the life cycle of *Cryptosporidium parvum* (After Fayer *et al.*, 1990).

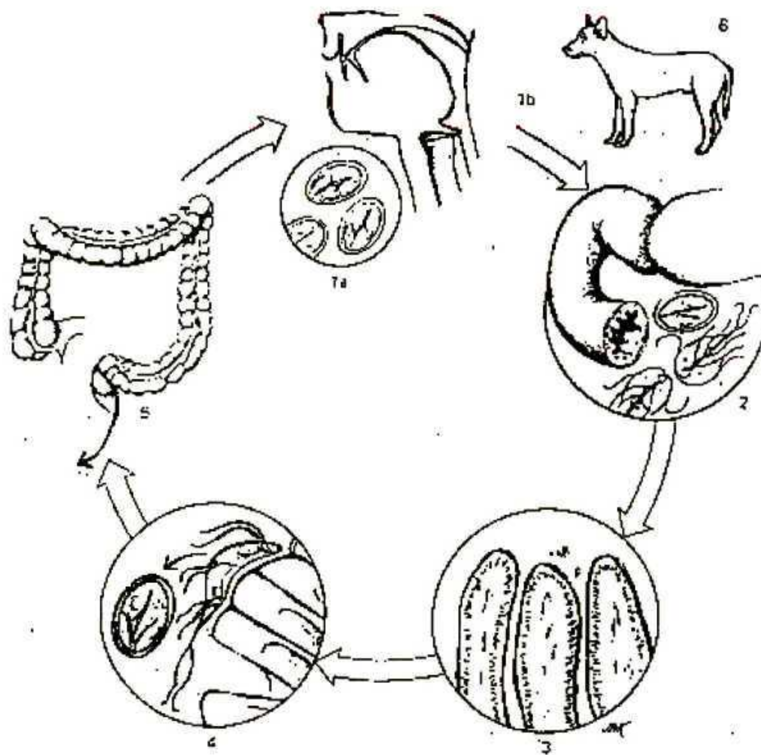


Figure 1.2. Diagram of the life cycle of *Giardia lamblia* (After Despommier and Karapelou, 1987).

1a *Giardia lamblia* cysts.

1b Cysts are ingested in faecally contaminated food or water.

2 Excystment occurs in the intestine giving rise to two trophozoites. Each trophozoite possesses six flagella.

3 The trophozoites live upon the surface of the villi of the small intestine.

4 *G. lamblia* adheres to the columnar cells by means of a disklike depression on its ventral surface, which functions as a sucker. Encystment occurs in the lumen of the small intestine, resulting in the production of infectious quadrinucleate cysts.

5 The cysts pass into the environment in the faeces.

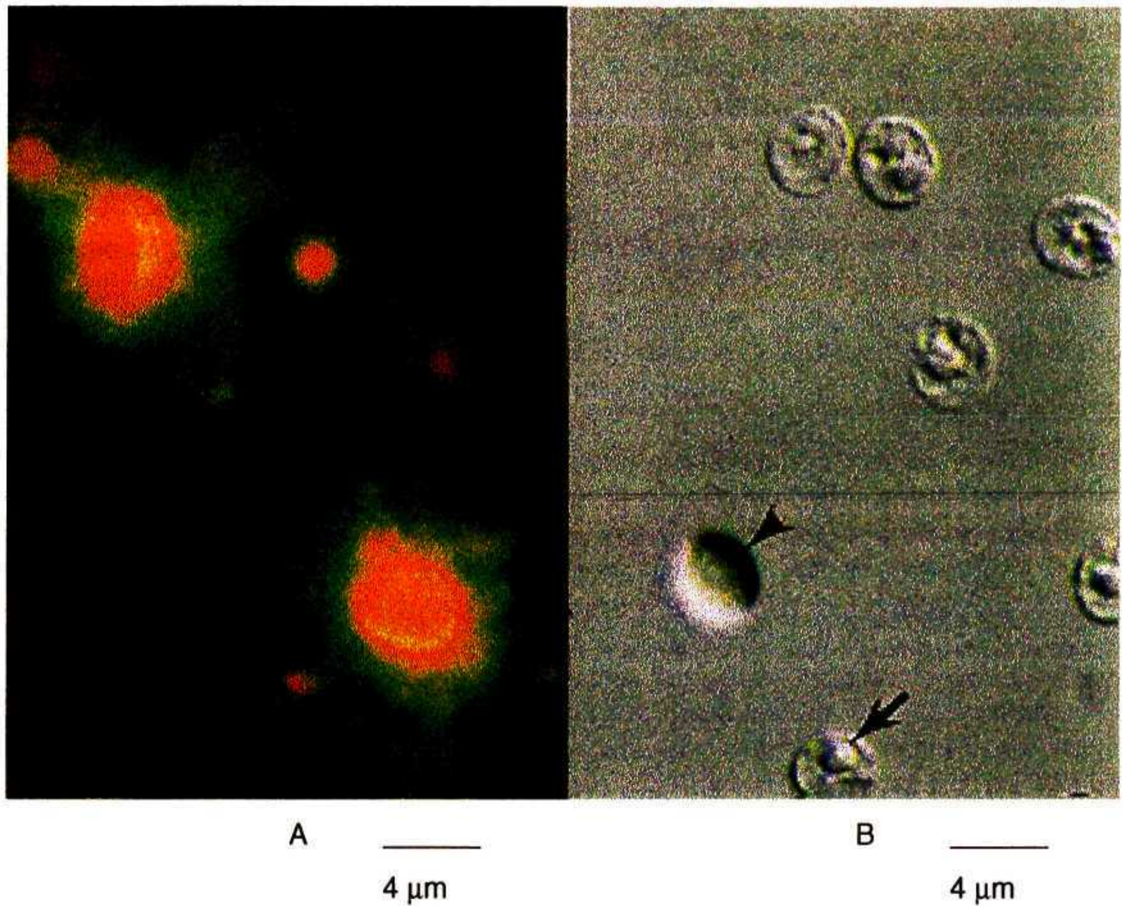


Plate 1.1. (A) Spherical shaped *Cryptosporidium* oocysts labelled by immunofluorescence using a monoclonal antibody-based assay; (B) Differential interference contrast (DIC) image of oocysts. Note the residual body (arrow) in the oocyst and yeast cell (arrowhead) (After Arrowood, 1997).

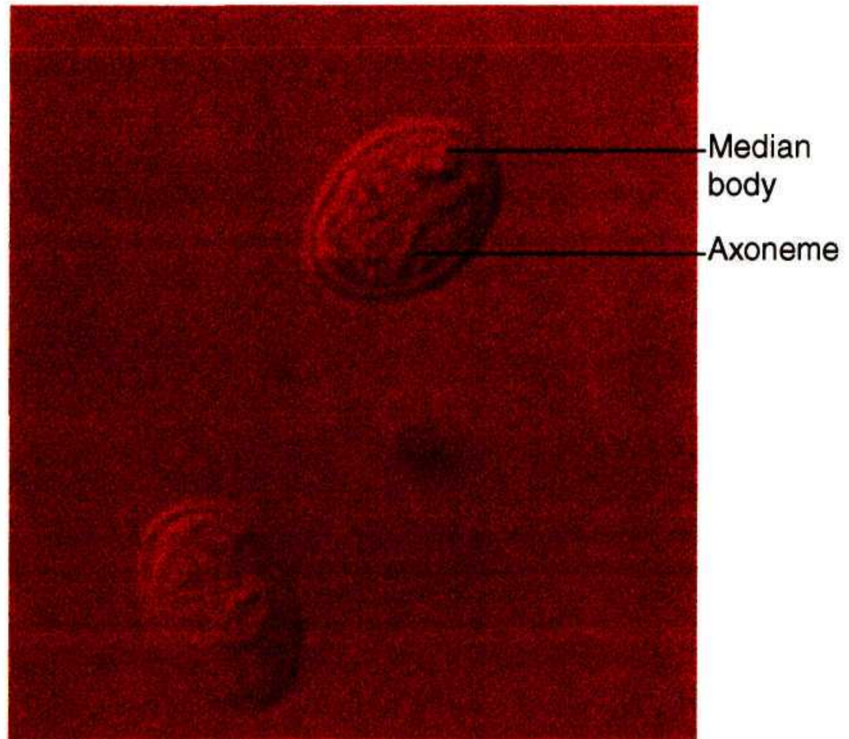


Plate 1.2. Differential interference contrast (DIC) image of a *Giardia* cyst.

Plate 1.3. *Giardia* cyst stained with fluorescein isothiocyanate (FITC) labelled antibody.

1.4. CONCENTRATION TECHNIQUES FOR *CRYPTOSPORIDIUM* AND *GIARDIA* FROM WATER

Measuring the concentrations of *Cryptosporidium* and *Giardia* at selected sampling locations can provide useful data if appropriate sampling and analytical strategies are adopted (Smith *et al.*, 1995). USA and UK nationally recommended procedures include the filtration of large volumes of water (100 - 1000 ℓ) 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 microscopy. This method is inefficient, time-consuming, labour-intensive and tedious (Ho *et al.*, 1995). A variety of different techniques have been evaluated and are also described below.

1.4.1 CALCIUM CARBONATE FLOCCULATION

In the calcium carbonate flocculation method of Vesey *et al.* (1993a), a floc of finely dispersed calcium carbonate is produced in the water sample by the addition of calcium chloride and sodium bicarbonate. The calcium carbonate is allowed to settle, 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, uses small volumes of water (10-20 ℓ), basic equipment and is less labour intensive than other methods (Vesey *et al.*, 1993a). It has been shown to have consistently high recoveries (71.3 - 76.0%) in deionised, tap and river water (Vesey *et al.*, 1993a; Shepherd and Wyn-Jones, 1996). A difficulty with this technique is that it concentrates particulates of a wide range of sizes resulting in a residue which may contain a greater amount of particulate matter (Vesey *et al.*, 1993a). It has also been stated that this method can reduce oocyst viability (Campbell *et al.*, 1994).

1.4.2 CARTRIDGE FILTRATION

In cartridge filtration a larger volume of water (100 – 1000 ℓ) can be passed through a porous or open textured medium in order to remove suspended particles or (oo)cysts by leaving them behind in the pores. Thereafter the (oo)cysts can be eluted from the filtering medium and further concentrated. A number of different media type cartridge filters have

been evaluated for their recovery efficiency of oocysts and cysts. It is difficult to compare various researchers' work with regard to recovery differences as individual elution and centrifugation techniques may affect the recovery after filtration.

a) *Polypropylene cartridge filters*

Recovery of *Cryptosporidium* oocysts from seeded tap water samples using 1.0 μm pore size polypropylene cartridge filters ranged from 8.0 to 50.0% (Rose *et al.*, 1986; Musial *et al.*, 1987; Gilmour *et al.*, 1991; Nieminski *et al.,* 1995) while recovery of *Giardia* cysts averaged 33% (Gilmour *et al.*, 1991). Whitmore and Carrington (1993) used a larger pore size polypropylene filter (5.0 μm) which further reduced the recovery of oocysts from seeded 100–litre tap water samples to 0.4 –5.5%.

b) *Cotton cartridge filters*

LeChevallier *et al.* (1995) filtered 100 ℓ of oocyst and cyst-spiked tap water through cotton cartridges (1.0 μm pore size) and had an average recovery of 75.0% for *Cryptosporidium* oocysts (7.0 - 129.0%) and 50.0% for *Giardia* cysts (31.0- 68.0%). The use of smaller pore size cotton cartridges (0.45 μm) had a lower recovery of *Giardia* cysts which averaged 14.0% from seeded tap water (10.0 - 21.0%) (Kfir *et al.*, 1995a).

c) *Borosilicate glass microfibre cartridge filters*

Whitmore and Carrington (1993) found the 2.0 μm pore size borosilicate glass microfibre cartridges had a mean recovery of oocysts from 100-litre seeded tap water of 8.8% while the mean recovery for seeded river water was 4.7%. A lack of filter integrity was encountered and elution of oocysts from the filter could only be achieved through backflushing the filter. Mechanical agitation of the filter disrupted the matrix to the extent that the fibres interfered with the subsequent analysis (Whitmore and Carrington, 1993).

d) *Vokes Polyfil cartridge filters*

Vokes polyfil cartridge filters, composed of individual pleated membranes of various pore sizes (2.0; 3.0 and 5.0 μm), were evaluated by Shepherd and Wyn-Jones (1996). A mean recovery of 11.2% (4.5 – 16.1%) and 9.4% (3.9 – 14.2%) was obtained for *Cryptosporidium* oocysts seeded into 100-litre tap and river water samples respectively. The mean percentage recovery from the same cartridges for *Giardia* cysts seeded into tap and river water was 17.1% (11.8 – 23.2%) and 16.3% (9.8 – 20.4%) respectively. The variation between organism recovery may be due to the larger size of *Giardia* cysts which are more likely to be retained in the filter cartridge than the *Cryptosporidium* oocysts (Shepherd and Wyn-Jones, 1996).

e) *Gelman Envirocheck cartridge filters*

More recently Gelman Envirocheck cartridges which are 1.2 μm pore size polysulfone absolute filters housed in a polycarbonate case were evaluated for their capacity to recover *Cryptosporidium* oocysts and *Giardia* cysts from seeded tap, synthetic turbid water (5 nephelometric turbidity units (NTU)) and natural surface water (115 NTU) (10 litre samples). The results showed that the Envirocheck cartridges were suitable for the range of samples as *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).

Increases in the volume filtered through cartridges of various media have little effect on recovery while a tenfold increase in flow rate decreased the filter retention (Musial *et al.*, 1987) and recovery of (oo)cysts decreases with increased turbidity (Nieminski *et al.*, 1995). Of the cartridge filters available, the Gelman Envirocheck cartridge had the best recovery of oocysts and cysts from a variety of water types although the cost ($ 95.00) of these cartridges may hinder their routine use.

1.4.3 MEMBRANE FILTRATION

Here 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 ℓ).

a) *Polycarbonate membrane filters*

The average recovery of oocysts and cysts from seeded tap water (10 ℓ) using 2.0 μm pore size polycarbonate membranes was 27.7% and 48.5% respectively whilst recovery of oocysts and cysts from seeded river water (10 ℓ) was 36.0% and 48.2 respectively (Shepherd and Wyn-Jones, 1996). Nieminski *et al.* (1995) had similar recovery of cysts from raw (50.0%) and treated waters (60.0%). The 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. An average of 101.1% of oocysts and 94.9% of cysts were recovered from tap water while 82.0% of oocysts and 113.0% of cysts were recovered from the turbid water samples. As the exact number of (oo)cysts is not known for seeding experiments, it can vary resulting in greater than 100% recoveries. Although the average recovery was high, the capability of the membrane was only 2.5 litres for the five NTU water sample making it impractical and expensive for use with environmental samples. When seeded tap water samples filtered through polycarbonate membranes (pore size 5.0 μm) the percentage of oocyst recovery decreased to approximately 5.0-20.0% (Ongerth and Stibbs, 1987).

b) *Polyethylsulfone membrane filters*

Polyethylsulfone membranes (nominal porosity 0.8 μm) were found to have a mean recovery of 24.0% for oocysts and 45.1% for cysts from seeded tap water (10 ℓ). Seeded river water had recoveries of 21.8% for oocysts and 41.2% for cysts. Samples were difficult to filter and cleaning of the membrane proved difficult due to compaction of particles onto the surface. This suggested that either 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).

c) ***Cellulose membrane filters***

Cellulose-acetate and cellulose-nitrate membranes of varying porosity have been evaluated for their recovery of oocysts and cysts from seeded tap and river water. Cellulose-acetate membranes (1.2 μm pore size) were able to recover 32.0 – 87.0% of oocysts from three to 10 litres of seeded tap water (Aldom and Chagla, 1995; Kfir *et al.*, 1995a; Shepherd and Wyn-Jones, 1996) whilst 4.5 – 51.3% of cysts were recovered from 10-litre seeded tap water (Kfir *et al.*, 1995a; 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). Clancy *et al.* (1997) evaluated the cellulose-acetate membrane dissolution method (8.0 μm pore size) for its ability to recover oocysts and cysts from seeded tap and synthetic turbid water (5 NTU) (10 ℓ). Water is filtered through the membrane which is then dissolved in acetone followed by 95% ethanol and 70% ethanol. Although the recovery of oocysts and cysts from tap water was 83.6% and 76.9% respectively, the recovery from the turbid water (5 NTU) was only 7.0% and 8.0% respectively. Clancy and colleagues (1997) found the cellulose-acetate membrane was only able to filter eight litres of turbid water prior to blocking which made this method expensive for use with environmental water samples. In comparison Shepherd and Wyn-Jones (1996) recovered 32.0 – 42.7 % of oocysts and 36.5 – 62.4% of cysts from seeded river water samples. This discrepancy in recoveries may be due to the difference in the turbidity of the water sampled. This method is suitable for small volumes of treated waters but is not suitable for turbid water samples due to the expense.

Cellulose-nitrate (pore size 3.0 μm) membranes had recoveries of 24.0 –36.0% for oocysts and 56.5 – 77.6% for cysts from seeded tap water (10 ℓ) whilst recovery of oocysts and cysts from 10-litre seeded river water was 25.3 – 36.0% and 47.1 – 60.0% respectively (Shepherd and Wyn-Jones, 1996).

d) ***Acrylic copolymer membrane filters***

Acrylic copolymer membranes (pore size: 1.2 μm) were found to recover 10.2 – 36.6% of *Cryptosporidium* oocysts in 10 ℓ of seeded potable water samples (Dawson *et al.*, 1993; Shepherd and Wyn-Jones, 1996) and 22.4 – 51.2% of *Giardia* cysts (Shepherd and Wyn-

Jones, 1996). The same pore size membrane recovered 29.3 – 41.3% of oocysts and 41.2 – 51.8% of cysts from seeded river water samples (Shepherd and Wyn-Jones, 1996). Similar recoveries of oocysts and cysts were achieved with the 3.0 μm pore size membranes as 17.3 – 28.0% of oocysts and 38.8 – 49.4% of cysts were recovered from seeded tap water. The recovery of oocysts from 10 ℓ of seeded river water ranged from 18.6 – 26.7% whilst the recovery of cysts ranged from 36.5 – 48.2% (Shepherd and Wyn-Jones, 1996).

1.4.4 OTHER FILTRATION METHODS

a) *Sand column filtration*

Sand column filtration was evaluated by pumping 2.5 ℓ of spiked water down a sand column at a predetermined rate (Whitmore and Carrington, 1993). The recovery of oocysts was a function of the loading rate as the slower the rate of loading (0.1 $m^3 m^{-2} h^{-1}$), the greater the oocyst recovery (98.4%). A high loading rate (19.8 $m^3 m^{-2} h^{-1}$) had poor oocyst recovery (2.3%).

b) *Vortex flow filtration*

Vortex flow filtration (VFF) is based upon Taylor vortices which are established in the apparatus by rotation of a cylindrical filter inside a second cylinder. The sample is fed under pressure between these two cylindrical surfaces forcing the liquid phase (permeate) across the filter into the inner cylinder, thus continuously concentrating the recirculating (retenate) fluid. Clogging of the filter is suppressed by the vortices generated between the cylinders. Using VFF with a polysulphone membrane cartridge (0.45 μm) Whitmore and Carrington (1993) recovered 30.0 - 40.0% of oocysts seeded in tap and river water samples. Fricker *et al.* (1997) concentrated up to 100 ℓ of *Cryptosporidium* and *Giardia* seeded tap and river water samples using VFF with hydrophilic polyacrylonitrile Ultrofilic™ membrane filters (nominal porosity of 0.05 μm). Recovery from seeded tap water (<0.1 NTU) was 87.5-90.4% while seeded river water (~5 NTU) had recoveries of 71.8 – 90.4%.

Membrex Ultrafilic polymer filters with VFF were evaluated by Clancy *et al.* (1997) for their ability to recover oocysts and cysts from tap, synthetic turbid water (5 NTU) and natural surface water (115 NTU). Recovery was low for both oocysts and cysts as an average of 9.4% of oocysts and 24.1% of cysts were recovered from 10-litre seeded tap water

samples, 28.0% of oocysts and 47.0% of cysts were recovered from 10-litre synthetic turbid water samples whilst 14.0% of oocysts and 19.0% of cysts were recovered from 10-litre surface water samples.

c) *Cross-flow filtration*

Cross-flow or tangential flow filtration is potentially a more efficient means of separating particles from a suspension as the bulk of the flow is parallel to the filter surface rather than perpendicular to it (Whitmore and Carrington, 1993). Part of the liquid phase passes through the filter whilst the remainder is recirculated. This has the effect of concentrating the solids in a decreasing volume of retenate in addition to maintaining the filter element clear of deposited material (Whitmore and Carrington, 1993). Using cross flow filtration Whitmore and Carrington (1993) recovered 73.0% and 86.0% of oocysts from seeded 10-litre deionised water samples, but this was not maintained in subsequent runs (37.0 - 41.0%) using 100-litre seeded tap water samples. It was noted that a film of deposited material was present on each membrane and this membrane fouling probably caused the deterioration in results. Efficient cleaning between trials or replacement of membranes is therefore necessary to maintain efficient recovery. Cross-flow filtration was found to be a rapid and comparatively efficient means of recovering *Cryptosporidium* oocysts. In addition the small volume of final retenate fluid facilitates the subsequent concentration by centrifugation (Whitmore and Carrington, 1993).

d) *Ultrafiltration*

Ultrafiltration is simply a low pressure selective fractionation of molecules by size. The ultrafiltration membrane concentrates higher molecular weight species while allowing lower molecular weight materials to pass through. An average recovery of 11.6% of cysts was obtained using ultrafiltration (pore size 40-50Å) with a range of 2.7 – 25.5% (Kfir *et al.* 1995a). They found ultrafiltration to be inefficient as only 10 ℓ was able to be filtered before flow difficulties occurred due to clogging of the filter.

1.4.5 CENTRIFUGATION

Whitmore and Carrington (1993) subjected small (10 ℓ) and large (100 ℓ) volumes of oocyst seeded water to continuous flow centrifugation. The recovery of oocysts was optimal from spiked tap (30.0%) and river water (11.0%) at a flow rate of 0.31 ℓ min^{-1}.

1.5. 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). Immunomagnetic separation, density gradient centrifugation and flow cytometry have been used to alleviate this problem.

a) 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 from different media (Olsvik *et al.*, 1994).

Bifulco and Schaefer (1993) used an antibody-magnetite method to selectively concentrate *Giardia* cysts introduced into prefiltered river water samples artificially mixed with green and blue-green algae to achieve turbidities of approximately 6 000, 600, 60 and 6 NTU. The magnetically labelled cysts were concentrated by high-gradient neodymium magnetic separation which recovered an average of 82% of the seeded cysts from water samples of various turbidities. Significantly higher cyst recoveries of approximately 97.0% were obtained from water samples with turbidities below 600 NTU.

Rossomando *et al.* (1995) evaluated the potential of IMS for the continuous immunocapture (CIC) of *Cryptosporidium* oocysts and *Giardia* cysts. Results indicated that capture was dependent on mixing time, flow rate and immunobead number. They concluded that antibody-antigen binding affinities are of sufficient strength to allow capture from flowing water. One hundred percent of oocysts and cysts were recovered from seeded buffer samples.

b) Density gradient centrifugation

A number of *Cryptosporidium* and *Giardia* concentration procedures include a density gradient to separate oocysts or cysts from debris in the concentrated sample. The specific gravity of the gradient is chosen so that the microorganisms 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 evaluated and recoveries of cysts from stools averaged 76.0, 77.0, 70.0, 68.0, 60.0 and 40.0% for potassium citrate, Percoll-sucrose (specific gravity 1.09), Sheather's sucrose flotation, 4/4 Sheather's sucrose flotation, 3/5 Sheather's sucrose flotation and zinc sulfate respectively (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 110.0% of cysts 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, 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.

c) Flow Cytometry

Flow cytometers 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 488nm laser light,

emits light at 525nm. This light energy 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-dimentional 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.

Often flow cytometry is unable to distinguish between oocysts and some autofluorescent plants, algae or mineral particles in water samples but Vesey *et al.* (1993b) have used flow cytometry and cell sorting (FCCS) to separate oocysts and cysts from background debris. This resulted in the recovery of oocysts and cysts seeded into river and reservoir water samples in excess of 92% (Vesey *et al.*, 1994). They found the technique enabled large volumes of sample to be tested rapidly and 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 personnel requirements for the running of the cytometer, the need to preconcentrate, the difficulty in obtaining the equipment and the need for confirmation of oocyst and cyst identity by microscopy (Jakubowski *et al.*, 1996).

1.6. DETECTION METHODS FOR *CRYPTOSPORIDIUM* AND *GIARDIA* IN WATER

Bright field microscopy for detecting *Giardia*, or conventional clinical staining using acid-fast stains such as modified Ziehl-Neelsen for identification of *Cryptosporidium* oocysts, is of little use in water-related studies because distinguishing oocysts and cysts amongst numerous extraneous particulates in water is impossible.

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). Alternative techniques, some of which are modifications to the standard immunofluorescence techniques or use very different technologies, have been developed.

a) Enzyme linked immunosorbent assay (ELISA) / enzyme immuno-assay (EIA)

Enzyme linked immunosorbent assay (ELISA) or enzyme immuno-assay (EIA) detects an antibody-antigen reaction using an enzyme and substrate reaction to produce a detectable colour. Kits that use labelled antibodies specific to *Cryptosporidium* and *Giardia* are available for use in environmental samples but are currently not used to a great extent by laboratories conducting environmental testing (Jakubowski *et al.*, 1996).

The EIA method detected < 10 fresh oocysts / cysts seeded into concentrated source water samples but sensitivity was adversely affected by turbid water samples and cross-reactivity with algae occurred (De la Cruz and Sivaganesan, 1994). In contrast Siddons *et al.* (1992) found EIA more sensitive than microscopy for detecting oocysts in water samples as the debris present in water that makes microscopy difficult, does not affect EIA.

ELISA / EIA has the advantages of rapid screening of numerous samples, minimal reagent preparation, being inexpensive, simple and able to be read visually or with a spectrophotometer (Scheffler and van Etta, 1994; Aldeen *et al.*, 1995). The use of a microplate reader allows for semi-quantitative testing and an ELISA analyser has the potential to automate oocyst / cyst detection. Previous studies with ELISA for *Giardia*

have shown the degree of non-specific binding to be insignificant (Goldin *et al.*, 1990; Rosenblatt *et al.*, 1993).

The disadvantages of ELISAs are the lack of viability detection (Jakubowski *et al.*, 1996), the lack of species detection (Graczyk *et al.*, 1996), non-specific antibody binding (De la Cruz and Sivaganesan, 1994) and inability to detect *Cryptosporidium* and *Giardia* simultaneously (Rochelle *et al.*, 1995). Two types of error may affect the performance of ELISA: operator error and variations due to changes in ambient conditions such as temperature fluctuations (Goldin *et al.*, 1993).

b) Immunofluorescence assay (IFA)

Immunofluorescence assays use fluorescent dyes coupled to antibodies specific to the antigen of interest. The conjugates combine with the antigen and can be visualised by epifluorescence microscopy.

Commercially available direct immunofluorescence (the antibody conjugated to the fluorochrome is applied directly to the antigen) and indirect immunofluorescence (unlabelled antibody is applied to the antigen and visualised by adding a fluorochrome conjugated anti-immunoglobulin) assays use fluorescein isothiocyanate (FITC) labelled 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; (c) size; (d) the presence of internal structures under DIC (LeChevallier *et al.*, 1995) (1.2). Tetramethyl red (TMR) labelled antibodies against *Cryptosporidium* or *Giardia* are also available which stain the (oo)cyst wall red (Waterborne Inc., Louisiana, USA).

According to Graczyk *et al.* (1996) there is no difference in the sensitivity and specificity between direct and indirect immunofluorescence although the testing of environmental samples may lead to misindentification of medically important isolates. Confirmation by differential interference contrast (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 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 putative (oo)cysts can be occluded (Johnson *et al.*, 1993). The nucleic acid stain 4', 6-diamidino-2-phenylindole (DAPI) used in conjunction with the IFA provides an easy, reproducible method for the detection and identification of sporulated oocysts as sporozoite nuclei are stained sky-blue. This reduces the likelihood of false positive or false negatives occurring (Grimason *et al.*, 1994).

c) Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is based on enzymatic amplification to detectable levels of target nucleic acid sequences that may be present in low numbers in water samples (Jakubowski *et al.*, 1996).

Recent advances in molecular techniques have included double PCR with primers to increase the chance of detecting *Giardia* cysts in wastewater samples which contain high amounts of the inhibitory factors humic and fulvic acid (Mayer and Palmer, 1996). Simultaneous running of *Cryptosporidium* and *Giardia* in one piece of equipment, which is made possible with PCR technology, would save significant amounts of time, expense and analyst fatigue in most laboratories (Swabby-Cahill *et al.*, 1994).

Viability determination has also been performed using a reverse transcription (RT)-PCR, which can detect the presence of a single viable oocyst in treated or turbid waters by the detection of mRNA from *C.parvum* heat shock protein (hsp) (Stinear *et al.* ,1996) while Mahbubani *et al.* (1991) were able to discriminate between live and dead cysts using the giardin gene, which constitutes the diagnostic ventral disk of *Giardia* (Peattie *et al.*, 1989), as the target.

Johnson *et al.* (1995) used oligonucleotide primers to routinely detect one to 10 oocysts in purified environmental preparations as shown by immunofluorescence and direct microscopic counts. However, the sensitivity of PCR in some seeded environmental water samples was up to 1 000 fold lower although this interference was reduced by flow cytometry and magnetic-antibody capture. Sensitivity was also improved 10 to 1 000 fold by probing the PCR product on dot blot with an oligonucleotide probe detected by chemiluminescence.

Limitations of PCR include the removal of inhibitors such as humic compounds which interfere with the activity of enzymes in the reaction and the fact that oocyst numbers can only be estimated by visual comparison of the intensities of the sample bands produced with the band produced by one viable oocyst (Stinear *et al.*, 1996).

The advantages of PCR are the decreased time and cost and increased sensitivity which allows for the detection of low numbers of target DNA and RNA usually found in environmental samples while the major disadvantages are the need for preconcentration and purification (Jakubowski *et al.*, 1996).

d) DNA Hybridization

Hybridization of full-length DNA probes to membrane bound target DNA extracted from concentrated water samples is a rapid method for the detection of *Cryptosporidium* oocysts and *Giardia* cysts (Rochelle *et al.*, 1995). However, reported sensitivities ranged from one to five *Giardia* cysts detected per mℓ (Abbaszadegan *et al.*, 1991) to 10 000 *Cryptosporidium* oocysts per mℓ at best (Johnson *et al.*, 1993).

The biggest hinderance of the probe used by Johnson *et al.* (1993) was its high degree of nonspecific detection in environmental samples. As a result false positive signals were detcted by autoradiography. They state that the advantage of hybridization techniques is that large samples can be analyzed simultaneously while the disadvantages are that it is less sensitive than immunofluorescence and can yield false positives with environmental samples.

e) Fluorescent In-Situ Hybridization (FISH)

The hybridization of fluorescently labeled oligonucleotide probes to single stranded ribosomal RNA (ss rRNA) forms the basis of FISH. The fluorescing target organisms are observed by fluorescence microscopy, confocal laser scanning microscopy (CLSM) or flow cytometry (Rochelle *et al.*, 1995).

FISH combined with CLSM detection is under development for identification and differentiating *C.parvum* oocysts from morphologically similar non-pathogenic *Cryptosporidium* spp. in water samples (Jabukowski *et al.*, 1996). The advantage of the CLSM technique is its ability to detect low intensity fluorescence signals with clear imaging of fluorescently labeled oocysts in semi-opaque detrital material occurring in turbid waters.

A comparison of oocysts percentage viability as determined by excystation to that determined using FISH indicated that FISH has potential to determine the viability status of oocysts although this needs be evaluated with oocysts inactivated by chemical disinfection (Vesey *et al.*, 1997).

The disadvantage is that the physiological conditions of the oocysts will affect the fluorescence intensity of FISH-positive oocysts while CLSM instrumentation is also expensive (Jabukowski *et al.*, 1996).

f) Ultraviolet-visible (UV-VIS) spectroscopy

This procedure utilizes optical information obtained from both UV and visible spectra on a spectrophotometer and is under development using purified oocyst or cyst concentrates (Jakubowski *et al.*, 1996). In the visible range of the spectrum (400 – 800 nm), light scattering provides information on the size and quantity of particles while in the UV region (200 – 400 nm), proteins and other cellular components produce a unique biochemical make-up or “fingerprint” thereby allowing species differentiation (Patten *et al.*, 1994).

Sample concentrations are varied by sucrose dilutions and five to seven spectral readings are obtained for each dilution in order to obtain spectra within the linear range of the spectrophotometer. Spectral deconvolution techniques are used to identify the microorganism. Negative aspects of this technique include losses of oocysts / cysts in the concentration procedure and background particles causing numerous spectral patterns which may mask deconvolution of the target fingerprint (Jakubowski *et al.*, 1996).

Patten *et al.* (1994) reported a sensitivity of 10 organisms per mℓ for purified and semi-purified samples. The method is quantitative and may be able to determine viability when oocysts / cysts are treated with fluorogenic dyes. It has the potential to be a rapid, automated analysis.

g) Electrorotation assay (ERA)

Based on technology developed in the UK, this assay operates on the principle that small particles within a rotating electrical field will rotate in a specific manner at specific frequencies (Jakubowski *et al.*, 1996). The rate of ERA is dependent on a number of factors such as the frequency of rotation of the field and the surface charge characteristics of the particles. The target organism possesses a characteristic rotational profile at a defined frequency that distinguishes it from other particles - an observation made using light microscopy (Jakubowski *et al.*, 1996).

This method, in which water samples (10 – 100 ℓ) are passed through a compressed foam filter prior to ERA, is developmental but may be able to distinguish between viable and nonviable oocysts and cysts, based on differences in their rotation rates at different frequencies. Water sources containing algae, debris or high turbidity may require clarification prior to use for the analysis of smaller portions of sample (Jakubowski *et al.*, 1996).

1.7. VIABILITY DETERMINATION OF *CRYPTOSPORIDIUM* AND *GIARDIA* IN ENVIRONMENTAL WATERS

The assessment of oocyst or cyst viability becomes important in examining survival in the environment, treatment processes and public health risks. Viability of oocysts or cysts may be measured by infectivity, excystation or dye inclusion or exclusion (Lisle and Rose, 1995).

1.7.1 INCLUSION / EXCLUSION DYES

a) Fluorescein diacetate (FDA) and propidium iodide (PI)

The inclusion dye fluorescein diacetate (FDA) and the exclusion dye propidium iodide (PI) have been used to determine viability of mammalian cells (Jones and Senft 1985). FDA is a nonpolar ester which passes through the cell membrane and is hydrolysed by intracellular esterases to produce fluorescein. Accumulated fluorescein exhibits green fluorescence when excited by blue fluorescent light, indicating a viable cell (Sauch *et al.*, 1991). FDA has been shown to be nontoxic to a wide variety of cell types ranging from primitive eukaryotes to mammalian cells and tissues (Rotman and Papermaster, 1966). PI is capable of passing through only damaged cell membranes where it intercalates with the nucleic acids of injured and dead cells to form a bright red fluorescent complex (Sauch *et al.,* 1991). Jones and Senft (1985) have shown PI to be nontoxic to cells.

Schupp and Erlandsen (1987) evaluated the efficacy of the fluorescein diacetate (FDA)-propidium iodide (PI) method for determining cyst viability by using animal infectivity as an *in vivo* indicator of the viability of the fluorogenic dye stained cysts. Their study demonstrated that FDA positive cysts are viable as determined by infectivity while PI-positive cysts were nonviable and incapable of producing infection. The procedure was found to be simple, inexpensive, quick and has the potential for determining the effects of disinfectants (physical or chemical) and other environmental factors on viability of oocysts and cysts.

Although PI exclusion is not suitable for determining the efficacy of chlorine or monochloramine disinfection (as the sites into which PI intercalates are masked or destroyed by these chemicals) it is useful for the determination of cyst viability in raw water or treated water supplies prior to the addition of chlorine or monochloramine (Sauch *et al.*, 1991).

Not all cysts that exclude the fluorogenic dye PI or incorporate the dye FDA may be able to undergo excystation (DeRegnier *et al.*, 1989). Both FDA and PI were found to overestimate viability after *Giardia* cysts were warmed to 60°C which kills cysts (Thiriat *et*

al., 1998). This author also found viability determination, using eosin exclusion, was overestimated.

b) Propidium iodide-immunofluorescence assay (PI-IFA)

Dowd and Pillai (1997) developed a propidium iodide-immunofluorescence assay (PI-IFA) staining method for the simultaneous detection and viability determination of oocysts and cysts. Nonviable (PI+) oocysts and cysts had characteristic fluorescent green cell walls surrounding an orange-red fluorescing centre. Viable (PI-) oocysts and cysts were distinguished by fluorescing green cell walls without the orange-red fluorescence internally.

c) 4', 6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI)

Campbell *et al.* (1992) developed a viability assay for *Cryptosporidium* oocysts based on the inclusion or exclusion of two fluorogenic dyes namely 4', 6-diamidino-2-phenylindole (DAPI) and propidium iodide. The results of the study demonstrated that oocysts whose walls were permeable to DAPI and not to PI following incubation, were viable as they excysted *in vitro.*

Thiriat *et al.* (1998) used FITC labelled antibodies to quantify *Giardia* cysts and the fluorogenic dyes DAPI and PI and differential interference contrast (DIC) to determine cyst viability. The DIC optics made it possible to compare the morphology of the cysts with strictly defined morphological criteria indicative of viability (refringent cytoplasm) or lack of viability (granulous and retracted cytoplasm or empty cysts). They found this method more accurate than FDA or PI, as only non-viable cysts would have stained after warming at 60°C for 20 minutes to ensure cyst death (Schaefer *et al.*, 1984). FDA and PI, as mentioned before, had false positives for viability under these conditions (Thiriat *et al.*, 1998).

d) FluoroBoro I (FBI)

Exclusion of the fluorescent dye 3-[dansylamido-phenyl boronic acid] (FluoroBora I – FBI) from *Giardia muris* cysts was compared with excystation as a measure of cyst viability (Hudson *et al.*, 1988). Non-viable *Giardia muris* cysts accumulated the dye and fluoresced green whereas viable cysts excluded the dye and showed no fluorescence.

This method was performed in 5 minutes and showed a higher degree of precision than excystation at viability levels above 60%. Since only fluorescence or non-fluorescence were observed, the method is less subjective than excystation and no further reference has subsequently been made (Hudson *et al.*, 1988).

1.7.2 ANIMAL INFECTIVITY

Animal studies have long been considered the method of choice for determining the infectivity of oocysts and cysts (Jakubowski *et al.*, 1996). However, infectivity studies fail to provide useful quantitative data for low concentrations (Jakubowski *et al.*, 1996) as the 50% infectious dose (ID_{50}) for *Cryptosporidium* oocysts ranges from 60 to 1 000 (Lindsay, 1997). Animal infectivity models are generally expensive, laborious and require special accommodations (Fayer *et al.*, 1997).

Animal infectivity assays have been useful in assessing the effectiveness of drinking water disinfection (Finch *et al.*, 1993). More recently, Swabby-Cahill and Cahill (1997) developed a murine model that is relatively inexpensive and offers an *in vivo* model to study infectivity and viability of *C. parvum* oocysts.

1.7.3 CELL CULTURE

The cell culture technique is able to detect oocysts and cysts by enducing them to excyst and pass through their respective developmental stages (Figures 1.1. and 1.2.) thereby determining infectivity and possibly speciation of oocysts or cysts. The disadvantages of this technique are the need for purified concentrates, optimal conditions for excystation, growth has to be examined microscopically or by nucleic acid methods and special

facilities and equipment are necessary for routine maintenance of tissue cultures (Jakubowski *et al.*, 1996).

Cell lines that have been successful for *in vitro* cultivation of *Cryptosporidium* include HCT-8 (Upton *et al.*, 1995; Battigelli *et al.*, 1997), MDBK, MDCR, RL95-2 (Upton *et al.* 1994), MDCK (Rosales *et al.*, 1993) and Caco 2 (Rochelle *et al.*, 1997). The Caco 2 cell line is able to support the growth of both *Cryptosporidium* and *Giardia,* offering the potential for a simultaneous infectivity assay.

1.7.4 *IN VITRO* EXCYSTATION

Excystation techniques rely on *in vitro* simulation of the epigastric conditions (elevated temperatures and acidic environment in the presence of bile salts) under which infective *Cryptosporidium* sporozoites or *Giardia* trophozoites emerge from the oocyst or cyst respectively. Excystation suspensions were viewed microscopically using fluorescence and DIC for quantification of viable oocysts (Campbell *et al.*, 1992).

Advantages of excystation include its ease of use, low cost and resemblance to the process of pathogenesis. Correlation of excystation with the viability stains DAPI and PI was shown to be statistically significant (Campbell *et al.*, 1992). Disadvantages include the need for concentration and purified samples, extended incubation periods and experimental variability. In addition, the estimation of *Giardia* cyst viability determined by excystation includes the free-swimming trophozoites and the partially emerged trophozoites. Many of the partially emerged forms appear never to complete the excystation process therefore counting partially emerged forms as viable cysts may overestimate the viability of the population (DeRegnier *et al.*, 1989). This method is not practical for application to environmental samples because large numbers of oocysts or cysts are necessary for conducting the assay (Jakubowski *et al.*, 1996).

1.8. OCCURRENCE OF *CRYPTOSPORIDIUM* AND *GIARDIA* IN ENVIRONMENTAL SAMPLES

Cryptosporidium oocysts and *Giardia* cysts, excreted by infected humans and animals, can contaminate waters either in faeces, in sewage effluent, in slurry discharges or in run-off from faecally contaminated land.

a) Occurrence of Cryptosporidium and Giardia in surface waters

Examination of surface water, comprised of streams, rivers, lakes or dams/reservoirs, concentrates from the UK and USA has indicated that both *Cryptosporidium* and *Giardia* have widespread occurrence. *Cryptosporidium* was detected in 24.0 - 100.0% of 600 surface waters tested in the USA. The concentration of oocysts ranged from 0.0025 - 484 ℓ^{-1} (Ongerth and Stibbs, 1987; Sterling, 1990; Hansen and Ongerth, 1991; LeChevallier *et al.*, 1991; Rose *et al.*, 1991; LeChevallier and Norton, 1995). In comparison, the UK occurrence of *Cryptosporidium* in surface waters was lower, ranging from 4.4 - 40.5% despite more samples being analysed (2 106) (Smith *et al.*, 1990; The National *Cryptosporidium* survey group, 1992; Humphreys *et al.*, 1995;). A survey of 22 Canadian surface waters did not detect *Cryptosporidium* (Roach *et al.*, 1993) while in Germany and Spain surface waters tested for the presence of *Cryptosporidium* were 77.8% (7/9) and 50.0% (4/8) positive respectively (Gornik and Exner, 1991; DeLeon *et al.*, 1993). Oocyst concentrations in Spanish waters were extrapolated to range from <0.0 - 0.31 ℓ^{-1} (DeLeon *et al.*, 1993).

In the USA, *Giardia* cysts were detected in 16.0 -81.2% of 488 surface waters tested, with levels extrapolated to range from 0.02 - 66 cysts ℓ^{-1} (LeChevallier *et al.*, 1991; Rose *et al.*, 1991). Of 53 samples tested in the UK, 33.0% were positive with cyst concentrations ranging from 0.01 - 1.05 ℓ^{-1} (Smith *et al.*, 1993). *Giardia* cysts were also detected in 7.0% of samples tested from 22 Canadian surface waters while 63.0% of Spanish waters had *Giardia* in low concentration (extrapolated to range from <0.01 to 0.21 cysts ℓ^{-1}) (Gornik and Exner, 1991; DeLeon *et al.*, 1993)

In South Africa, the presence of *Giardia* cysts ranged from zero to 460.0 10 ℓ^{-1} and exceeded the number of *Cryptosporidium* oocysts in surface waters (0.0 - 250.0 10 ℓ^{-1}). This may have been due to the presence of cysts of non-human origin as the immunofluorescence assay used was pan specific and reacted with all species of *Giardia* (Kfir *et al.*, 1995b).

Giardia cysts were detected in five of 10 (50.0%) surface water samples collected in the Western Cape (Harding and Genthe, 1998). Levels ranged from 40.0 - 390.0 cysts ℓ^{-1} while *Cryptosporidium* oocysts were not detected. One hundred and fourteen raw water samples were analysed by Gericke *et al.* (1996) over a 12 month period. *Cryptosporidium* oocysts were detected in 59 of the samples (52%) while *Giardia* cysts were detected in 75 (66.0%) of the samples. *Cryptosporidium* and *Giardia* were concurrently detected in 39.0% of the raw water samples (42 / 114). Oocyst and cyst levels ranged from 50.0 - 700.0 per 100 ℓ and 70.0 - 500.0 per 100 ℓ respectively with higher averages occurring during summer months.

Harding and Genthe (1998) collected 17 raw water samples from 10 impoundments in the Western Cape of which nine (52.9%) were positive for *Giardia* and two (11.8%), from the same source, were positive for *Cryptosporidium*. The mean number of *Giardia* cysts detected was 1.04 per litre while an average of 0.8 oocysts per litre was detected for *Cryptosporidium.*

In the UK and USA, examination of raw waters indicated that the concentrations of oocysts and cysts detected are generally low and depend upon the variety of contributors and their associated activities performed in the catchment area. In developing countries, as a result of the likely contamination of water by human and animal wastes, the occurrence and concentration of these organisms in surface water is likely to be higher (Smith *et al.*, 1995).

b) Occurrence of Cryptosporidium and Giardia in potable waters

Examination of drinking water in the UK and USA has revealed the presence of *Cryptosporidium* oocysts and *Giardia* cysts although outbreaks have not been associated with all communities served by these waters.

In the USA, the occurrence of *Cryptosporidium* in 118 potable waters tested ranged from 17.0 - 26.8% with levels of 0.005 - 0.017 oocysts ℓ^{-1} while *Giardia* cysts were detected in zero to 16.9% of samples (LeChevallier *et al.*, 1991; Rose *et al.*, 1991). In the UK, one of 15 potable water samples analysed was positive for *Cryptosporidium* with 0.006 oocysts ℓ^{-1} being detected (Humphreys *et al.*, 1995). Of the 106 treated water samples analysed from Scotland, *Giardia* cysts occurred in 19.0% of samples with concentration levels ranging from 0.01 - 1.67 ℓ^{-1} (Smith *et al.*, 1993).

Thirty-three percent of nine potable water samples from Spain were positive for *Cryptosporidium* with oocyst levels ranging from <0.01 - 0.02 ℓ^{-1} whilst 22.0% were positive for *Giardia* with cyst levels ranging from <0.01 -0.03 ℓ^{-1} (DeLeon *et al.*, 1993).

Kfir et al. (1995b) examined selected drinking waters in South Africa and detected 0.0 - 4.0 *Giardia* cysts per 10 ℓ and 0.0 -1.0 per 10 ℓ *Cryptosporidium* oocysts whilst Gericke *et al.* (1996) did not detect *Cryptosporidium* oocysts, but did detect *Giardia* cysts (4.0 cysts per 100 ℓ) in one of eight samples tested monthly or a year.

c) Occurrence of Cryptosporidium and Giardia in groundwater

Contamination of groundwater, derived from wells and boreholes, by microbes is infrequent although limited study results have shown that *Cryptosporidium* and *Giardia* do occur. In the US, *Cryptosporidium* oocysts were found in 10.1% (22/217) of the sites including 4.7% (7/149) of the vertical wells, 14.3% (5/35) of the springs, 50.0% (2/4) of the infiltration galleries and 45% (5/11) of the horizontal wells (Rose *et al.* 1991; Hancock *et al.*, 1998). *Giardia* cysts were found in 6.1% (12/199) of the sites, including 1.3%

(2/149) of vertical wells, 14.3% (5/35) of the springs, 25.0% (1/4) of the infiltration galleries and 36.4% (4/11) of the horizontal wells.

Laboratory studies in which *Cryptosporidium* oocysts were seeded into three soil types confirmed the potential for groundwater contamination associated with rainfall where livestock waste has been applied to land (Mawdsley *et al.*, 1996).

d) Occurrence of Cryptosporidium and Giardia in cistern water

Cistern or rainwater roof catchment systems are used as a primary water supply throughout the U.S. Virgin Islands (Lye, 1992) where most of the population relies on cistern water as the potable water supply. As these systems are open to the environment they are susceptible to contamination from the droppings of birds, rodents and other animals as well as debris from trees. Crabtree *et al.* (1996) determined the occurrence and concentrations of the human enteric protozoa, *Cryptosporidium* and *Giardia*, in cisterns. A total of 45 samples was collected from cisterns over a one year period and *Cryptosporidium* oocysts and *Giardia* cysts were detected in privately owned and public cisterns. The average number of *Cryptosporidium* oocysts detected was 2.41 oocysts per 100 ℓ (range <1.0-70.3 oocysts) and 48.0% of the samples were positive. The average number of *Giardia* cysts was 1.1 cysts per 100 ℓ (range <1-3.79 cysts) and 26.0% of samples were positive.

e) Occurrence of Cryptosporidium and Giardia in wastewater

Nine treated sewage effluent (activated sludge) plants in the US were determined by Madore *et al.* (1987) to contain *Cryptosporidium* with levels of 140 to 3 960 oocysts ℓ^{-1}. Lower numbers of oocysts were found when activated sludge treatment was followed by sand filtration (4 - 2 560 oocysts ℓ^{-1}).

In the UK, nine treated sewage effluents tested were found to contain *Giardia* cysts with an average concentration of 358.60 cysts ℓ^{-1} (Gilmour *et al.*, 1991). Treated effluent samples tested in South Africa contained zero to 450 oocysts per 10 ℓ and 0 - 3 910 cysts per 10 ℓ (Kfir *et al.*, 1995b).

1.9. SOURCES OF CONTAMINATION IN WATERBORNE OUTBREAKS OF *CRYPTOSPORIDIUM* AND *GIARDIA*

Approximately 50% of the outbreaks of giardiasis have occurred where the treatment of the water implicated is minimal whereas outbreaks of cryptosporidiosis seem to have occurred where there has been an unusual occurrence in the water supply, either due to irregularity in procedure or treatment, a greater than usual exposure to oocysts at the treatment plant or from post-treatment contamination. That outbreaks of waterborne giardiasis occur frequently from pristine water supplies further suggests that the zoonotic contribution may be significant (Smith *et al.*, 1995). Five well-documented outbreaks of cryptosporidiosis in drinking water in the US affected from 500 to 403 000 persons. Lakes, springs and ground water have been implicated as the source (Rose *et al.*, 1997).

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). Oocyst numbers in outbreak implicated water has ranged from 0.002 - 77.0 ℓ^{-1} (Richardson *et al.*, 1991; MacKenzie *et al.*, 1994). A number of other factors may influence the possibility of outbreaks from low levels of contamination. These may include the immune status of the population at risk, the number of individuals not previously exposed and therefore have little resistance, numbers of oocysts or cysts in the water and their viability and virulence status (Smith *et al.*, 1995).

The concentration of oocysts and cysts in potable water depends upon a number of factors such as the concentration of oocysts and cysts in the raw water at the treatment plant, the treatment components in use at the treatment plant, the combined removal efficiency of the treatment plant and the integrity of the distribution system (Rose *et al.*, 1997).

1.10. *CRYPTOSPORIDIUM* AND *GIARDIA* REGULATIONS

No drinking water regulations in the USA specifically address *Cryptosporidium* in potable water supplies although a number of related regulations provide some protection (Rose *et al.,* 1997). The Enhanced Surface Water Treatment Rule (ESWTR) draft includes a

watershed control programme for *Cryptosporidium* and *Giardia*, a sanitary survey of public water systems every three to five year and 3 - 6 log treatment based on raw water levels with a minimum treatment of 2 log removal of *Cryptosporidium* and 3 log removal for *Giardia* (99.9%) (USEPA, 1994).

Proposals have been put forward in England and Wales for a legal requirement to ensure treated water contains no more than one oocyst per ten litres. Failure to maintain this standard may result in unlimited fines against the water supplier (Water Quality International, 1998). In South Africa, the Department of Water Affairs and Forestry (DWAF) recommend < 1 (oo)cysts per 10 ℓ in potable water (DWAF, 1996).

1.11. SUMMARY AND OBJECTIVES

Although there are many causes of diarrhoea, the enteric protists *Cryptotosporidium parvum* and *Giardia lamblia* have been recognised as important causes of both outbreak-related and sporadic diarrhoea in humans (Casemore, 1990). Whilst the major route of transmission is from person-to-person, water is a potential source of *Cryptosporidium* and *Giardia* worldwide and risk assessment models suggest that this contamination is contributing to endemic levels of disease (Lisle and Rose, 1995).

In most individuals both *Cryptosporidium* and *Giardia* infections are self-limiting, however, in some immunocompromised individuals cryptosporidiosis can cause death (Smith *et al*., 1995). Whilst several drugs are available for the treatment of giardiasis, no effective drug therapy is presently available for treating cryptosporidiosis (Blagburn and Soave, 1997). The low infectious dose for both protists means that few viable (oo)cysts need to be ingested for infection to establish in susceptible hosts.

Population and laboratory-based surveys of the prevalence of cryptosporidiosis and giardiasis depend on the identification of (oo)cysts in faecal specimens. Many surveys have been biased towards children and completed over too short a time period to adequately address the possibility of seasonal or temporal variation (Meinhardt *et al*., 1996). Despite these biases, both population and laboratory-based surveys do provide valuable information on the ubiquity of the organisms (Current, 1994).

Cryptosporidium and *Giardia* concentrations in water are generally low and depend upon various activities in catchments. However, the occurrence and concentration of these organisms in surface waters is likely to be higher in developing countries where contamination of water by human and animal wastes is likely to be more prevalent (Smith *et al.*, 1995). Several waterborne outbreaks of cryptosporidiosis and giardiasis have been reported worldwide and suspected sources of contamination in outbreaks have included raw sewage, runoff from agricultural land, treated wastewater from boats, faults in operational procedure or mechanical and operational difficiencies at water treatment plants. Properly operated conventional treatment of water can, however, remove 99.0% or more (oo)cysts (Nieminski, 1994).

Environmental monitoring of both parasites is made problematic by their small size, their relatively low concentrations in most waters, the inability to augment their numbers by *in vitro* culture and the difficulty in identifying them amongst other particles and debris (Smith *et al.*, 1995). In addition. the ideal concentration and detection technique for *Cryptosporidium* and *Giardia* has not yet been identified. Procedures include a concentration step which either requires the filtration of large volumes of water through a membrane or cartridge filter or the flocculation of smaller volumes of water. Thereafter the trapped parasites are either eluted from the filter matrix or released from the floc and further concentrated by centrifugation to a smaller volume. Clarification of turbid samples is possible using density gradients, IMS or flow cytometry, with varying degrees of success. Examination and identification of the final concentrate or a proportion thereof is generally by fluorescence microscopy using labelled antibodies specific to *Cryptosporidium* or *Giardia* although other methods such as ELISA, DNA hybridization, PCR, UV-VIS spectroscopy, ERA and FISH have been evaluated. However, the identification of (oo)cysts in water concentrate can be difficult, particularly when they are either obscured by debris, contain no identifiable internal contents or are misshapen because of environmental degradation, method processing or both (Smith and Hayes, 1997).

The assessment of *Cryptosporidium* oocyst and *Giardia* cyst viability is important in examining survival in the environment, treatment processes and public health risk. *In vitro* excystation, vital dye staining and animal infectivity have been used in this regard with varying degrees of success. While animal infectivity has proved the most successful, this is expensive and time-consuming, therefore quicker and cheaper methods are required for routine viability determination. Other techniques such as *in vitro* excystation or dye

inclusion / exclusion are easy to use and relatively inexpensive and their correlation has been shown to be statistically significant (Campbell *et al.*, 1992).

Many researchers question the validity of concentration and detection methods used to isolate and quantify these protists from water so the search for novel techniques continues. Sensitivity and specificity are paramount when determining the occurrence and significance of (oo)cysts in water concentrates (Smith *et al.*, 1995) therefore ideal concentration and detection techniques need to be automated to reduce analyst error and time.

In addition, technologies to remove and/or inactivate these organisms in water treatment are under scrutiny therefore the monitoring of environmental waters needs to be instituted to minimise the risk of infection to the public. Results from monitoring pose questions such as the need to determine the source of the (oo)cysts in the environment and the means to eradicate the contamination.

Future research may involve the elucidation of the virulence status of (oo)cysts which may also assist in developing new methods of (oo)cyst capture and inactivation. As present techniques for monitoring waters are time-consuming, indicators of possible contamination or the production of an early warning system would be beneficial. On-site continuous monitoring of water to a treatment plant would be ideal and would allow immediate action should (oo)cysts be present in raw water.

Few countries have regulations regarding the monitoring of these organisms in potable waters. Stringent regulations in the United Kingdom state that failure to maintain the standard of < 1 oocyst per ten litres may result in unlimited fines against the water purveyor. South Africa recommend that < 1 oocyst per ten litres be present in potable water, but at present non-compliance does not result in prosecution.

This research programme will incorporate an epidemiological study using data collected from pathology laboratories to ascertain the significance of these protists in KwaZulu-Natal populations. The occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in

environmental water samples in the Umgeni Water Operational area will also be determined. As the ideal concentration and detection technique for *Cryptosporidium* and *Giardia* in water has not yet been identified, various currently used concentration techniques for the recovery of (oo)cysts from potable and environmental waters will be selected on their reported recovery efficiencies and evaluated. The detection of (oo)cysts in turbid waters can prove difficult therefore methods aimed at enhancing their recovery and identification will be evaluated. Detection techniques that are easy-to-use and inexpensive will also be developed during this research programme as will a novel viability stain.

*CHAPTER 2
UBIQUITY OF THE WATERBORNE PATHOGENS, *CRYPTOSPORIDIUM* AND *GIARDIA*, IN KWAZULU-NATAL POPULATIONS

2.1. INTRODUCTION

Inadequate water supply and sanitation is largely responsible for more than 800 million estimated cases of diarrhoeal disease and 4.5 million associated deaths in developing countries every year (Esrey *et al.*, 1990). In South Africa more than 12 million people (30% of the population) did not have access to an adequate potable water supply in 1994 and nearly 21 million (53.8%) lacked basic sanitation (Dept. Water Affairs and Forestry, 1994) that further highlights the potential of infection by waterborne disease. Although there are many causes of diarrhoea, the enteric protists *Cryptosporidium parvum* and *Giardia lamblia* have been recognised as important causes of both outbreak-related and sporadic diarrhoea in humans (Casemore, 1990). Their major means of transmission is the faecal-oral route while water and zoonotic transmission are also of importance and *Giardia* has been reported to be sexually transmitted (Phillips *et al.*, 1981). High risk categories for infection by both protists include young children (< 5 years) and immunocompromised patients (Current and Haynes, 1983). In South Africa 11 out of 110 children infected with cryptosporidiosis died, according to a study by Moodley *et al.* (1991a) and relatively high mortality in patients with diarrhoea due to *Cryptosporidium* have also been reported by Smith and van den Ende (1986) (22.6%) and Wittenberg *et al.* (1987) (23%). In economic terms, diarrhoeal disease in South Africa is estimated to cost R 3 375 million/year and in KwaZulu-Natal R 785 million/year (Pegram *et al.*, 1998).

The occurrence of cryptosporidiosis and giardiasis is probably higher than recorded as only one in fourteen people with diarrhoea in South Africa seek formal treatment from a health practitioner, clinic or hospital every year, while approximately 43 000 South Africans die every year from diarrhoea (Pegram *et al.*, 1998). Many symptomatic people do not seek medical treatment as they cannot get to a hospital or they visit traditional healers. Medical treatment is also not sought if the infection is mild or due to ignorance of the symptoms.

*This chapter forms part of a paper which has been submitted for publication in Water SA

A need to determine the prevalence of *Cryptosporidium* and *Giardia* in KwaZulu-Natal required a survey of hospital and private pathology laboratory recordings of their occurrence. Laboratory-based surveys are, however, subject to a variety of biases; ascertainment will inevitably vary according to specimen selection criteria (clinical and laboratory), diagnostic test method, availability of facilities, and reporting systems (Casemore, 1991). This chapter presents the number of cryptosporidiosis and giardiasis cases recorded from January 1996 - March 1998. Results were evaluated with respect to rainfall, race, gender and distribution of occurrence in diarrhoeic patients. Results of the presence or absence of the protists in a group of HIV positive patients with and without diarrhoea, were evaluated and the percentage occurrence was calculated.

2.2. METHODS AND MATERIALS

2.2.1 Source of *Cryptosporidium* and *Giardia* data

The information used in this study was obtained from two government hospital pathology laboratories and a private pathology laboratory from 1996 - March 1998. This represents 9.7% (3 / 31) of laboratories in KwaZulu-Natal that test for *Cryptosporidium* and 6.0% (3 / 50) of laboratories testing for *Giardia* (Haynes, pers comm.). These laboratories, however, perform most of the testing from throughout KwaZulu-Natal and are situated in the metropolitan areas.

The study population varied with respect to race and income as the private pathology laboratory tests non-formed stools from patients of a higher income bracket and predominantly from the White and Indian population in the Durban and Pietermaritzburg metropolitan areas, while government hospitals test non-formed stools from patients of various race and socio-economic backgrounds within KwaZulu-Natal.

The test criteria required for the analysis of stools for *Cryptosporidium* and *Giardia* at Government hospital laboratories differed. The one laboratory tests all stools for *Cryptosporidium* while *Giardia* analyses are only performed on request from the doctor. The other government hospital laboratory tests all stools received from patients less than two years of age or on request from the doctor for the presence of *Cryptosporidium* while *Giardia* testing is performed on all stools received. The private pathology laboratory analyses for *Cryptosporidium* in non-formed stools of children younger than 5 years, or on

request from the doctor while stools are only analysed for *Giardia* on request from a doctor or those tested for occult blood or reducing agents.

The government laboratories in this study either stain *Cryptosporidium* oocysts by the modified Ziehl-Neelsen technique (Henricksen and Pohlenz, 1981) or by Sheather's flotation (Moodley *et al.*, 1991b) while the private pathology laboratory uses the phenol auramine O stain (Nichols and Thom, 1984). The cysts of *Giardia* are not stained but are detected using bright field microscopy (Wahlquist *et al.*, 1991).

Data indicating positive and negative *Cryptosporidium* and *Giardia* results were used to determine the incidence of the protists (Figures 2.1. and 2.2.).

2.2.2 Rainfall data to determine seasonal variations in the occurrence of *Cryptosporidium* and *Giardia*

Rainfall data was compared with seasonal variation in the occurrence of *Cryptosporidium* and *Giardia.* The assumption was made that high run-off from faecal contaminated land or farm slurry can contaminate water sources resulting in the infection of those using the water further down-stream.

Rainfall for each month from January 1996 to March 1998 was averaged from data collected at two points namely Durban Heights Waterworks, Durban (coastal) and Darvill Wastewater Works (a meteorological station), Pietermaritzburg (inland).

2.2.3 Statistical analyses

Pearson's correlation coefficient was used to examine possible relationships of the incidence of *Cryptosporidium* or *Giardia* with respect to rainfall, month and year, from results recorded by laboratories in the Pietermaritzburg (inland) and Durban (coastal) areas. The relationship between the prevalence of *Cryptosporidium* and *Giardia* was also examined.

2.3. RESULTS

2.3.1 Occurrence of *Cryptosporidium* and *Giardia*

During 1996, 2.9% (96 / 3 274) of patients that were tested for cryptosporidiosis were positive while 3.0% (102 / 3 426) were positive for giardiasis (Figure 2.1.).

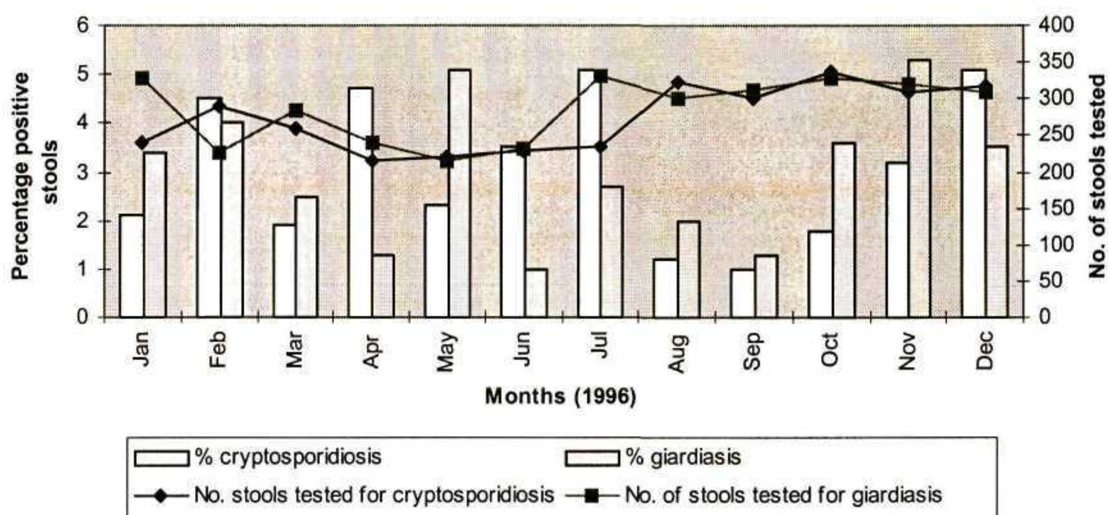


Figure 2.1.

The percentage of *Cryptosporidium* and *Giardia* positive cases recorded each month during 1996.

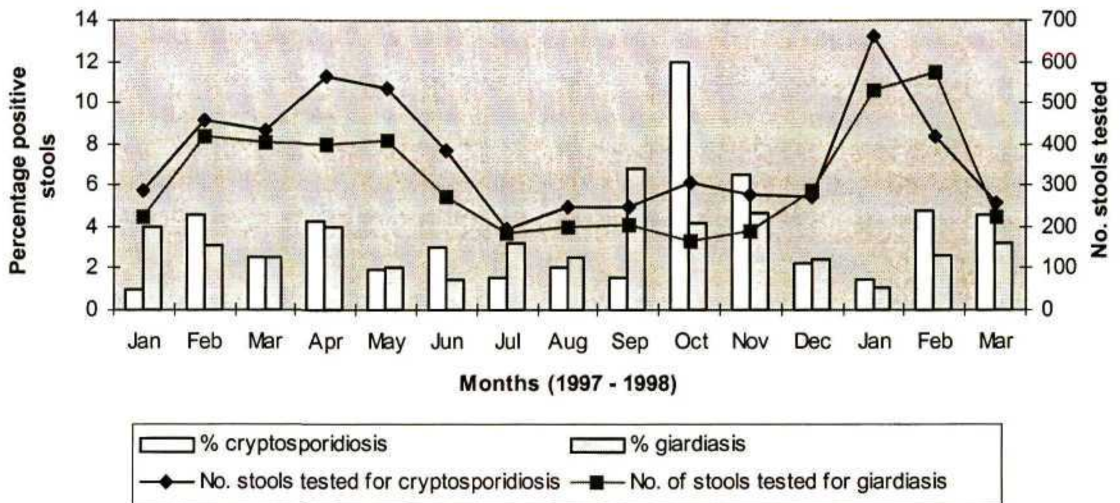


Figure 2.2.

The percentage of *Cryptosporidium* and *Giardia* positive cases recorded each month from January 1997- March 1998.

From January 1997 - March 1998, 3.7% (193 / 5 161) of stools analysed were positive for *Cryptosporidium* and 2.9% (135 / 4 683) were positive for *Giardia* (Figure 2.2.).

2.3.2 Rainfall data to determine seasonal variations in the occurrence of *Cryptosporidium* and *Giardia*

Cryptosporidium and *Giardia* occurred throughout 1996 and monthly prevalence varied between 0.7 - 5.1% and 0.9 - 5.3% respectively (Figures 2.3. and 2.4.). Incidence of cryptosporidiosis infections was higher in February, April, July and December while giardiasis infection prevalence was high in February, May and November. The number of diarrhoeal samples analysed increased during January, August, September, October, November and December while high rainfall occurred in January, February, November and December.

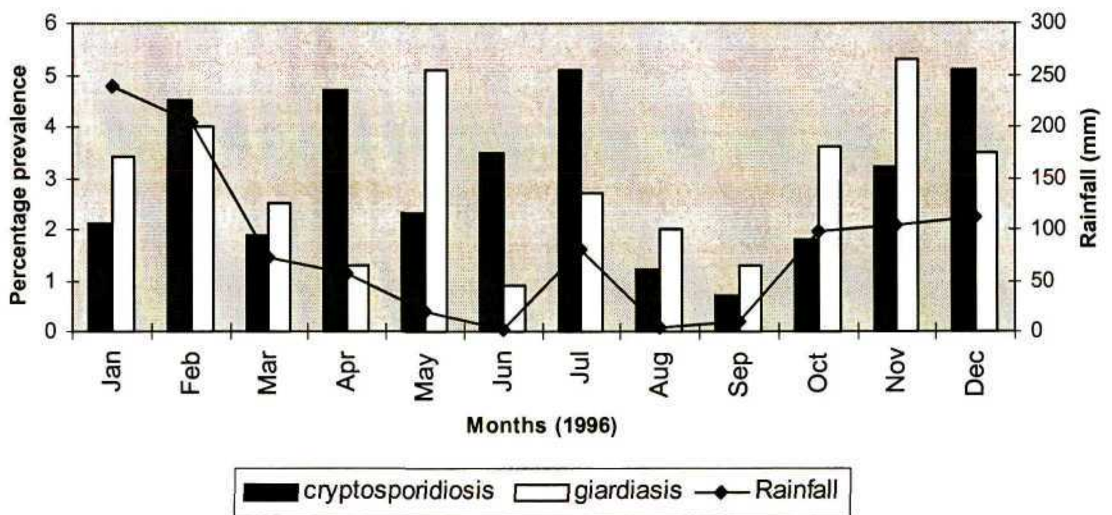


Figure 2.3.

The prevalence of cryptosporidiosis and giardiasis, in patients tested, with respect to rainfall during 1996.

Cryptosporidiosis prevalence was high during January, September, October and November of 1997 while giardiasis prevalence was higher during February, April, October and November. The prevalence of diarrhoeal samples submitted for analysis increased during February, March, April and May while rainfall increased in January, March, April and November. During 1998 *Cryptosporidium* and *Giardia* cases were more prevalent in February and March while diarrhoeal prevalence and rainfall also increased during these months.

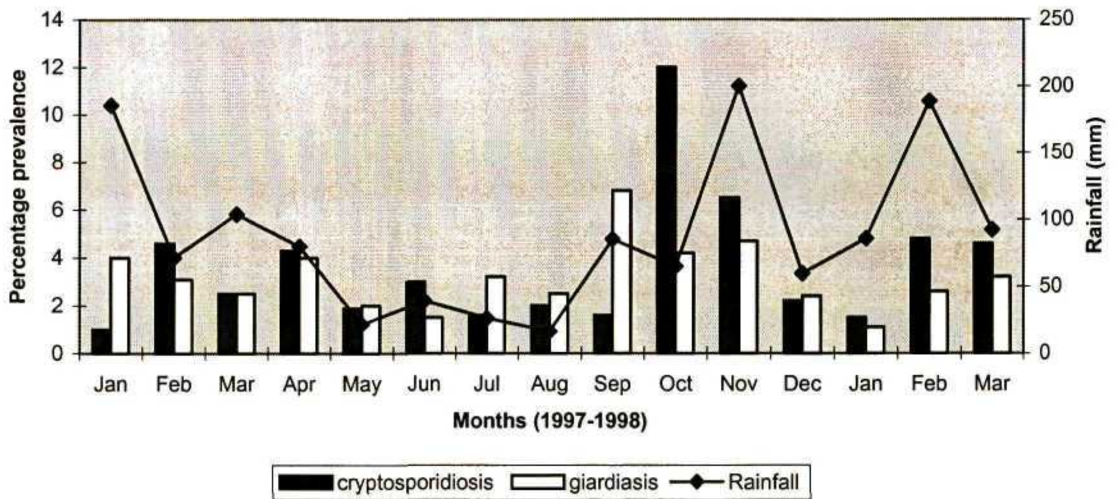


Figure 2.4.

The prevalenceof cryptosporidiosis and giardiasis, in diarrhoea patients tested, with respect to rainfall from January 1997 to March 1998.

2.3.3 Age of patients infected with *Cryptosporidium* or *Giardia*

Of the cryptosporidiosis cases recorded for children < 5 years, the highest percentage (39.3%) (1100 / 2 800) occurred in the < 1 year age group (Figure 2.5.) with the fewest cases occurring in the 1 - 2 year and 2 - 3 year age group (200 / 2 800).

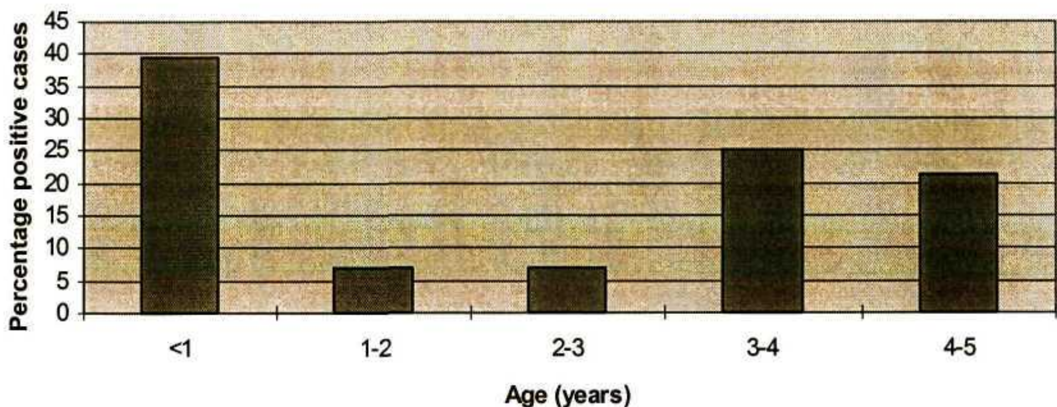


Figure 2.5.

Distribution of cryptosporidiosis cases in diarrhoeic children under 5 years of age tested.

The highest occurrence (38.5%) (500 / 1 300) of giardiasis cases was in the 3 - 4 year age group (Figure 6) while lowest number of cases occurred in the 1 - 2 year age group (100 / 1 300).

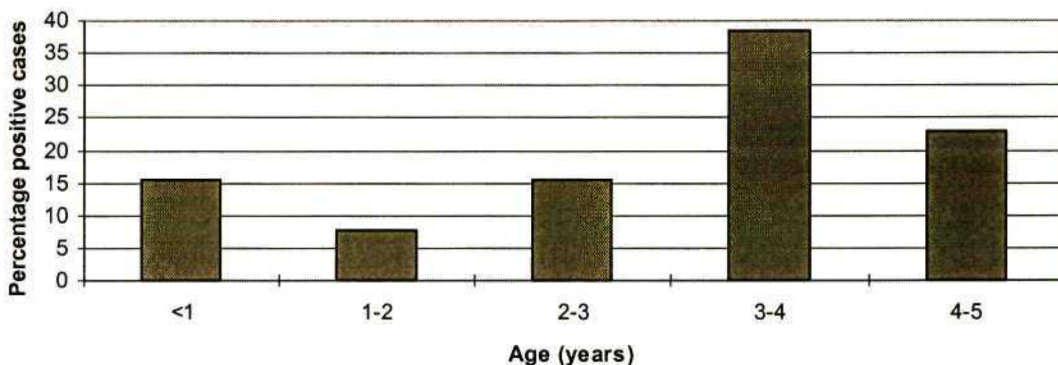


Figure 2.6.

Distribution of giardiasis cases in diarrhoeic children under 5 years of age tested.

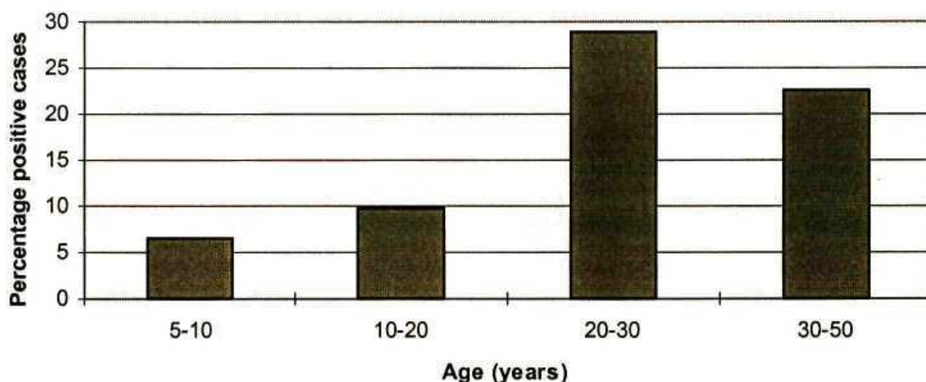


Figure 2.7.

Distribution of cryptosporidiosis cases in diarrhoeic patients older than 5 years tested.

Cryptosporidiosis cases were highest in the 20 - 30 year age group (29.0%) while the lowest number of recorded cases occurred in the 5 - 10 year age group (6.5%).

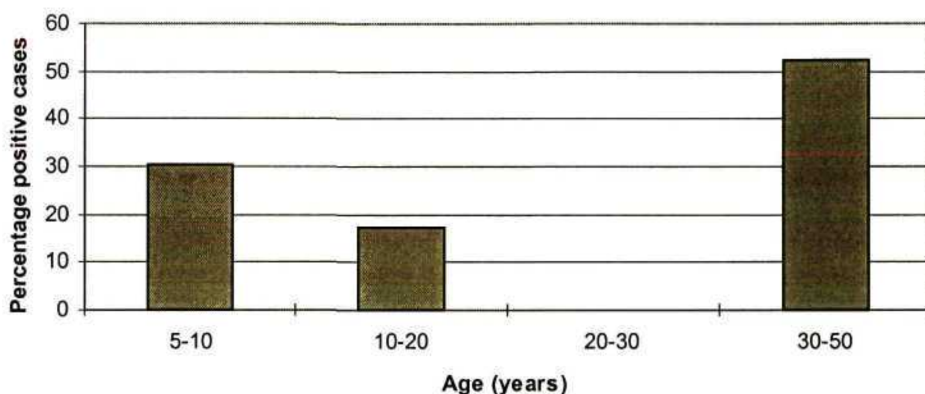


Figure 2.8.

Distribution of giardiasis cases in diarrhoeic patients older than 5 years tested.

Giardiasis was most prevalent in the 30 - 50 year age group (52.2%). No data were recorded for the 20 - 30 age group while the lowest number of giardiasis cases occurred in the 10 - 20 year age group (17.4%).

2.3.4 Race group of patients infected with *Cryptosporidium* or *Giardia*

The race group of sixty-four percent of patients tested for cryptosporidiosis was recorded (2 690 / 4 201), of which 93.0% of the patients tested were Black (2 502 / 2 690), 2.5% were Asian (67 / 2 690) and 5.0% were White (134 / 2 690). Of the 151 cryptosporidiosis positive cases, 75.5% of the positive patients were Black (114 / 151), 13.2% were Asian (20 / 151) and 11.3% were White (17 / 151). Of the *Giardia* positive patients, 80.9% were Black (76 / 107), 14.9% were White (14 / 107) and 4.3% were Asian (4 / 107).

2.3.5 Gender of patients infected with *Cryptosporidium* or *Giardia*

From data that recorded the gender of the patient, 47.7% (210 / 440) of females had cryptosporidiosis compared to 52.3% (230 / 440) of males while 63.6% (140 / 220) of female cases and 36.4% (80 / 220) of males were positive for giardiasis.

2.3.6 HIV patients infected with *Cryptosporidium* or *Giardia*

Of the HIV patients who exhibited diarrhoea, 4.5% (6 / 133) were positive for cryptosporidiosis (symptomatic) while 2.4% (3 / 124) without diarrhoea had oocysts in their stools (asymptomatic). Seven of the 133 HIV patients with diarrhoea (5.3%) (symptomatic) had giardiasis while about 0.8% (1 / 124), without diarrhoea, were *Giardia* positive (asymptomatic).

2.3.7 Statistical analyses

The distribution of the data was tested and found to follow the Poisson distribution. The incidence of *Cryptosporidium* or *Giardia* was found not to be significantly related to climatic factors such as rainfall, month or year despite the large sample size (8 435 during 1996 and 8 109 from January 1997 - March 1998). *Cryptosporidium* and *Giardia* were also not significantly related ($p < 0.05$). This points, therefore to some other factor(s), social or environmental, which are controlling the incidence of these diseases.

2.4. DISCUSSION

2.4.1 Laboratory-based survey

The recording of data associated with *Cryptosporidium* and *Giardia* testing by most laboratories was found to be insufficient. Often only samples that tested positive were recorded and not those found to be negative. Less than eighty percent of the data recorded made no reference to race, gender or the age of the patient.

The rates of stool positivity vary according to diagnostic techniques employed (Bogaerts *et al.*, 1984; Crawford and Vermund, 1988) making it difficult to gain an accurate picture of incidence rates or of prevalence. However, the data do indicate the ubiquity of the parasite (Current, 1994). Reports of laboratory-confirmed *Cryptosporidium* incidence in developed countries generally yield figures of approximately 1.0 - 2.0% overall (Fayer and Ungar, 1986). *Cryptosporidium* rates are generally higher in developing countries (2.5 to >30.0%; mean 8.5%), especially in children younger than 2 years, in whom it may be symptomatic or asymptomatic (Casemore *et al.*, 1997). A four year study at GaRankuwa Hospital, Pretoria, from October 1985 to September 1989, indicated a yearly incidence from 3.13 - 5.52% (Fripp *et al.*, 1991). Similarly *Cryptosporidium* and *Giardia* results in

KwaZulu-Natal were 2.9-3.7% and 2.9-3.0% respectively indicating that infection due to these protists is endemic.

Pathology laboratories should be encouraged to include *Cryptosporidium* diagnostic techniques with the routine diagnosis of stool specimens as 62.0% (31 / 50) of laboratories do not test for *Cryptosporidium*. There is a need for conformity in testing and a standard diagnostic test needs to be devised so that the results are comparable countrywide, a view shared by Moodley *et al.* (1991b). Recording of socio-economic data would also provide further information for use in the investigating the causes or contributing factors affecting the prevalence of diseases.

2.4.2 Occurrence of *Cryptosporidium* and *Giardia* with respect to seasonality

Cryptosporidium and *Giardia* occurred throughout each year and the monthly incidence varied between 0.7 - 6.8% and 0.7 - 11.8%, of diarrhoeal cases submitted for protozoan analysis, respectively (Figures 2.3. and 2.4.).

High numbers of *Cryptosporidium* and *Giardia* cases were generally recorded during the summer rainfall months of November, December, January, February and March each year. *Giardia* was found to be more prevalent, although sporadic outbreaks of *Cryptosporidium* occurred every year. It is possible that contamination of rivers by stormwater run-off, containing faeces of infected humans and animals, resulted in the higher numbers of cryptosporidiosis and giardiasis cases during high rainfall months, if water transmission occurred. Moodley *et al.* (1991a) also found *Cryptosporidium* infections in children, admitted to a hospital in Durban, KwaZulu-Natal, to be more prevalent during high rainfall seasons while Fripp *et al.* (1991) showed a peak detection during the late summer months towards the end of the rainy season.

Results from the analysis of diarrhoeic samples indicated that cholera, salmonella, amoebic dysentery, *Shigella,* or enteropathic *E.coli* were often the cause of diarrhoea in samples testing negative for *Cryptosporidium* or *Giardia.*

2.4.3 Age of patients infected with *Cryptosporidium* or *Giardia*

The difference in prevalence of the two diseases was dependent on the accurate recording of data that was often limited and therefore may not represent the actual occurrence within the age groups.

Cases of cryptosporidiosis were found to be most prevalent in the < 1 year age group whereas Walters *et al.* (1988) found 100% positivity for cryptosporidiosis (8/8) in the <1 year age group attending a day-care centre in Durban. Surveys in the United Kingdom and in some developing countries have also found *Cryptosporidium* to be common in children <1 year (Casemore, 1990). High occurrence of cryptosporidiosis infection in children < 2 years of age is consistent with the findings of others such as Shahid *et al.* (1988) and Smith and Van den Ende (1986) who analysed samples from Bangladesh and Durban respectively.

This high occurrence of cryptosporidioisis may be attributed to malnourished babies with immunocompromised systems, unsterilised feeding bottles or faecal-oral transmission by care-givers who change the baby's nappies. Faecal-oral transmission by care-givers has been reported by others (Walters *et al.*, 1988).

The high occurrence of *Giardia* in the 3 - 4 year age group could be as a result of faecal-oral transmission particularly at crèches where children play together in sand-pits and handle toys. In rural situations this infection may arise as a result of contact with animals. Contaminated water could also be a major source (Nimri and Batchoun, 1994).

Parents, care-givers, crèches and hospital personnel need to be well informed of the risks of transmission in order to prevent the spread of infection.

The high prevalence of cryptosporidiosis in the 20 - 30 year age group (29.0%) and 30 - 50 year age group (22.6%) and the high prevalence of giardiasis in the 30 - 50 year age group (52.2%) could be due to inadequate potable water supply and sanitation, poor hygiene practices and the prevalence of immunocompromised individuals in these age groups. The prevalence of cryptosporidiosis in individuals between 20 and 30 years of age may be due to the high incidence of HIV infection in this age group (Abdool Karim and Abdool Karim, 1999).

2.4.4 Race of patients infected with *Cryptosporidium* or *Giardia*

Although a larger portion of the Asian and White race groups tested positive for cryptosporidiosis and giardiasis compared with the Black patients, the sample size of the Black population was approximately ten times greater. It is possible that fewer Asian and White people suffer from diarrhoea or are not tested for the aetiology of diarrhoeal disease, but are merely prescribed anti-diarrhoeic medicines.

However, overall a higher percentage of Black patients were positive for *Cryptosporidium* and *Giardia*. This is probably due to socio-economic conditions, as many people in KwaZulu-Natal do not have access to an adequate potable water supply or sanitation.

2.4.5 Gender of patients infected with *Cryptosporidium* or *Giardia*

Male and female patients tested for cryptosporidiosis had similar positive percentages. Giardiasis on the other hand was found to be more prevalent in female patients tested. A study of hospitilised children in Delhi found *Cryptosporidium* infection to be predominant in males (Mahajan *et al.*, 1992) while an increased prevalence was found for females in Nigeria (Okafor and Okunji, 1994). In rural communities of developing countries, UNICEF found that girls may be more susceptible to diarrhoeal disease as they are often fed less, given less nutritious food, provided less health care and given more work (Doyle, 1995).

2.4.6 HIV patients infected with *Cryptosporidium* or *Giardia*

A low percentage of *Cryptosporidium* and *Giardia* positive cases were recorded in the HIV patients sampled, but the percentages were similar in both symptomatic and asymptomatic patients. Asymptomatic *Cryptosporidium* infections have been documented previously in AIDS cases (Zar *et al.*, 1985). The prevalence of asymptomatic infections has not been determined and its potential may be underestimated. The 4.5% positive cases for cryptosporidiosis in HIV patients was comparable with 4.2 - 6.2% reported by Sorvillo *et al.* (1994).

2.4.7 Statistical analyses

As rainfall, season or year did not appear to be correlated with the incidence of *Cryptosporidum* or *Giardia*, waterborne transmission does appear to be the primary route and other factors such as personal hygiene, sanitation, education or potable water probably have a more significant impact on the prevalence of these diseases.

2.5. CONCLUSIONS

Cryptosporidium and *Giardia* were found to be endemic in KwaZulu-Natal with laboratory-confirmed prevalence ranging from 2.9 - 3.7% and 2.9 - 3.0%, of cases submitted for analysis, respectively. *Cryptosporidium* and *Giardia* incidence did not appear to correlate statistically with rainfall, month or year, indicating that waterborne transmission is not important and other factors such as personal hygiene, potable water supply, sanitation and education probably have a more significant impact on their incidence. However, the graphs indicated that the incidence of diarrhoea and infection by these pathogens increased when rainfall increased. Increases in the number of diarrhoeal samples submitted for *Cryptosporidium* or *Giardia* analysis were independent of the incidence of either protozoan pathogen. Female and male diarrhoeal patients had similar percentages of cryptosporidiosis while giardiasis was more prevalent in female patients. *Cryptosporidium* and *Giardia* incidence in children under 5 years indicated that *Cryptosporidium* was most prevalent (39.3%) in the <1 year age group while *Giardia* was most prevalent in the 3 - 4 year age group (38.5%). Cryptosporidiosis cases were highest in the 20 - 30 year age group (29.0%) while giardiasis was most prevalent in the 30 - 50 year age group (52.2%). A low percentage of *Cryptosporidium* and *Giardia* positive cases were recorded in symptomatic (4.5% and 5.3% respectively) and asymptomatic (2.4% and 0.8% respectively) HIV patients. Clear recording and storage of data including gender, age, race, environment (location) and socio-economic factors of the patients is essential so that trends and outbreaks may be easily identified. Detection techniques need to be enhanced and standardised so that results are comparable.

CHAPTER 3

RECOVERY OF *CRYPTOSPORIDIUM* AND *GIARDIA* FROM POTABLE AND TURBID WATER SAMPLES USING CALCIUM CARBONATE FLOCCULATION, MEMBRANE FILTRATION OR MEMBRANE DISSOLUTION

3.1. INTRODUCTION

Current UK and USA methods for concentrating *Cryptosporidium* oocysts and *Giardia* cysts from water samples involve the filtration of large volumes (100 – 1 000 ℓ) of water through cartridge filters, elution of the microorganisms from the filter followed by centrifugation and clarification using a Percoll-sucrose density gradient (LeChevallier *et al.*, 1995). However, these methods have recoveries of *Cryptosporidium* oocysts from potable water ranging 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). Large variations in recovery efficiencies using this method have been reported between laboratories and even within a single laboratory (Clancy *et al.*, 1994; LeChevaller *et al.*, 1991). Recoveries from turbid waters using this method was often less than 5% (Vesey *et al.*, 1993a). 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).

As the above described method is time-consuming, labour intensive and requires a large degree of analytical expertise (LeChevallier *et al.*, 1995), innovative methods including calcium carbonate flocculation or membrane filtration and dissolution of membranes for (oo)cyst recovery from small volumes (10 - 20 ℓ) of water have been described (Vesey *et al.*, 1993a; Aldom and Chagla, 1995; Shepherd and Wyn-Jones, 1996). Calcium carbonate flocculation has reported recoveries from seeded deionized, tap and river water in excess of 68% (Vesey *et al.*, 1993a) while membrane filtration using cellulose acetate membranes had recoveries of *Cryptosporidium* oocysts and *Giardia* cysts averaging 38.1 - 51.3 % from seeded tap and river water samples (Shepherd and Wyn-Jones, 1996). Aldom and Chagla (1995) described a membrane dissolution method with an average recovery of *Cryptosporidium* of 70.5% from spiked water. As these methods required

further evaluation using a wider range of environmental water samples, it was undertaken in this study to subject a variety of waters to these concentration procedures to determine their recovery efficiencies and reproducibilities.

3.2. MATERIALS AND METHODS

3.2.1 *Cryptosporidium* and *Giardia* reference counts for use in seeding experiments

For the recovery of oocysts and cysts following different concentration methods a known number of oocysts or cysts is required. This was determined in the following manner:
A 1:5 working dilution of purified *Cryptosporidium* oocysts (Human AZ-1 strain, produced in mice and purified from faeces by sodium chloride centrifugation gradients, PRL DyNAgenics, Arizona, USA) and a 1:10 working dilution of purified *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) Five microlitres of each protozoon dilution were applied to each of six wells on two multiwell microscope slides, air-dried and acetone fixed (20 $\mu\ell$). The slide well contents were stained and enumerated as described in 3.2.3. The average number of oocysts / cysts and the standard deviation were calculated.

3.2.2 Sample seeding

Tap water samples

Eighteen 10-litre (oo)cyst-free tap water samples (0.1 NTU) were each seeded with approximately 10^3 (oo)cysts and homogenised by stirring. Each of six seeded samples were concentrated by calcium carbonate flocculation (APPENDIX 1), membrane filtration (APPENDIX 2) or membrane dissolution (APPENDIX 3). Instead of scraping the membrane following filtration as described by Shepherd and Wyn-Jones (1996), the membrane was sonicated and the eluate was further concentrated (APPENDIX 2). Three 10-litre tap water samples were not seeded but subjected to each of the concentration procedures as negative controls.

Turbid water samples

Eighteen 10-litre (oo)cyst-free tap water samples for each method evaluated, were mixed with Loamy soil, from a greenhouse where *Cryptosporidium* and *Giardia* were unlikely to be present, to achieve six turbidity readings of 10, 15 and 20 nephelometric turbidity units (NTU) respectively using a Hach 50 turbidimeter (Hach, Colorado, USA) according to the method described in Standard Methods (1995). The turbid water samples were seeded with approximately 10^3 purified (oo)cysts and homogenised by stirring for five minutes with an automatic stirrer (Millipore Corporation, Massachusetts, USA). The samples equilibrated overnight at room temperature Six samples at each turbidity were subjected to calcium carbonate flocculation, membrane filtration or membrane dissolution. Three 10-litre turbid water samples at each turbidity were not seeded but subjected to each of the concentration procedures as negative controls. All samples were centrifuged in 250 mℓ polypropylene flat-bottomed and 50 mℓ polypropylene conical-shaped centrifuge bottles (Bibby Sterilin, Staffordshire, UK) using a Juoan CR422 centrifuge (Juoan, Cedex, France) fitted with a swinging-bucket rotor. The brake was switched off during centrifugation.

Following the respective concentration techniques, 20 μℓ samples were air-dried in triplicate onto multiwell slides, fixed with a drop of acetone (20 μℓ) and stained for enumeration as described in 3.2.3.

3.2.3 Staining and enumeration of oocysts and cysts

The concentrated samples were fixed to glass multiwell slides as in 3.2.1 and 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 each well and incubated at 37°C for 30 minutes according to the manufacturer's instructions (APPENDIX 4). A Zeiss Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany) fitted with differential interference contrast (DIC) and selected excitation of 450 - 490nm and barrier filter of 520nm was used with X400 magnification to examine the stained samples. Oocysts and cysts were identified by shape, size and internal structures as described in 1.6b.

Stools, previously identified a positive for oocysts and cysts by pathology laboratories, were stained and used as positive controls while the negative controls were uncontaminated tap water.

The number of *Cryptosporidium* oocysts and *Giardia* cysts per volume of water sampled was calculated as follows:

$N \times V / v$

Where N = number of (oo)cysts counted

V = total volume of concentrate ($\mu\ell$)

v = volume of concentrate examined ($\mu\ell$)

3.3. RESULTS

3.3.1 *Cryptosporidium* and *Giardia* reference counts for use in seeding experiments

The average number of oocysts per 5 $\mu\ell$ of the 1:5 dilution was determined to be 80 with a standard deviation of 9.94 (APPENDIX 5). The average number of cysts per 5 $\mu\ell$ of the 1:10 dilution was determined to be 59.2 with a standard deviation of 13.44 (APPENDIX 5).

3.3.2 Recovery of oocysts and cysts using different concentration procedures

Oocysts and cysts were not detected in any of the negative controls although non-specific fluorescence of irregular shaped particles was seen. Distinct particulate matter that did not fluoresce was also seen using epifluorescence microscopy.

Percentage recovery of oocysts and cysts from seeded tap water samples using different concentration methods ranged from 0.0 - 78.5% (Table 3.1.). A small amount of particulate matter was collected following centrifugation of the samples after flocculation

or sonication and was stained for viewing. Dissolution of the membrane was not always successful making pipetting of the sample onto slide wells difficult and recovery of (oo)cysts poor (Tables 3.2.; 3.3.; 3.4.). Detailed results from individual experiments are incorporated in appendices six to nine.

Table 3.1. Average percentage of oocysts and cysts recovered following the concentration of 10-litre seeded tap water samples by different methods.

Concentration method (n = number of replicates)	**Average % oocysts recovery (% range)**	**Average % cyst recovery (% range)**
$CaCO_3$ flocculation (n = 6)	71.1% (64.0 – 78.5%)	73.1% (69.0 – 78.0%)
Membrane filtration + sonication (n = 6)	34.7% (28.0 – 40.5%)	52.8 (42.0 – 61.0%)
Membrane dissolution (n = 6)	7.5% (0.0 – 15.0%)	16.2% (0.0 – 34.0%)

The pellet that formed following centrifugation of the calcium carbonate flocculated turbid samples was tightly packed and had to be manually resuspended using an orange stick prior to pipetting for the IFA. Although all particulate matter was concentrated using calcium carbonate flocculation, the oocysts and cysts were clearly visible using microscopy and their recovery was high (Tables 3.2.; 3.3.; 3.4.).

Table 3.2. Average percentage recovery of oocysts and cysts following the concentration of 10-litre seeded water samples (10 NTU) by various concentration methods.

Concentration method (n = number of replicates)	Average % oocyst recovery (range)	Average % cyst recovery (range)
$CaCO_3$ flocculation (n = 6)	69.8% (62.0 – 74.9%)	72.5% (68.0 – 75.3%)
Membrane filtration + sonication (n = 6)	21.8% (14.0 – 29.0)	31.5% (20.0 – 40.6%)
Membrane dissolution (n = 6)	1.4% (0.0 – 2.5%)	4.6% (0.0 – 10.0%)

Table 3.3. Average percentage recovery of oocysts and cysts following the concentration of 10-litre seeded water samples (15 NTU) by various concentration methods.

Concentration method (n= number of replicates)	Average % oocyst recovery (range)	Average % cyst recovery (range)
$CaCO_3$ flocculation (n = 6)	67.5% (61.4 – 71.6%)	70.1% (65.0 – 72.3%)
Membrane filtration + sonication (n = 6)	48.5% (31.3 – 45.9%)	47.8% (45.9 – 51.8%)
Membrane dissolution (n = 6)	2.0% (0.0 – 4.2%)	3.1% (0.0 – 6.3%)

Table 3.4. Average percentage recovery of oocysts and cysts following the concentration of 10-litre seeded water samples (20 NTU) by various concentration methods.

Concentration method (n = number of replicates)	**Average % oocyst recovery (range)**	**Average % cyst recovery (range)**
$CaCO_3$ flocculation (n = 6)	64.6% (58.0 – 69.3%)	65.2% (59.3 – 68.5%)
Membrane filtration + sonication (n = 6)	32.3% (24.0 – 39.0%)	40.1% (30.0 – 50.6%)
Membrane dissolution (n = 6)	0.7% (0.0 – 1.9)	1.6% (0.0 – 3.8)

3.4. DISCUSSION

Concentration techniques

Concentration techniques for *Cryptosporidium* and *Giardia* need to be reproducible and have a high recovery of oocysts and cysts from environmental water samples. As the raw waters received by the Umgeni Water's water treatment plants, which supply the greater Durban and Pietermaritzburg metropolitan areas, average eight to 16 NTU, the range of turbidities evaluated for their effect on the recovery of (oo)cysts using different concentration techniques was 10, 15 and 20 NTU.

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. Despite the lack of clarification procedures used, consistency between tap and turbid waters was demonstrated although a decrease in recovery was experienced the more turbid the water sample. However, Nieminski *et al.* (1995) demonstrated a decreasing oocyst and cysts recovery efficiency with increasing water turbidity regardless of the method used. The collection of 10-litresamples for this method lends itself to regular routine analysis of water samples.

The loss of (oo)cysts is possibly due to their being discarded in the supernatant or remaining attached to the side of the glass centrifuge tube. (Oo)cysts may become associated with other particles and lost (Veal *et al.*, 1997) although in higher turbidity (> 1 NTU) water, the presence of debris may help to pull (oo)cysts out of suspension assisting in their entrapment during centrifugation (LeChevallier and Norton, 1995). In addition, the identification of (oo)cysts in a water concentrate is difficult when obscured by debris (Smith and Hayes, 1997).

Despite this method having good recoveries of greater than 64.0%, it also been shown to have significant reductions ($P < 0.05$) in viability (Campbell *et al.*, 1994) should this parameter need to be assessed. This reduction in viability may be due to the pH change to 10 as research by Robertson *et al.* (1992) indicated that although oocysts are extremely robust, they are sensitive to extremes of pH, particularly exposure to pH 9 at room temperature.

While (oo)cyst concentration by flocculation has proved to be effective using fresh (oo)cysts, the recovery of aged oocysts (four months), kept in a water sample for two days prior to concentration, has been shown to decrease by 32.2% (Veal *et al.*, 1997). Therefore results from this experimentation which used (oo)cysts of a month old may overestimate actual recovery efficiencies of (oo)cysts that have been present in the environment for some time.

Cellulose-acetate membranes were chosen for use in the filtration of water samples as these have been shown to have good recoveries of oocysts and cysts from tap and turbid waters compared to membranes of other media (Shepherd and Wyn-Jones, 1996). Despite the good recoveries possible with polycarbonate membranes, these membranes blocked quickly and were not recommended for use with environmental water samples (Clancy *et al.*, 1997). Although the membrane filtration technique was quicker than the calcium carbonate flocculation, it was expensive as filtering of 10-litre water samples required two or more membranes when the turbidity was 10 NTU or higher. As the turbidity increased from 10 to 20 NTU more membranes were required. When the concentrated sample was viewed microscopically, some oocysts and cysts had distorted cell walls that may have been as a result of the sonication being too rough.

Recoveries were low for the membrane dissolution technique as (oo)cysts may have remained embedded in debris which did not dissolve in the acetone. The membrane dissolution technique proved to be more expensive than the calcium carbonate flocculation method, although the concentration time for each sample was low (2.5 hours). Poor recoveries of oocysts and cysts using this method have also been experienced by other researchers (Clancy *et al.*, 1997). Losses occurring during centrifugation depend upon the volume and shape of the centrifuge tubes. As flat-bottomed large volume centrifuge bottles have been shown to be less effective with poor recoveries than conical centrifuge bottles (Smith and Hayes, 1997), 250 mℓ flat-bottomed bottles were only used initially followed by 50 mℓ conical tubes. This, however, has the drawback of the packed pellet being difficult to resuspend after centrifugation (LeChevallier *et al.*, 1995). Their results suggested that bottle configuration made little difference in the recovery of (oo)cysts. They also found a slight benefit (ranging from 3.0 - 5.0%) to centrifuging with the brake off although this increases the turn-around time of the analysis as one has to wait for the rotor to slow by itself.

A range of recoveries was experienced between different methods that may have resulted not only from losses occurring during the concentration procedures, but the variation in seed level, water chemistry, particulate matter and quality of the oocysts or cysts.

Immunofluorescence assay

The *in vitro* direct immunofluorescence method used to detect the oocysts and cysts was quick and easy to use. The Crypto/Giardia IF test kit has been analysed by the manufacturers for cross-reactivity against a variety of enteric bacteria and helminths and found to be negative. However, the assay is pan-specific and detects oocysts of *C. parvum*, *C. baileyi* and *C. muris* as well as *Giardia* cysts from humans, rats, dogs, cattle and sheep.

The positive control was used to ensure that the kit was working adequately while the negative control indicated that the monoclonal antibodies did not bind to any other organisms. In both the positive and negative samples, much non-specific binding occurred which fluoresced with an intensity of 4+ but did not have any distinctive shape. In water samples algae, such as *Microcystis*, which is similar in size and shape to

Cryptosporidium, pollen or fungal spores could interfere with the detection of the protozoa resulting in false positives. Thus presumptive *Cryptosporidium* oocysts and *Giardia* cysts, identified by their characteristic 2+ to 3+ intensity staining, shape and size, were confirmed using DIC and the presence of internal structures.

3.5. CONCLUSIONS

The calcium carbonate flocculation proved to have the best recoveries for tap and turbid waters and therefore lends itself to routine monitoring of smaller volumes of water. Membrane filtration together with sonication had fair recoveries but required more than one membrane for turbid water samples and is therefore not suitable for use with environmental samples. Membrane dissolution had very poor recoveries for both tap and turbid water samples and is not recommended for use.

IFA combined with DIC microscopy allowed for the identification and confirmation of *Cryptosporidium* oocysts and *Giardia* cysts although this step is time-consuming and tiring thus highlighting the need for automated systems.

Despite the good recoveries of *Cryptosporidium* and *Giardia* from water using the calcium carbonate flocculation technique, improvements to the method which would simplify the detection of (oo)cysts whilst enhancing their recovery would be beneficial in protecting the consumer from these pathogens.

CHAPTER 4
AN EVALUATION OF METHODS TO ENHANCE THE RECOVERY OF OOCYSTS AND CYSTS FROM TURBID WATER

4.1. INTRODUCTION

The calcium carbonate flocculation procedure was determined to have consistently higher recoveries of *Cryptosporidium* oocysts and *Giardia* cysts from a variety of water types than other methods described (Chapter 3), although losses of oocysts and cysts escalated with increasing turbidity. These losses are further exacerbated as the recovery efficiency is more variable when the number of organisms is low. This may result in underestimation of both the percentage of positive samples and the concentration of (oo)cysts in water samples (Smith and Hayes, 1997).

Among the methods described in Chapter 3, some researchers have evaluated density gradients such as sucrose flotation in an attempt to clarify water samples and improve recoveries of (oo)cysts (Rose *et al.,* 1988; Nieminski *et al.,* 1995; Shepherd and Wyn-Jones, 1995) while LeChevallier *et al.* (1995) used a range of pH values in an effort to improve desorption of oocysts and cysts from cartridge filters.

The following methods were evaluated here for their ability to enhance the recovery of oocysts and cysts from turbid water samples: pre-filtration prior to flocculation to remove larger debris which could mask (oo)cysts during microscopy (Smith *et al.*, 1995), pH adjustments to change (oo)cyst isoelectric points thereby removing them from other particles or sucrose flotation following flocculation to clarify the concentrated sample. A Visser helminth filter which is used to concentrate ova of the intestinal helminth *Ascaris lumbricoides* from sewage sludge (Melmed and Comninos, 1979), was used in the pre-filtration step while sucrose flotation was performed according to standard techniques and pH adjustment of the calcium carbonate method ranged from pH 6 ± 0.5 to pH 8 ± 0.5.

4.2 METHODS AND MATERIALS

4.2.1 *Cryptosporidium* and *Giardia* reference counts and sample seeding

Purified deactivated *Cryptosporidium* oocysts (Human AZ-1 strain, produced in mice and purified from faeces by sodium chloride centrifugation gradients) and *Giardia* cysts (CH-3 strain, produced in Mongolian gerbils and purified from faeces by zinc sulphate) were purchased from PRL DyNAgenics (Arizona, USA).Protozoa counts were determined as described in 3.2.1. Six turbid water samples for each turbidity (10 $\pm$ 0.5; 15 $\pm$ 0.5; 20 $\pm$ 0.5 NTU) were prepared and seeded as described in 3.2.2.

4.2.2 Prefiltration of turbid water samples prior to calcium carbonate concentration

A Visser helminth filter apparatus (Visser Filters, Pretoria, South Africa) was used as a prefiltration step for the turbid water samples to remove larger particulates and debris prior to concentration and detection. The Visser filter apparatus consists of three consecutive filter sleeves which fit together with a 100 micron mesh inner filter enclosed by an 80 micron mesh and then a 35 micron mesh filter (Figure 4.1.).

Each of eighteen 10-litre seeded turbid water samples was passed through the Visser filter, the eluate was collected in 500 mℓ beakers and transferred to a 20 ℓ flat bottomed plastic bucket. The eluate was then concentrated by the calcium carbonate flocculation procedure (APPENDIX 1) and the recovery of the seeded oocysts and cysts was determined as described in 3.2.3. The particulate matter trapped by each filter was also collected, stained and (oo)cysts quantified according to 3.2.3.

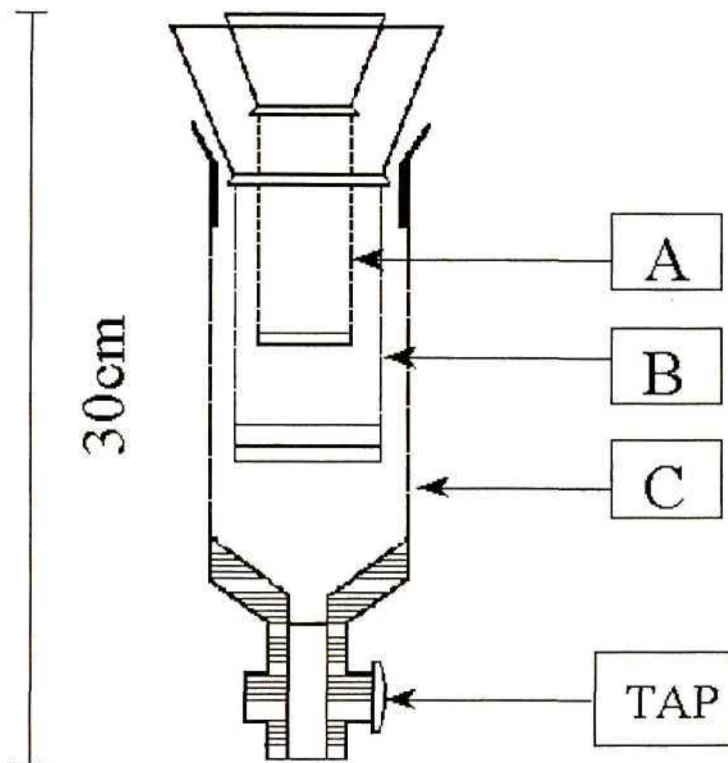


Figure 4.1. Diagram of the Visser helminth filter.
A= 100 μm; B= 80 μm; C=35 μm

4.2.3 pH adjustment of water concentrates following calcium carbonate flocculation

Six water samples at each turbidity (10 ± 0.5; 15 ± 0.5; 20 ± 0.5 NTU) were flocculated (APPENDIX 1) and pH was measured using a pHScan WP1/2 meter (Eutech, Singapore). The pH meter was calibrated with two standards (pH 7 and pH 4) prior to use. Each flocculated water sample had a reading between pH 2.0 - 2.2 which was adjusted to 6.0 ± 0.5, 7.0 ± 0.5 and 8 ± 0.5 with the addition of 1 M NaOH. Six replicates were made at each pH. The concentrated samples were centrifuged as described in APPENDIX 1. Oocysts and cysts recovered by this technique were stained and quantified as described in 3.2.3.

4.2.4 Clarification of the calcium carbonate concentrate using sucrose flotation

The debris in six water samples for each turbidity (10 ± 0.5; 15 ± 0.5; 20 ± 0.5 NTU) was flocculated using calcium carbonate (APPENDIX 1). After centrifugation at 3 000 *g*, the supernatant was vacuum aspirated and the pellets were resuspended, pooled and transferred to 50 mℓ conical polypropylene centrifuge tubes (Bibby Sterilin, Staffordshire, UK). Each pellet was underlayed with sucrose (specific gravity of 1.18) by carefully injecting 10 mℓ of the sucrose solution into the base of the centrifuge tube and centrifuging at 1 000 *g* (5 minutes)(Juoan CR422; Juoan, Cedex, France)(Anon., 1990). The supernatant including the interface was removed with a Pasteur pipette, placed in clean 50 mℓ conical polypropylene centrifuge tubes and centrifuged at 1 500 *g* (10 minutes)(Anon., 1990). Oocyst and cyst recovery following sucrose flotation was determined as described in 3.2.3. In addition, 20 μℓ samples were taken from the pellet of each turbid water concentrate following sucrose flotation. Samples were stained and (oo)cysts enumerated as described in 3.2.3.

4.3 RESULTS

4.3.1 Pre-filtration of turbid waters prior to calcium carbonate flocculation

Pre-filtration of the turbid water samples made microscopy easier as larger particulate matter that may obscure oocysts and cysts was removed. Oocyst and cyst recovery decreased as the turbidity increased despite the removal of larger particulate matter by the Visser helminth filter (Table 4.1.)(APPENDIX 10). However, although oocysts and cysts passed through the 100μm pore size filter, a few were trapped by the 80 μm and 35 μm pore size filters (Table 4.2.; 4.3.)(APPENDIX 11). The results were compared and contrasted with those in Tables 3.2. - 3.4. which indicate concentration by calcium carbonate flocculation without a pre-filtration step.

Table 4.1. Percentage cysts recovered from the seeded turbid water samples using pre-filtration and calcium carbonate flocculation.

Turbidity (NTU)	Mean percentage oocyst recovery after pre-filtration (range)	Mean percentage cyst recovery after pre-filtration (range)
10 ± 0.5 (n = 6)	68.1% (3.1 - 73.0%)	68.7% (64.5 - 71.3%)
15 ± 0.5 (n = 6)	62.0% (59.7 - 65.0%)	67.2% (63.0 - 70.9%)
20 ± 0.5 (n = 6)	63.0% (57.9 - 69.4%)	65.5% (62.9 - 69.2%)

n = number of replicates

Table 4.2. Percentage oocysts trapped from the seeded turbid water samples by different porosity Visser helminth filter components.

Filter pore size	Average percentage of oocysts trapped by the filters for each turbid water sample evaluated (range)		
	10 ± 0.5 NTU (n = 6)	**15 ± 0.5 NTU (n = 6)**	**20 ± 0.5 NTU (n = 6)**
100 μm	0.0%	0.0%	0.0%
80 μm	2.8% (1.2 - 3.2%)	4.1% (2.4 - 5.7%)	74% (4.2 - 10.0%)
35 μm	6.7% (5.8 - 7.7%)	6.5% (4.9 - 7.2%)	6.2% (5.4 - 7.0%)

n = number of replicates

Table 4.3. Percentage cysts trapped from the seeded turbid water samples by different porosity Visser helminth filter components.

Filter pore size	Average percentage of cysts trapped by the filters for each turbid water sample evaluated (range)		
	10 NTU (n = 6)	15 NTU (n = 6)	20 NTU (n = 6)
100 µm	0.0%	0.0%	0.0%
80 µm	1.6% (1.1 - 2.0%)	1.9% (1.4 - 2.4%)	2.8% (2.4 - 3.1%)
35 µm	1.9% (1.0 - 2.7%)	2.1% (1.6 - 2.6%)	2.5% (1.8 - 3.2%)

n = number of replicates

4.3.2 pH adjustment following calcium carbonate flocculation

Varying the pH of the calcium carbonate concentrate had little effect on the recovery of *Cryptosporidium* oocysts and *Giardia cysts* from turbid water samples. Average recovery results using pH 7 ± 0.5 and pH 8 ± 0.5 were the same as those in Tables 3.2 - 3.4. The highest overall recovery from the different turbidity water samples occurred at pH 6 ± 0.5 with the average recovery ranging from 0.2 - 2.4% higher than without a pH adjustment (APPENDIX 12).

4.3.3 Clarification of the calcium carbonate concentrate using sucrose flotation

Samples from the supernatant and sucrose / concentrate interface when viewed by epifluorescence microscopy were clear of most particles although some non-specific fluorescence occurred. This made microscopy easier and oocysts and cysts were distinctly visible (Table 4.4.)(APPENDIX 13). The samples from the pellet that remained after sucrose flotation were brown in colour with distinct particulate matter when viewed by epifluorescence microscopy. Oocysts and cysts were found to be present in the pellet (Table 4.5.)(APPENDIX 14).

Table 4.4. Recovery of oocysts and cysts following sucrose flotation of the concentrate.

Turbidity (NTU)	Mean percentage recovery of oocysts following sucrose flotation (range)	Mean percentage recovery of cysts following sucrose flotation (range)
10 ± 0.5 (n = 6)	15.0% (7.0 - 18.0%)	17.0% (13.0 - 21.0%)
15 ± 0.5 (n = 6)	13.0% (10.0 - 21.0%)	18.4% (14.0 - 25.0%)
20 ± 0.5 (n = 6)	23.0% (19.0 - 31.0%)	25.0% (17.0 - 35.0%)

n = number of replicates

Table 4.5. Recovery of oocysts and cysts remaining in the pellet after sucrose flotation.

Turbidity (NTU)	Mean percentage recovery of oocysts in pellet (range)	Mean percentage recovery of cysts in pellet (range)
10 ± 0.5 (n = 6)	18.0% (14.0 - 25.0%)	17.0% (15.8 - 21.0%)
15 ± 0.5 (n = 6)	23.2% (17.2 - 28.0%	21.6% (19.0 - 27.0%)
20 ± 0.5 (n = 6)	26.6% (24.0 - 32.0%)	28.0% (19.0 - 33.0%)

n = number of replicates

4.4. DISCUSSION

Similar oocyst and cyst recoveries were obtained irrespective of whether pre-filtration was carried out despite losses occurring in the 80 μm and 35 μm pore size filters. However, pre-filtration using the Visser helminth filter made microscopy both easier and less time-consuming as larger particulates were removed, thereby enhancing the

detection of (oo)cysts. Although the flow of water through the Visser filters may also have aided the detachment of (oo)cysts from larger particulate matter some oocysts or cysts may have been embedded in the smaller particulate matter trapped by the filters. In addition, the recovery of (oo)cysts may be overestimated if the overnight equilibration of the seeded water samples did not allow adequate (oo)cyst association with particulate matter. Other researchers have stated that (oo)cysts may become associated with particulate matter present in environmental water samples, thereby reducing their recovery (Veal *et al.*, 1997).

The recovery of (oo)cysts was found to increase slightly with a pH adjustment to 6 $\pm$ 0.5 after concentration by flocculation. Similar findings by LeChevallier *et al.* (1995) indicated that desorption of oocysts and cysts from filter material was highest between pH's 6.0 and 7.4. *Cryptosporidium* has a negative zeta potential in water matrices above pH 3 which may have resulted in attraction to and repulsion from various particles present in water thereby increasing or decreasing their recovery (Drozd and Schwardzbrod, 1996). As the soil used in these experiments was loamy, it had a negative charge (Johnston pers. comm.) which probably assisted in the repulsion of (oo)cysts thereby increasing their recovery at pH 6.0.

Clarification of the water concentrate is necessary to remove interfering debris without concomitant loss of organisms (Rose *et al.*, 1988). However, high turbidity water samples may inhibit the effectiveness of the sucrose flotation procedure as the presence of debris may help to pull cysts and oocyst out of suspension and trap them in the pellet (LeChevallier *et al.*, 1995).

The recovery of (oo)cysts using sucrose flotation for clarification was poor, in relation to pre-filtration and pH adjustment, at all turbidities evaluated (Table 4.4.). Other researchers found recoveries of oocysts from turbid water samples fell dramatically when sucrose flotation techniques (specific gravity not stated) were applied in conjunction with calcium carbonate flocculation (Shepherd and Wyn-Jones, 1995). In contrast, LeChevallier *et al.* (1995) had 67.0% and 96.0% recoveries of oocysts and cysts respectively from seeded environmental water samples using a sucrose gradient with a specific gravity of 1.15. The specific gravity may play an important role as LeChevallier *et al.* (1995) have shown that oocyst recovery improved by 35% when sucrose with a

specific gravity of 1.15 was used in lieu of one with a specific gravity of 1.0. It is possible that the use of a sucrose gradient with a higher specific gravity of 1.18 in this study was also inefficient in the recovery of (oo)cysts.

Reasons for the variable and often inefficient recoveries attributed to sucrose flotation may include the biological state of the organisms. Viable organisms have intact, self-regulating surfaces and membranes while non-viable organisms do not. In addition, non-viable organisms have a molecular architecture different from viable organisms allowing the former to interact with a greater variety of particulates (Smith and Hayes, 1997). Bukhari and Smith (1995) assessed the usefulness of sucrose flotation for concentrating *C. parvum* oocysts finding that recovery efficiencies were dependent on the proportion of viable: non-viable oocysts in the sample. Viable oocysts are more likely to float on the interface and non-viable oocysts are more likely to pass through into the pellet during centrifugation which explains why oocysts and cysts were detected in the pellet during this experiment as the (oo)cysts purchased had been inactivated with formalin and were not viable (PRL DyNAgenics, Arizona, USA). In contrast, LeChevallier *et al.* (1995) found improved recovery of aged preparations of oocysts and cysts compared to those of freshly seeded samples which may suggest that empty (oo)cysts are preferentially concentrated by the sucrose gradient. The difference in density between viable and non-viable (oo)cysts has the potential to be a diagnostic tool to be used for determining viability of these organisms in environmental samples, but was not investigated in this study.

Excess sucrose also reduces the ability of (oo)cysts to adhere onto glass (Smith and Hayes, 1997) therefore further oocysts and cysts may have been lost from the microscope slides due to the use of sucrose with a slightly higher specific gravity (1.18).

4.5 CONCLUSIONS

Pre-filtration of turbid water samples prior to calcium carbonate flocculation made microscopy easier as larger particulate matter that may obscure oocysts or cysts was removed. However, some losses of oocysts and cysts occurred as they were trapped amongst debris retained by the 80 µm and 35 µm pore size filters. Pre-filtration is

therefore not recommended for routine use with turbid waters where recovery of (oo)cysts needs to be maximised, but the relatively low (oo)cyst losses make this method feasible where time is an important consideration. Recovery of (oo)cysts was poor after sucrose flotation although this may have been affected by the non-viable status of the (oo)cysts. The adjustment of pH to 6 ± 0.5 following calcium carbonate flocculation improved the recovery of (oo)cysts slightly and is recommended when turbid water samples are to be assessed using this method.

*CHAPTER 5

A SIMPLE AND ECONOMIC METHOD FOR DETECTING *CRYPTOSPORIDIUM* AND *GIARDIA* IN WATER

5.1. INTRODUCTION

Cryptosporidium and *Giardia* detection techniques such as flow cytometry (Vesey *et al.*, 1994) 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.

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 $\mu\ell$) 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).

*A condensed version of this chapter was published in the Water Quality International 1998 Proceedings

5.2. MATERIALS AND METHODS

The basic procedure from which all SIA variations derive involves the steps presented in Figure 5.1. 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.

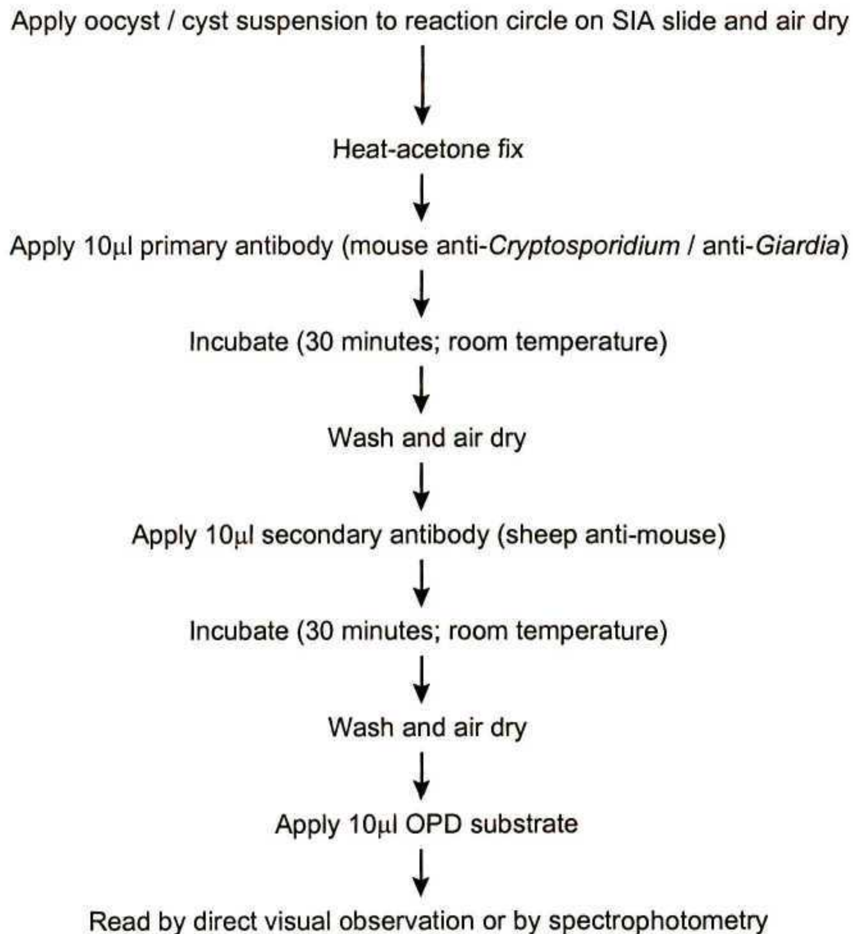


Figure 5.1. Flow diagram depicting the steps involved in SIA.

5.2.1 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 $\mu\ell$ 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 $\mu\ell$ 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 $\mu\ell$) 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 $\mu\ell$). The negative control was 10 $\mu\ell$ 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 $\mu\ell$ of 30% hydrogen peroxide added just prior to use) substrate solution (10 $\mu\ell$) 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 5.1. 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 that produced a visually detectable colour reaction.

5.2.2 The 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 $\mu\ell$ 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 $\mu\ell$). 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 1.6b.

5.2.3 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 prepared as described in 3.2.2. 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 5.1., using optimised reagent concentrations as determined in 5.2.1. The negative control was 10 $\mu\ell$

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.

5.2.4 Multiple phase SIA

5.2.4.1 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 $\mu\ell$ 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 $\mu\ell$) 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 $\mu\ell$ 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.

5.2.4.2 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 5.2.).

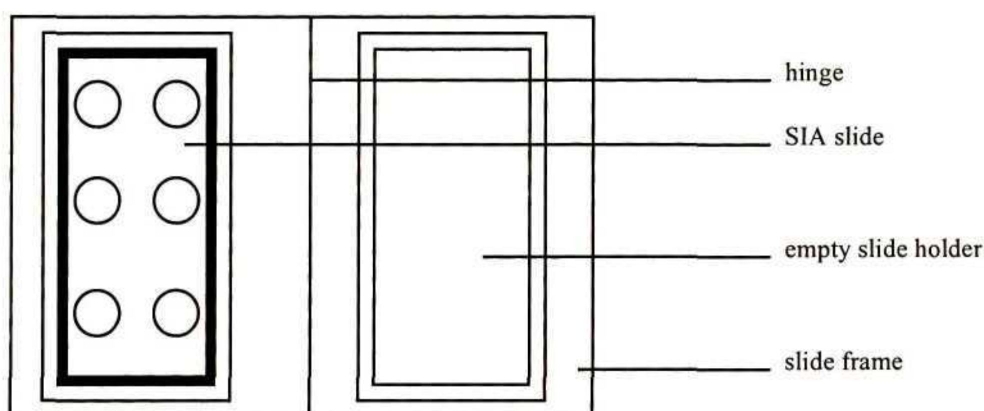


Figure 5.2. Diagram of the basic multiple phase slide module.

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. The closed module was incubated at room temperature for 5 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 antibody* (liquid phase) was applied to each reaction circle of the antigen slide and incubated at room temperature for a further 5 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 $\mu\ell$) was added to each reaction circle being tested and the slides were returned to the humid chamber and read spectrophotometrically as stated above.

5.2.4.3 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 of 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 above experiment, fresh substrate slides were made up and stored at 4°C. The experiments were repeated 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.

5.2.4.4 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. All costs are expressed in South African currency.

5.3. RESULTS

5.3.1 Calibration of the SIA system

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 that 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 5.3.).

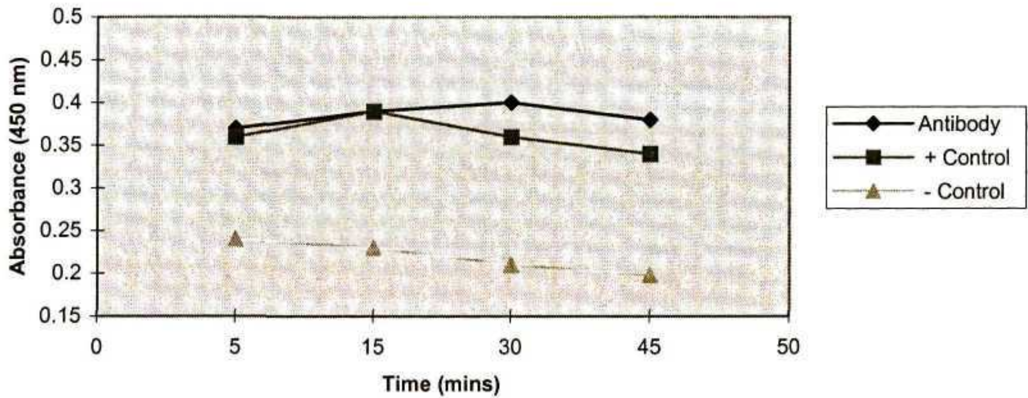


Figure 5.3. Absorbance readings using 5 μℓ of a 1:5 dilution of *Cryptosporidium* combined with a 1:50 dilution of primary and secondary antibody between 5-45 minutes.

The 5 μℓ of a 1:25 dilution of *Cryptosporidium* did not produce a colour that 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 5.4.).

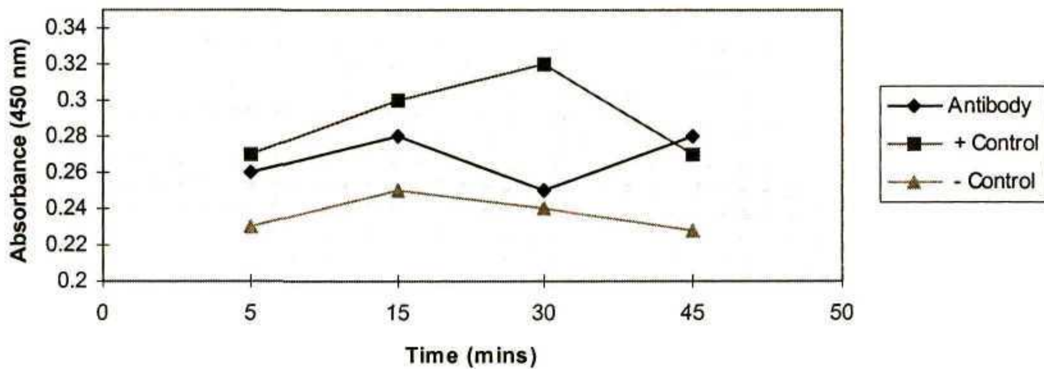


Figure 5.4. Absorbance readings using 5 μℓ of a 1:25 dilution of *Cryptosporidium* combined with a 1:50 dilution of primary and secondary antibody.

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 5.5. and 5.6. respectively).

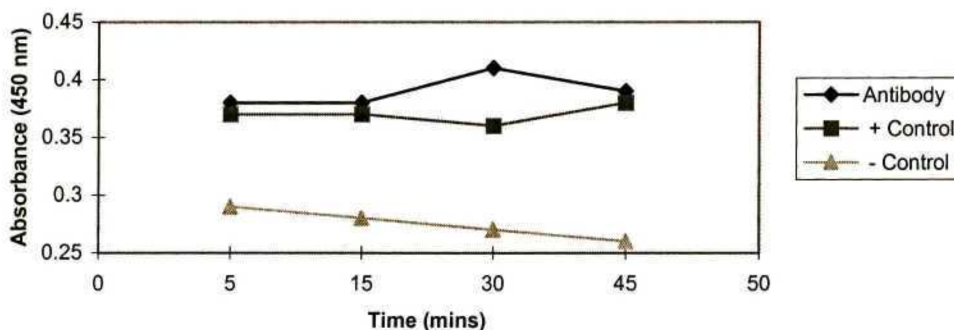


Figure 5.5. Absorbance readings using 5 µℓ of a 1:5 dilution of *Giardia* combined with a 1:5 dilution of primary antibody and a 1:50 dilution of secondary antibody.

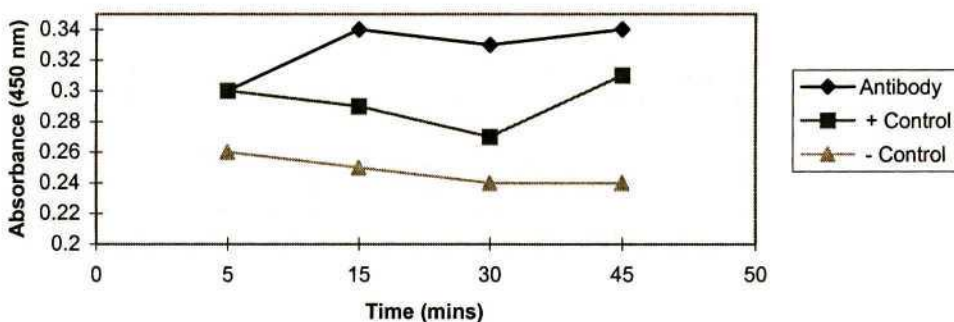


Figure 5.6. Absorbance readings using 5 µℓ of a 1:10 dilution of *Giardia* combined with a 1:5 dilution of primary antibody and a 1:50 dilution of secondary antibody.

5.3.2 The 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.

5.3.3 Detection of *Cryptosporidium* and *Giardia* in turbid water samples using SIA

The SIA was successful with all the turbid samples seeded with *Cryptosporidium* and *Giardia*. A pale yellow was visible to the naked eye after five minutes for all the seeded water samples. After 15 minutes incubation a yellow colour was visible with the *Cryptosporidium* - seeded 600 NTU samples while dark yellow was visible with the *Cryptosporidium* - seeded 4000 NTU samples. The lower turbidity *Cryptosporidium* samples remained pale yellow in colour (0.1 NTU; 6 NTU; 60 NTU). After 30 minutes these samples turned mid-yellow while the 600 NTU and 4000 NTU water samples turned dark yellow. The colour reactions were easily distinguishable from the negative controls that remained pale yellow.

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 that remained pale yellow.

5.3.4 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 5.7.) The *Giardia* absorbance readings (0.30 - 0.78) were also above background (0.15 - 0.34)(Figure 5.8.). 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.

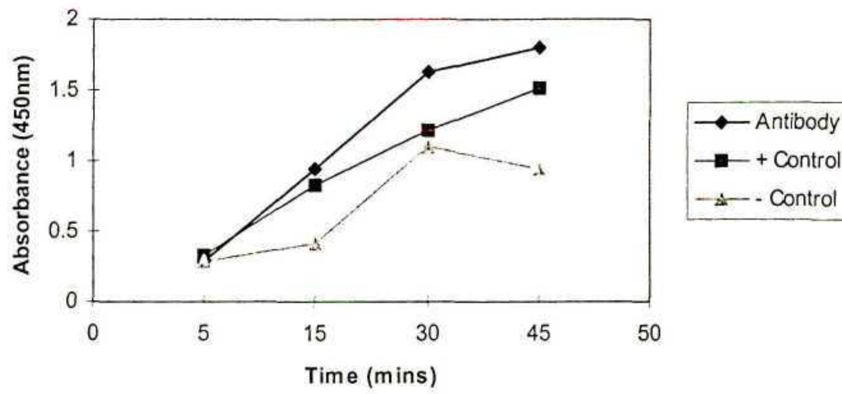


Figure 5.7. Absorbance readings of a 1:5 dilution of *Cryptosporidium* in liquid phase when used in combination with solid phase primary and secondary antisera and solid phase substrate.

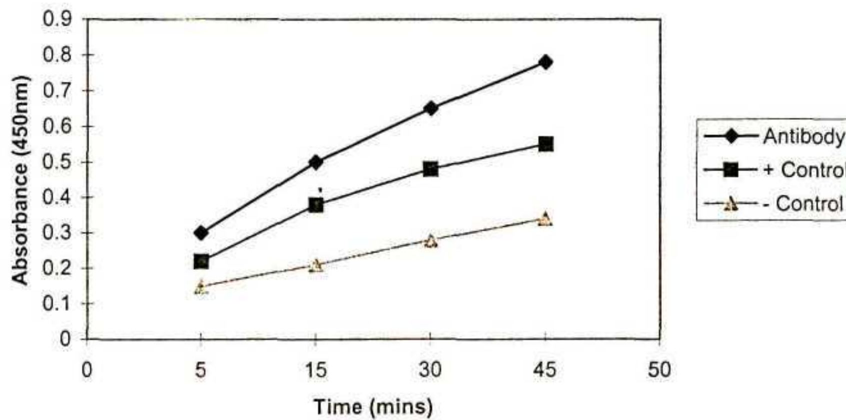


Figure 5.8. Absorbance readings of a 1:5 dilution of *Giardia* in liquid phase when used in combination with solid phase primary and secondary antisera and solid phase substrate.

b) The use of the secondary antibody in the liquid phase produced a colour reaction that could not be distinguished from the negative control. The use of the freshly prepared substrate produced a yellow colour reaction that could be seen by direct visual observation of the 1:5 dilution of *Cryptosporidium* and the 1:5 dilution of *Giardia*. The *Cryptosporidium* absorbance readings (0.10 - 0.14) were above the background (0.09 - 0.10)(Figure 5.9.) as were the *Giardia* absorbance readings (0.12 - 0.16)(Background: 0.11 - 0.16)(Figure 5.10.). 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* that increased in intensity with time and was most intense from 15 to 30 minutes (Maximum absorbance: 0.24)(Figures 5.11. and 5.12.).

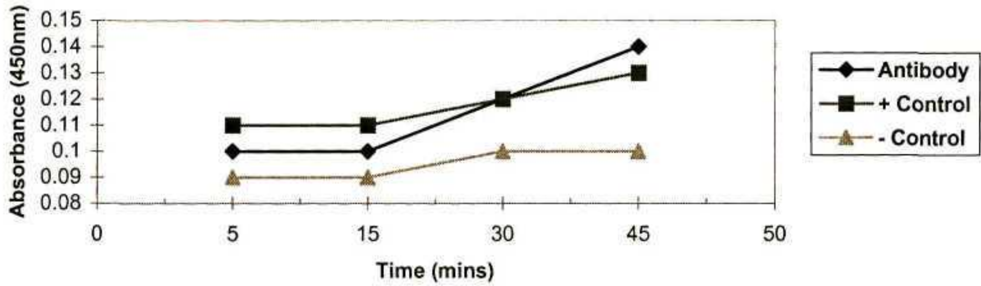


Figure 5.9. Absorbance readings of a 1:5 dilution of *Cryptosporidium* in liquid phase when used in combination with solid phase primary antisera, liquid phase secondary antisera and solid phase substrate.

The positive control (10 $\mu\ell$) absorbance readings may be lower than that of the antisera reactions (5 $\mu\ell$) due to the unavailablity of antigenic sites as a result of clumping of the oocysts / cysts in the positive control (Figures 5.7., 5.8. and 5.11).

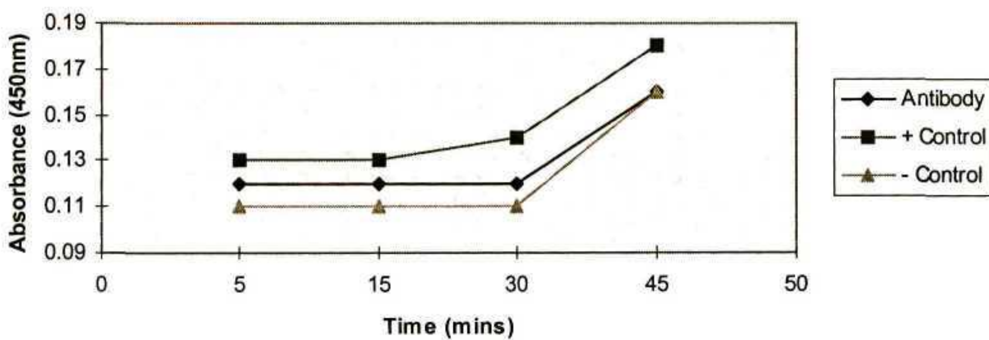


Figure 5.10. Absorbance readings of a 1:5 dilution of *Giardia* in liquid phase when used in combination with solid phase primary antisera, liquid phase secondary antisera and solid phase substrate.

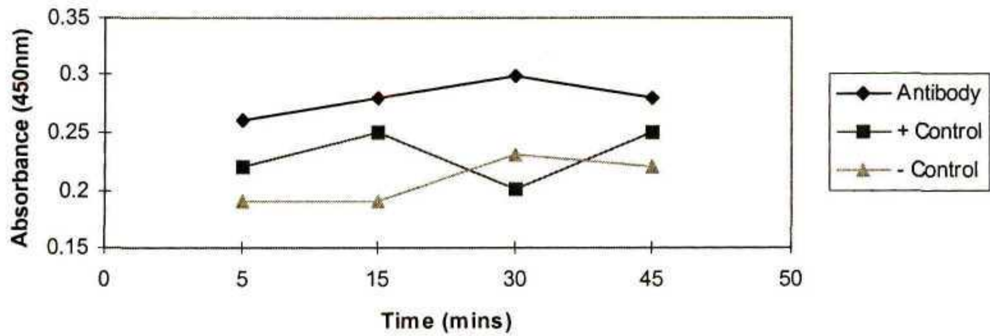


Figure 5.11. Absorbance readings of a 1:5 dilution of *Cryptosporidium* in liquid phase when used in combination with solid phase primary and secondary antisera and liquid phase substrate.

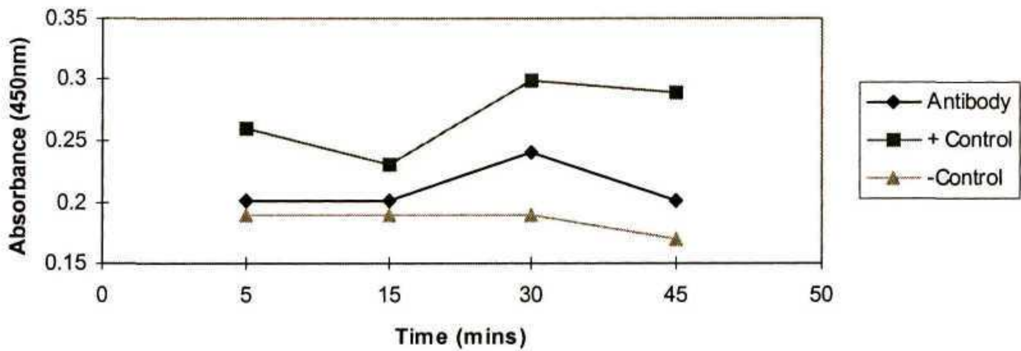


Figure 5.12 Absorbance readings using a 1:5 dilution of *Giardia* in liquid phase when used in combination with solid phase primary and secondary antisera and liquid phase substrate.

5.3.5 COSTING ANALYSIS

The total cost to perform a SIA analysis for the detection of *Cryptosporidium* or *Giardia* in water samples was R 30-00 (APPENDIX 15).

5.4 DISCUSSION

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 that 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). Other techniques such as IFA use FITC labelled antibodies that alone cost approximately R 85-00 per sample.

The optimum fixation method was determined to be a heat-acetone combination that allowed for the fixation of the most protists with uniform distribution that covered 50% of the surface of each reaction circle and exposure of the maximum number of available antibody-binding sites.

The optimum reagent concentrations required for visual colour detection of *Cryptosporidium* were a 1:50 dilution of primary antibody against *Cryptosporidium* and a 1:50 dilution of peroxidase labelled secondary antibody. Using these dilutions 154 oocysts could be detected in five microlitres. For visual colour detection of *Giardia* the best reagent concentrations were a 1:5 dilution of primary antibody against *Giardia* and a 1:50 dilution of peroxidase labelled antibody. Using these dilutions 74 cysts could be detected in five microlitres. Lower numbers of oocysts and cysts could be detected spectrophotometrically as the absorbance values were above those of the background but this was not determined.

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.

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 protists 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).

(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.

SIA is suitable for use with water samples of varying turbidity as the colour reactions were distinguishable from the negative control for both *Cryptosporidium* and *Giardia*. In its present form the SIA technique could be used to detect and monitor oocysts and cysts at contamination sources. These could include runoff points from farms or nearby abattoirs or communities lacking formal water and sanitation amenities where the protozoa are in high quantity. Refinement would be necessary to increase the sensitivity for detection for raw and potable water samples. A calibration curve for spectrophotometric quantification could also be constructed.

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.

The optimum multiple phase combination for visual colour detection of *Cryptosporidium* and *Giardia* resulted when using solid phase primary and secondary antibody with liquid phase substrate. Previous experiments substituting liquid phase substrate with pre-prepared solid phase substrate produced good colour reactions initially but when repeated two weeks later with the same reagents colour reactions were not visually distinguishable from the negative control.

The slide frame used in the multiple liquid-solid phase SIA ensured exact superposition of circles with matching reagents separated by a gap 1mm thick. This gap was occupied by the drops of water that formed a liquid column and allowed interaction of reagents (Conway de Macario *et al.*, 1987).

Further experiments need to be performed to refine the use of multiple liquid-solid phases as the preliminary experiments indicate that the substrate is unstable in a solid phase form and decays after three weeks. If this substrate is to be used in a SIA kit, it would have to be provided in a liquid state or separate powder form for dilution as required.

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).

5.5. 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 $\mu\ell$ 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 immunomagnetic apparatus is being designed to concentrate oocysts and cysts from environmental water samples prior to application of SIA.

Further tests using environmental samples of water, sludge and faeces will be performed to determine sensitivity but the SIA technique shows great potential as it has been found to be more sensitive than indirect immunofluorescence or ELISA and more reliable due to the virtual absence of background interference (Conway de Macario *et al.*, 1983).

SIA is a simple tool that is less time-consuming and requires less capital expenditure than other methods. It also only requires small volumes of reagent that 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 with DC current (using solar rechargeable batteries) 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.

*CHAPTER 6

A NOVEL METHOD FOR QUANTIFICATION OF VIABLE *GIARDIA* CYSTS IN WATER SAMPLES

6.1. INTRODUCTION

Assessment of *Giardia* viability is a major requirement for public health purveyors and the water industry. Cyst viability has been evaluated by methods such as *in vivo* infectivity (Faubert *et al.,* 1986), *in vitro* excystation (Smith and Smith, 1989), dye inclusion and exclusion (Schupp and Erlandsen, 1987; Ongerth *et al.*, 1989), animal infectivity (Isaac-Renton *et al.*, 1996). and cyst morphology using Normarski DIC microscopy (DeRegnier *et al.*, 1989).

Many of the methods for viability determination are impractical for routine use in laboratories due to time constraints. Excystation and animal models are impractical to routine utilities for viability determination as they are expensive and unreliable due to variability in results (Dowd and Pillai, 1997). Therefore a combined detection-viability method is required that is quick and simple to perform.

For this reason fluorescein diacetate (FDA), which was previously found to be suitable for staining viable cysts (Schupp and Erlandsen, 1987), was combined with cyst identification using *Giardia* specific tetramethylrhodamine(TMR)-labelled antibodies. Viability staining of *Cryptosporidium* oocysts using this combination was not performed as positive stool quantities were minimal.

6.2. MATERIALS AND METHODS

6.2.1 Purification of *Giardia* cysts from stools

Stools received from hospitals, which had determined them to be positive for *Giardia* by bright-field microscopy, were purified according to a sucrose gradient procedure as described by Roberts-Thompson *et al.* (1976) (APPENDIX 16). The purified cysts were stored in distilled water at 4°C prior to use within one week of obtaining samples.

*A condensed version of this chapter has been accepted for publication in Water Research

6.2.2 Viability staining using FDA

A stock solution of fluorescein diacetate (FDA)(Molecular Probes, Oregon, USA) was made by dissolving 10 mg FDA in 1mℓ acetone and stored at 4°C in the dark. A working dilution of 1:1000 was made in phosphate buffered saline (PBS) as required and stored at 4°C. A fresh working solution was made just prior to use.

Twenty-five microlitres of purified *Giardia* suspension was incubated in a clean tube in the dark with 5 μℓ FDA for five minutes at room temperature. Excess stain was washed off by centrifuging at 850 *g* for five minutes in 1 mℓ PBS. Five microlitres of the stained solution were applied to a microscope slide and viewed by epifluorescence microscopy using a 450-490 nm exciter filter attached to a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany). Oval shaped organisms which were 5-15 μm in width, 8-18 μm in length and fluoresced green were identified as presumptive cysts. The slide was also viewed microscopically using the triple band microscope filter with excitations of 400/ 450/ 570 nm and the colour of the cysts under both filters were compared.

6.2.3 Confirmation of *Giardia* identity

TMR-labelled *Giardia* antibody (Waterborne, Inc., Louisiana, USA) was diluted 1:20 and 1:40 in PBS. Twenty-five microlitres of each dilution of labelled antibody were applied to 25 μℓ of purified *Giardia* suspension in a clean tube and incubated for one hour in the dark at 4°C. Excess stain was washed off by centrifuging once at 850 *g* (5 minutes) in 1 mℓ PBS. Five microlitres of the stained suspension were applied to a microscope slide and viewed by epifluorescence microscopy using filters with excitations of 400/ 450/ 570 nm and 450-490 nm respectively. Cysts were identified by distinct red staining of the wall and morphology as described above.

6.2.4 Determination of cyst viability

A method combining FDA and TMR was devised and purified stool suspensions were quantified for viable and non-viable cysts as follows:

Twenty-five microlitre suspensions were incubated with 5 $\mu\ell$ FDA followed by 25 $\mu\ell$ of a 1:40 dilution of TMR-labelled antibody as described in 6.2.3. Five microlitres of stained suspension were applied to microscope slides and viewed under epifluorescence microscopy using the 400/ 450/ 570 nm triple band excitation filter. Cysts that stained red and fluoresced green internally were presumed viable. These cysts were then viewed under the 450-490 nm excitation filter and those fluorescing green were confirmed as viable *Giardia* cysts while others were considered to be non-viable.

6.3. RESULTS

6.3.1 Viability staining of *Giardia* cysts using FDA

Under the 450-490 nm excitation wavelength approximately 2.5% of the cyst-like structures (as observed under bright-field microscopy) in three samples showed distinct green fluorescence with 3+ to 4+ intensity. Due to the dark background using the 400/ 450/ 570 nm triple band excitation filter, the green fluorescence of the cytoplasm was enhanced indicating viable cysts. However, numerous samples contained cyst-like structures that did not fluoresce green after FDA staining indicating non-viability.

6.3.2 Confirmation of *Giardia* identity

The 1:20 dilution of TMR-labelled antiserum produced a red background and cysts were not easily distinguished. The 1:40 dilution of labelled antibody allowed distinct red staining of the cyst wall with 2+ to 4+ intensity using the triple band microscope filter with excitations of 400/ 450/ 570 nm. Under the 450-490 nm excitation filter the cysts were less easily distinguishable from the background, having a light yellow outline.

6.3.3 Determination of cyst viability

Combined staining of several purified cyst suspensions with FDA and TMR showed distinct red staining of cyst walls using the triple band microscope filter with excitations of 400/ 450/ 570 nm allowing positive identification of *Giardia* (Plate 6.1.). When viewed using the 450-490 nm filter these cysts were not visible, indicating non-viability.

In three of the samples cysts exhibited red staining of the walls while some cysts also fluoresced green internally with 3+ intensity indicating viability, when using the triple band microscope filter with excitations of 400/ 450/ 570 nm (Plate 6.2.). Non-specific red staining of debris occurred but this was distinguishable from the cysts by size and shape. When the red and green stained cysts were viewed using the 450-490 nm filter they exhibited green fluorescence of 1+ to 2+ intensity confirming viability whilst the non-viable cysts were not visible at this wavelength.

Of the three samples analysed, approximately 2.5% of the cysts were viable (38/1512 cysts). Many other *Giardia* positive samples supplied by the hospitals had *Giardia* cysts that reacted negatively to the FDA stain but positively to the TMR.

6.4. DISCUSSION

The simultaneous detection and determination of *Giardia* cyst viability was possible by staining with a 1:1000 dilution of FDA followed by a 1:40 dilution of TMR-labelled *Giardia* antibody and viewing by epifluorescence microscopy using a triple band microscope filter with excitations of 400/ 450/ 570 nm.

In combination with TMR the intensity of the FDA stained cysts diminished from 4+ to 2+. However, green and red stained cysts were still easily identifiable as viable *Giardia* although FDA was reported to overestimate viability (Thiriat *et al.*, 1998) for reasons discussed in Chapter 1.

The optimal dilution of TMR-labelled antiserum was found to be 1:40 as this reduced background staining and allowed red stained cysts to be easily differentiated from other background debris.

As FDA stains viable cysts (Schupp and Erlandsen, 1987), other viable and morphologically similar structures that did not react with TMR-labelled antibodies might be

considered to be false-positives. TMR-labelled antibodies specific to *Giardia* allowed confirmation of cysts identity despite some non-specific binding to debris.

If the viability status of cysts is required, the method for concentrating the cysts from water needs to be carefully chosen as Campbell *et al.* (1994) showed that oocyst viability following calcium carbonate flocculation was 46.5% opposed to 75.5% using centrifugation techniques. As the viability of (oo)cysts in potable water is of most importance to public health, it is suggested that these water samples, which have low turbidities (<1 NTU), be concentrated by centrifugation to reduce viability loss.

Few of the cysts purified from the stool samples were viable, indicating that although patients excrete high numbers, few are potentially dangerous. Other researchers have stated that a proportion of cysts did not stain with either FDA or PI although these became FDA positive over time (Schupp and Erlandsen, 1987) while Smith and Smith (1989) found non-staining cysts despite excystation and attributed this to structural and biochemical differences, either in the cysts and / or trophozoite membrane, from those which do stain. Robertson *et al.* (1992) found a relatively high proportion of *Cryptosporidium* oocysts in stool samples were initially impermeable to DAPI, suggesting that a component of faeces, possibly a mucopolysaccharide, might insert into the oocyst wall thereby decreasing permeability. Despite this, ingestion of low numbers of cysts are able to cause giardiasis (Smith *et al.*, 1995) thus highlighting the public health requirements for establishing cyst viability in the provision of potable water.

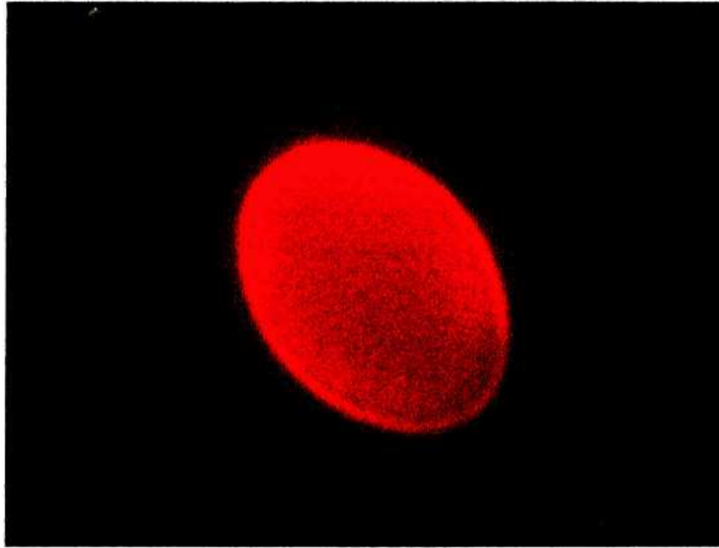
This FDA-TMR method holds potential for monitoring viability of *Giardia* cysts in potable and environmental water samples as results using seeded distilled water were reliable. This technique could be used in combination with FITC / PI (Thiriat *et al.*, 1998) as confirmation of viability status.

6.5. CONCLUSIONS

The simultaneous detection of *Giardia* cysts and determination of their viability was possible by staining with FDA followed by TMR-labelled *Giardia* antibody and viewing by epifluorescence microscopy using a triple band microscope filter. However, viability is overestimated by FDA therefore another combination of FITC / PI should be used in conjunction with this method as a confirmation of cyst viability. Although routine analyses to date have concentrated on detection of these pathogens (Chapter 3), overestimation of viability using this technique errs in favour of safety, but brings one a step closer to determining the risk posed should (oo)cysts be detected in drinking or recreational waters.

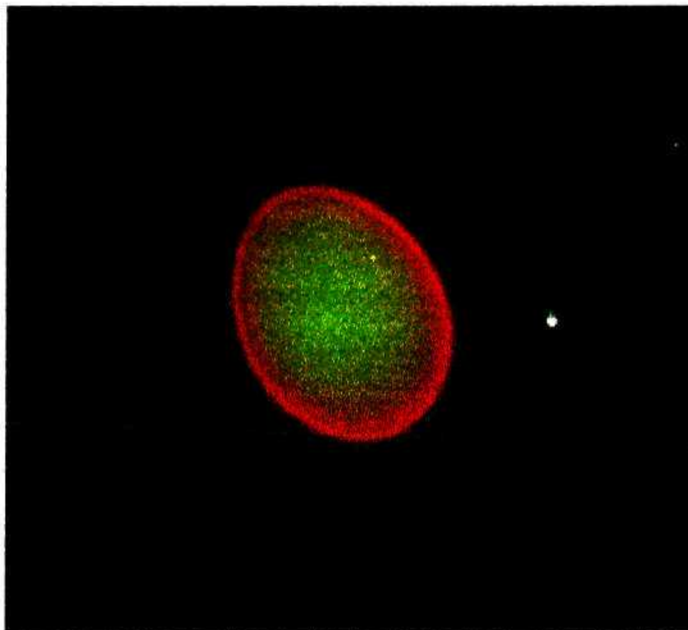
Using the triple band microscope filter, viable cysts stained green internally with a red wall while non-viable cysts only stained red. This method was simple, reliable and quick and could be used to differentiate *Giardia* cysts in potable and environmental water samples following standard concentration techniques excluding calcium carbonate flocculation. The use of this combination stain with *Cryptosporidium* oocysts using TMR-labelled antibodies specific to *Cryptosporidium* needs to be evaluated.

The overestimation of viability errs in favour of safety, but brings one a step closer to determining the risk in drinking and recreational waters as routine analyses to date have only concentrated on the detection of *Cryptosporidium* and *Giardia*.



8 μm

Plate 6.1. Photomicrograph showing distinct red staining of a non-viable *Giardia* cyst viewed under oil immersion by epifluorescence microscopy, fitted with a triple band filter with excitations of 400/ 450/ 510 nm.



8 μm

Plate 6.2. Photomicrograph of a viable *Giardia* cyst with a red staining wall and internal green fluorescence viewed under oil immersion by epifluorescence microscopy, fitted with a triple band filter with excitations of 400/ 450/ 510 nm.

*CHAPTER 7

OCCURRENCE OF *CRYPTOSPORIDIUM* OOCYSTS AND *GIARDIA* CYSTS IN KWAZULU-NATAL WATERS AND THEIR CORRELATION WITH OTHER WATER QUALITY PARAMETERS

7.1. INTRODUCTION

In KwaZulu-Natal, a large proportion of the population has inadequate sanitation and/or water supplies, which could lead to the spread of waterborne disease (Pegram *et al.*, 1998). In addition, the occurrence of *Cryptosporidium* and *Giardia* in KwaZulu-Natal waters is unknown due to the lack of environmental testing, despite the monitoring of waters for other causative agents of disease.

The direct detection of pathogenic bacteria and viruses is costly and time consuming, therefore indicator microorganisms are used as surrogates to measure the extent of faecal contamination of water as well as the efficiency of disinfection procedures. Similarly, the complexity and cost associated with the concentration and detection of *Cryptosporidium* and *Giardia* from aquatic systems suggests that an indirect way of monitoring would be beneficial (Chauret *et al.*, 1995).

Coliform bacteria are useful for determining the quality of potable and recreational water while the presence of the faecal coliform, *E. coli*, indicates the presence of faecal matter from warm-blooded animals (Bitton, 1994). Coliphages are viruses that infect and replicate in coliform bacteria and are more resistant to chlorination than the coliforms. They may thus be better indicators of disinfection efficiency than coliform bacteria (Kott *et al.*, 1974). Faecal streptococci are regarded as secondary indicators of faecal pollution and are used to assess the significance of coliforms in a water sample in the absence of *E. coli* (The Bacteriological Examination of Drinking Water Supplies, 1982).

Algal levels can be considered an index of eutrophication in a water body while pH is a measure of the degree of acidity in an aqueous solution and turbidity indicates the amount of suspended matter, such as clay, silt, finely divided organic and inorganic

*A condensed version of this chapter was published in the 1997 International Symposium on Waterborne *Cryptosporidium* proceedings

matter, soluble coloured organic compounds and microscopic organisms present in water (Standard Methods, 1995). In addition, some algae may be of similar size and shape to *Cryptosporidium* and *Giardia* and may cross-react with the IFA that could result in misidentification (Nieminski *et al*., 1995). pH and turbidity are two water quality indices which may have an effect on *Cryptosporidium* or *Giardia*. Suspension of (oo)cysts for prolonged periods at certain pH levels has resulted in loss of viability while the turbidity level may be useful as an indicator of possible contamination of these protozoons.

In this chapter, the occurrence of *Cryptosporidium* and *Giardia* in dam, river, raw and potable waters, treated effluents and wastewater sludges in the Umgeni Water operational area in KwaZulu-Natal were investigated. Raw and potable water samples were also monitored for *E. coli*, coliforms, faecal streptococci, algae, temperature, pH and turbidity. In addition, the presence of somatic coliphages was determined in all potable water samples. Dam and river water samples were monitored for *E. coli*, coliforms, faecal streptococci, algae, temperature, pH and turbidity while treated effluent samples were monitored for *E.coli*, somatic colipages and temperature. This information was used in statistical correlations between the protozoon concentrations and these indicators of water quality to determine their potential for use as indicators for indirect monitoring of possible *Cryptosporidium* or *Giardia* contamination.

7.2. MATERIALS AND METHODS

7.2.1 Study area

Midmar Dam, situated on the uMngeni River, is one of the major storage units serving the metropolitan areas from Pietermaritzburg to Durban. It is also a popular recreational facility, sporting sailing, swimming and fishing.

Raw and potable water samples were randomly collected from nine Umgeni Water-owned waterworks in KwaZulu-Natal. These waterworks supply potable water to the Greater Durban metropolitan area, Pietermaritzburg and surrounding areas and parts of the KwaZulu-Natal South coast. The main processes used to treat raw water received from dams in the surrounding catchment include coagulation, sedimentation and rapid sand

filtration. Disinfection of the treated water is by chlorination or chloramination. One plant, Wiggens Waterworks (Durban), ozonates the raw water received from Inanda Dam, both prior to coagulation and following filtration to oxidise taste and odour compounds present in the water.

Darvill Wastewater Works (Pietermaritzburg) is primarily a domestic wastewater works that also treats some industrial waste from Pietermaritzburg. Raw sewage is treated by anaerobic digestion and activated sludge processes. Some waste-activated sludge is further treated by dissolved air flotation (DAF) prior to land disposal with the primary digested sludge while the treated effluent from the activated sludge is disinfected with chlorine before flowing into the uMsunduze River.

7.2.2 Protozoon analyses

Ten-litre water samples were collected sporadically in 10 ℓ plastic bottles between July 1995 and September 1996, from Midmar Dam main basin. Ten-litre samples were collected periodically from the river inflows to the dam from February to September 1996. Raw and potable waters were sampled (10 ℓ) on three to nine occasions at each of the nine waterworks between January 1998 and December 1998. Ten-litre treated effluent samples were collected periodically, between May 1995 and December 1996, in 10 ℓ plastic bottles while pre- and post-thickener sludge samples were collected in 250 mℓ plastic bottles between June 1995 and December 1996.

All samples were stored at 0-10°C until analysis within 48 hours of sample collection. The 10-litre water samples were poured into 20 ℓ flat-bottomed plastic buckets and concentrated according to the calcium carbonate flocculation method of Vesey *et al.* (1993a) (APPENDIX 1). After dissolution of the floc with sulphamic acid and prior to centrifugation, the pH of the concentrate was increased to 6.0 $\pm$ 0.5 as described in 4.2.3. Oocysts and cysts were detected and enumerated as described in 3.2.3.

Twenty millilitre wastewater sludge samples were washed through the Visser helminth filter and the eluates were collected in beakers as described in 4.3.1. Oocysts and cysts were detected and enumerated as described in 3.2.3.

7.2.3 Other water quality parameters and statistical analyses

For bacterial analyses, water samples were aseptically filtered onto 0.45μm pore size cellulose acetate membranes (Millipore Corporation, Massachussets, USA) and placed on selective media as described in The Bacteriological Examination of Drinking Water Supplies (1982) and in Collins and Lyne (1985). Water samples for algal analyses were enumerated using membrane filtration according to Standard Methods (1992) while samples for coliphage detection were cultured using a double agar-layer technique according to Grabow *et al.* (1993). Turbidity and pH readings were performed as described in Standard Methods (1995).

Pearson correlation analyses, using MSExcel, were used to determine whether these water quality parameters indicated possible *Cryptosporidium* and / or *Giardia* contamination.

7.3. RESULTS

7.3.1 Protozoon analyses

Of the water samples collected from Midmar Dam main basin, 71.4% (5/7) were positive for *Cryptosporidium* oocysts (ranging from 0-800 10 ℓ^{-1}) while 28.6% (2/7) contained *Giardia* cysts ranging from 0-200 10 ℓ^{-1} (APPENDIX 17). The highest number of oocysts and cysts were recorded during the summer rainfall months of December and January. While *Cryptosporidium* oocysts were detected in 23.5% (4/17) of river water inflow samples, 35.3% of the river water samples were positive for *Giardia* cysts (Figures 7.1. and 7.2.; APPENDIX 17).

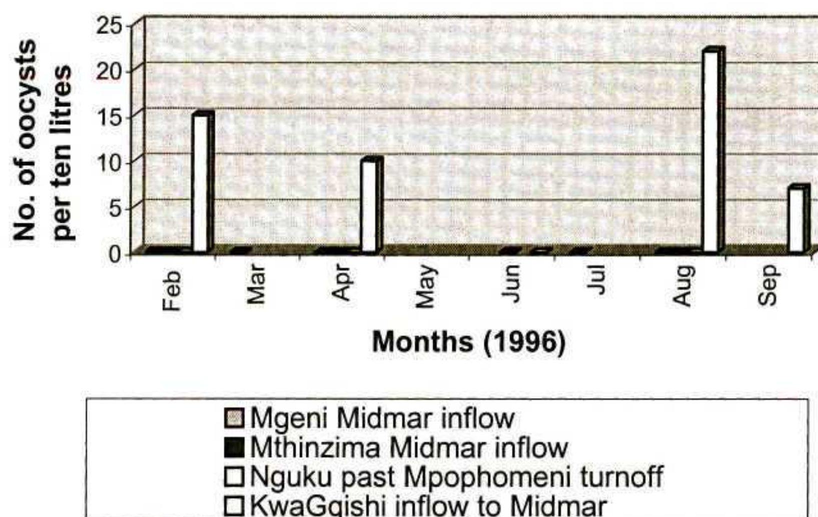


Figure 7.1. Occurrence of *Cryptosporidium* oocysts in rivers flowing into Midmar Dam.

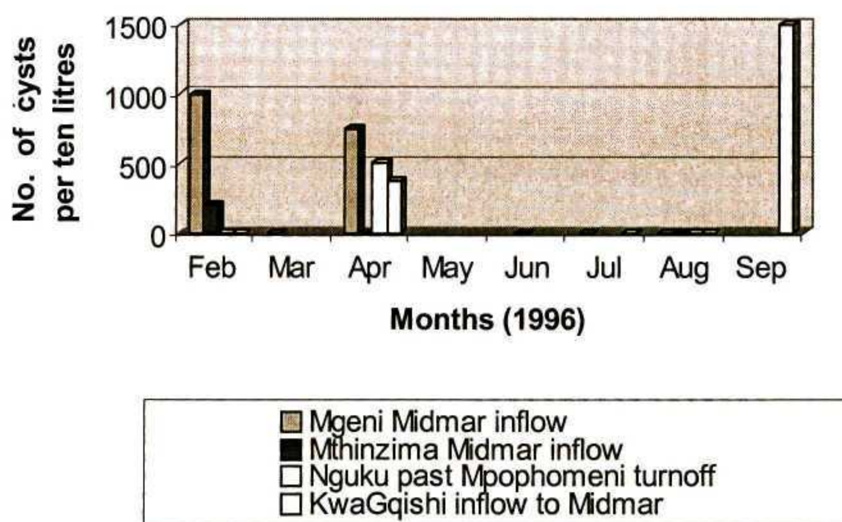


Figure 7.2. Occurrence of *Giardia* in rivers flowing into Midmar Dam.

Of all the raw water samples collected from the different waterworks, 4.3% (2/47) were positive for the presence of *Cryptosporidium* and *Giardia* and 2.1% (1/47) were positive for *Giardia* only. Both samples positive for *Cryptosporidium* contained 50 oocysts 10 ℓ^{-1} while two of the *Giardia* positive samples contained 10 cysts ℓ^{-1} and one contained 40 cysts ℓ^{-1}.*Cryptosporidium* or *Giardia* occurred twice in the raw water samples collected at one waterworks, but were not detected in re-samples which were collected two days after the original sample was received. All potable waters were free of *Cryptosporidium* and

Giardia and other indicators of faecal contamination. The turbidity and pH were maintained at <1NTU and pH 7.3 0- 9.0 respectively while some final waters had low quantities of algae (1 – 31 cells $m\ell^{-1}$).

Of chlorinated effluent samples, *Cryptosporidium* was detected in 80% of the samples (8/10) while 60% were positive for *Giardia* (6/10). The concentration of oocysts ranged from 0 – 1 500 oocysts 10 ℓ^{-1} while cyst concentrations ranged from 0 – 675 cysts 10 ℓ^{-1} (APPENDIX 18). High oocyst and cyst numbers were recorded in the pre- and post-thickener sludge samples (Figures 7.3. and 7.4.). A 30 -100% reduction in oocyst and cyst concentrations occurred between the pre- and post-thickener sludge.

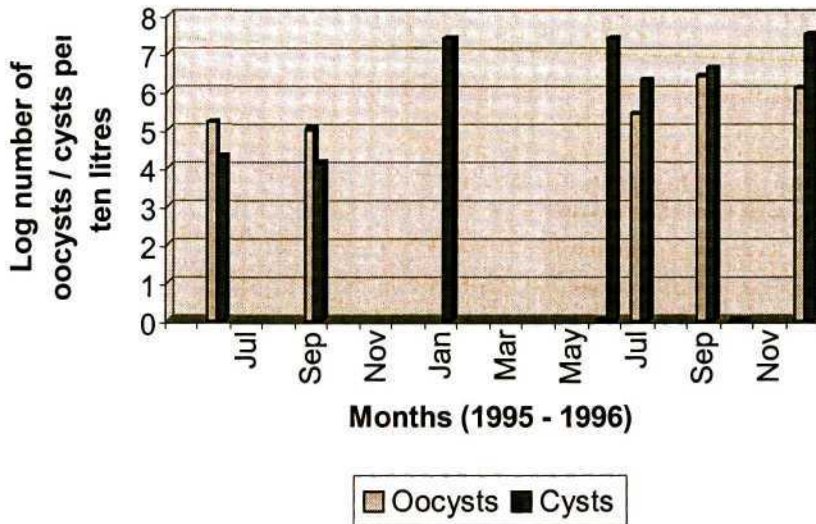


Figure 7.3. *Cryptosporidium* oocysts and *Giardia* cysts detected in Darvill pre-thickener sludge.

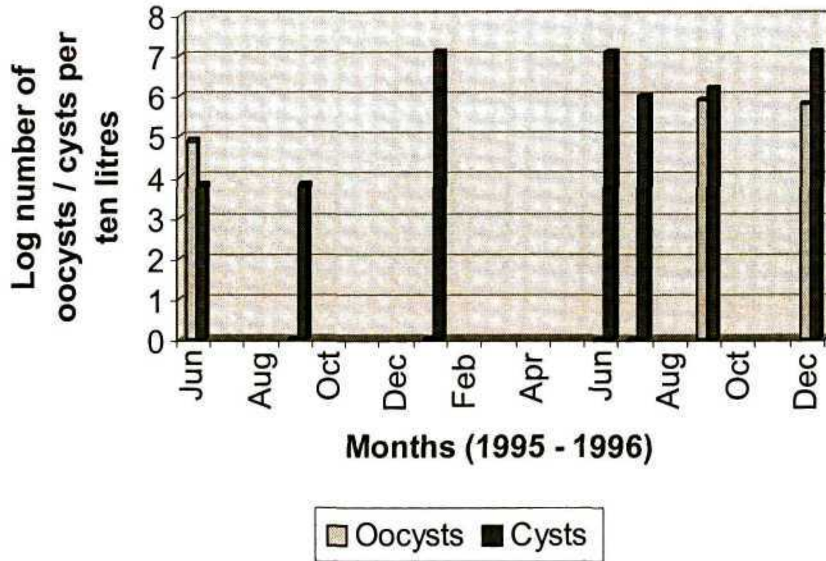


Figure 7.4. *Cryptosporidium* oocysts and *Giardia* cysts detected in Darvill post -thickener sludge.

7.3.2 Other water quality parameters and statistical analyses

A significant correlation (0.5) was found between the presence of *Cryptosporidium* and *Giardia*, however the results of this study did not yield any significant correlation between the protozoon parasites and other water quality parameters (APPENDIX 19).

7.4. DISCUSSION

Cryptosporidium and *Giardia* were detected regularly in Midmar dam and its river inflows, in treated wastwater effluents and sludges. Detection of these organisms also occurred during winter and summer further highlighting their temporal and spatial distribution.

The oocysts and cysts were in low concentration or were not detected in raw water received by water treatment plants, possible due to the long retention time in the dams from which the raw water is drawn. It is also possible that a variation in (oo)cyst load or non- point source of contamination occurred which random sampling missed. As oocysts

or cysts were not detected in re-samples of the raw waters that were initially positive, the source of contamination probably ceased to contaminate the water.

Cryptosporidium oocysts, *Giardia* cysts, *E.coli*, coliforms, faecal streptococci and coliphages were never detected in potable water samples indicating that the water treatment processes were adequate for the load received. However, due to the losses of oocysts and cysts during the concentration process (Chapter 4), negative *Cryptosporidium* or *Giardia* results do not necessarily mean the absence of oocysts from individual samples.

Oocysts were regularly detected in the iGqishi River which is bordered by dairy farms, chicken farms and a nature reserve, but was not detected in the other Midmar dam river inflows. *Giardia* cysts were detected in all the rivers flowing into Midmar Dam. These rivers are surrounded by cattle and dairy farms or informal settlements without formal water supplies and sanitation that may be the source of contamination. In fresh waters, microbial antagonism may be the main mechanism by which (oo)cyst survival is threatened. One study suggests that *Cryptosporidium* was affected by *Serratia marcescens*, which could apparently degrade the oocysts by chinolytic activity (Zuckerman *et al.*, 1997). The presence of oocysts and cysts in Midmar Dam may also be cause for concern to public health due to the water sports available. Body-contact recreation is a known non-point source of faecal contamination in lakes and reservoirs (Moore *et al.*, 1994; Kramer *et al.*, 1996) and strong associations between recreator activity and contracting gastrointestinal illness by bathers has been reported (Kramer *et al.*, 1996).

The high numbers of oocysts and cysts detected in the sludge from the wastewater works, at which agricultural wastes can be considered a minimum input, indicates that both protozoons are present in the Pietermaritzburg community. This sludge that is disposed of onto land could result in the contamination of grazing animals and herdsmen. The reduction in oocysts (30.0 – 100.0%) and cysts (33.0 – 50.0%) between the pre- and post-thickener suggests that the thickener is able to remove some of the protists although the viability of the remaining (oo)cysts is unknown. Darvill effluent, which enters the uMsunduze River and contains high quantities of both protists, could also contribute to infection of those using the river as a source of water. The variation in (oo)cysts numbers

during the months surveyed may be due to the levels of infection and disease in the community.

The correlation studies showed that the presence of *Cryptosporidium* oocysts were significantly correlated with the presence of *Giardia* cysts as did Rose *et al.* (1991). However, none of the indicator organisms surveyed (*E.coli;* coliforms; faecal streptococci; coliphages) could be used as indicators of either *Cryptosporidium* or *Giardia*. Although Chauret *et al.* (1995) found significant correlation between faecal streptococci and *Cryptosporidium* and between *Giardia* and both coliphages and algae in surface waters, these were found to be watershed dependent.

7.5. CONCLUSIONS

Cryptosporidium oocysts and *Giardia* cysts were found to be ubiquitous in KwaZulu-Natal, having been detected in a variety of waters tested (dam, river and raw waters, wastewaters) irrespective of seasonal changes. There were, however, increases in (oo)cyst numbers during summer months possibly due to run-off from faeces contaminated land or broken sewers following heavy rainfall. *Cryptosporidium* or *Giardia* were not detected in treated water samples indicating that the treatment processes were adequate. However, it should be kept in mind that losses of (oo)cysts occur throughout the concentration process as indicated in Chapter 3 and very low numbers of (oo)cysts may not be detected. Both the activated sludge process and the anaerobic digestion treatment of sewage did not remove oocysts or cysts adequately and the viability status of the remaining (oo)cysts should be evaluated. Sludge disposed of onto land needs to be monitored for parasitic protozoan concentrations and should not be used for recreational or agricultural purposes. Treated effluents need to be monitored before discharge into rivers that may act as a source of water for both humans and animals. Correlation between the presence of *Cryptosporidium* and *Giardia* or the water quality parameters *E.coli*, coliforms, faecal streptococci, coliphages, algae, pH or turbidity were not found to be significant. Correlations between *Cryptosporidium* and *Giardia* presence were significant.

CHAPTER 8
GENERAL DISCUSSION

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 public health threat has led to much interest in the monitoring of water for the presence of *Cryptosporidium* oocysts and *Giardia* cysts. However, 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).

In South Africa, diarrhoeal disease is a common occurrence in the population although little is known about the role of *Cryptosporidium* and *Giardia* as a cause of this disease. Therefore a laboratory-based epidemiological study of stools submitted for analysis, was undertaken to determine the occurrence of these pathogens in the KwaZulu-Natal population. The data collected indicated that both parasites are endemic in KwaZulu-Natal which may indicate moderate to high immunity levels within the population which results in sporadic cases and asymptomatic infections. The incidence of *Cryptosporidium* and *Giardia* did not appear to correlate with climatic factors such as rainfall, season or year, possibly indicating that waterborne transmission is not the predominant route of infection and other factors such as personal hygiene, potable water supply, sanitation and education have a more significant impact. Contrary to expectation, a low percentage of *Cryptosporidium* and *Giardia* positive cases were recorded in symptomatic (4.5% and 5.3% respectively) and asymptomatic (2.4% and 0.8% respectively) HIV patients. *Cryptosporidium* and *Giardia* incidence in young children (< 5 years) indicated that *Cryptosporidium* was most prevalent in the < 1 year age group while *Giardia* was most prevalent in the 3 - 4 year age group. Female and male patients tested for cryptosporidiosis had similar positive percentages while giardiasis was more prevalent in female patients. These differences may, however, not be representative of the actual occurrence within age groups as the data was dependent on separate and sparse recording of patient information. In addition, different detection methods were used which

have been shown to have varied recoveries (Moodley *et al.*,1991b) and a standard diagnostic test needs to be devised so that the results are comparable countrywide.

Despite the fact that the data were only sourced from 9.7% of all laboratories that test for *Cryptosporidium* and 6.0% of all laboratories that test for *Giardia*, the two hospitals from which the data were collected serve the entire province of KwaZulu-Natal while the private pathology laboratory receives stools from various towns in the province. As a database has been setup in the province in order to monitor the occurrence of a number of bacterial pathogens that cause diarrhoea, it would be beneficial to incorporate data on cryptosporidiosis and giardiasis so that epidemics can be easily predicted and preventative measures speedily put in place.

As *Cryptosporidium* and *Giardia* were found to be present in the KwaZulu-Natal population, the monitoring of these pathogens in water supplies was considered a necessity. Methods for their concentration from water have varied recovery rates, therefore three recent methods which offer improved recovery of oocysts and cysts were evaluated: calcium carbonate flocculation, membrane filtration and membrane dissolution. Experiments revealed that (oo)cyst losses occurred with each method. Membrane filtration combined with sonication required more than one membrane for turbid water analysis making it an expensive procedure for routine analyses while membrane dissolution had very poor recoveries from turbid waters. The flocculation procedure proved to have the best and most reproducible recoveries (between 64.6 and 73.1%, from tap and turbid waters) but suffers from the drawback that only relatively small volumes of water can be analysed.

Due to the deleterious effects of accumulated debris and other interfering substances on the recovery of oocysts and cysts from turbid water samples, methods to enhance their recovery during flocculation were investigated. These techniques included a pre-filtration step using a Visser helminth filter prior to flocculation, clarification using sucrose flotation after calcium-carbonate flocculation, and pH adjustment of the water concentrates following flocculation. In relation to pre-filtration and pH adjustment, recovery of (oo)cysts following clarification by sucrose flotation was poor, possibly due to the non-viable status of the (oo)cysts which caused them to pass through the density gradient into the pellet. Pre-filtration was successful in reducing the number of larger particulates which may have

masked (oo)cysts during microscopy, although (oo)cyst losses occurred as the water samples passed through some of the differential filters. pH adjustment to 6 ± 0.5 following flocculation and prior to centrifugation improved recoveries of (oo)cysts from turbid water samples although this may be dependent on the soil type and the charge of the soil.

Detection of oocysts and cysts in water concentrates is tedious and requires expensive microscopes fitted with suitable filters and halogen lamps. To avoid this requirement, a simple and economic method using the slide immunoenzymatic assay (SIA) (Conway de Macario *et al.*, 1986) was adapted to detect *Cryptosporidium* and *Giardia*. This method proved to be suitable for detecting (oo)cysts in treated water by direct visual observation and spectrophotometry 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 by direct visual observation of colour. A lower minimum detection level would be possible using a spectrophotometer capable of handling microscope slides, or more concentrated antibody specific to *Cryptosporidium* or *Giardia*. 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 while a calibration curve for spectrophotometric quantification could be constructed. Visual confirmation of identity is also possible as the (oo)cysts are mounted on microscope slides. Although SIA results were reproducible, further development of the multi-phase SIA system is required to optimise the dry ready-to-use reagents prior to its evaluation with environmental samples.

Knowledge of the viability of oocysts or cysts provides further information regarding their likely impact on public health should viable organisms pass through treatment processes (Smith *et al.*, 1995). In addition, viability assays are suitable for determining the effectiveness of chemical disinfection regimes (Black *et al.*,1996).

A combined detection-viability technique for *Giardia* cysts, which may be applied to *Cryptosporidium* oocysts, was developed. This method used fluorescein diacetate (FDA) to stain viable cysts and tetramethyl red labelled anti-*Giardia* monoclonal antibodies (TMR) for confirmation of identity. As a result of FDA staining, green fluorescence of viable cysts occurred while cyst identification was confirmed by red staining of the cyst wall. Non-viable cysts stained red only. In addition to microscopic examination, this simple

and quick method lends itself for use with flow cytometry and could be complemented with fluorescein isothiocyanate / propidium iodide (FITC / PI) as a confirmation of viability status. As the flocculation procedure reduces viability of (oo)cysts (Campbell *et al.*, 1994) and this is of concern in treated waters, it is suggested that potable water samples be concentrated by centrifugation or a suitable immunomagnetic technique be developed. In addition, FDA has been reported to overestimate viability (Thiriat *et al.*, 1998) therefore any error that may occur in monitoring of treated waters would err in favour of public health safety.

Using the calcium carbonate flocculation concentration technique and commercially available immunofluorescence assay for detection, *Cryptosporidium* and *Giardia* were detected in the dam, river, raw and waste water effluent samples collected. Positive samples were collected during winter and summer indicating their temporal distribution although higher concentrations occurred during the summer rainfall months. These may pose a risk to those using the rivers as a source of drinking water, or the dam and rivers for recreational purposes. The high numbers detected in the rivers could be due to run-off from agricultural farms or informal settlements with poor sanitation conditions. *Cryptosporidium* and *Giardia* were not detected in the treated drinking water, indicating that the treatment processes were adequate. However, it must be kept in mind that losses occur during the recovery procedure and low numbers of (oo)cysts present may not be detected.

Regular routine monitoring (weekly or monthly) of raw and treated waters as well as selected river sites which may be affected by faecal contamination should be performed. This knowledge of the likely sources of (oo)cysts which might contaminate a catchment is necessary in determining the potential impact of (oo)cysts at a water treatment plant. In addition, information accrued from sampling can assist in formulating catchment control strategies (Carrington and Miller, 1993).

The high numbers of *Cryptosporidium* and *Giardia* detected in the sludge from wastewater works indicates that both protists are present in the Pietermaritzburg community and surface run-off. This sludge which is disposed onto land could result in the contamination of grazing animals and herdsmen. The reduction in protozoon numbers between the pre- and post-thickener sludge indicates that the anaerobic digestion which

takes place is only able to remove some of the protists. Sludge disposed onto land needs to be monitored for protozoan concentrations and the contaminated land should not be used for recreational or agricultural purposes. Treated effluents also need to be monitored before discharge into rivers which may act as a source of water for both humans and animals.

Concentration techniques that are reproducible and have a high recovery of oocysts and cysts from environmental samples are still required. Ideally the detection method should also have a rapid turnabout time, be inexpensive and simple to perform, indicate viability while allowing simultaneous detection of oocysts and cysts.

A combination of calcium carbonate flocculation and SIA has the potential for routine use in assessing water samples due to its ease of use and fairly rapid turnabout (±7 hours). In addition, the small quantity of reagents required for SIA without the requirement of a fluorescence microscope make this detection option cheaper than the commercially available IFA kits. A drawback with this method is that it is only semi-quantitative and does not determine viability, limiting it to the detection of massive contamination during routine monitoring rather than determining the safety of drinking water. This method does lend itself to the production of dry ready-to-use reagents which could be used in field studies or small laboratories should simpler concentration methods be developed which do not require sophisticated equipment. Unfortunately, due to the loss of viability during the calcium carbonate concentration procedure, other concentration methods such as immunomagnetic separation or centrifugation need to be used if viability is to be determined using the TMR / FDA method developed in this study, and/or FITC / PI.

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APPENDIX 1

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

1. Collect a 10 ℓ water sample and place sample in a flat-bottomed polypropylene bucket.

2. Mix thoroughly and add 100 mℓ of 1 mol $ℓ^{-1}$ calcium carbonate and 100 mℓ of 1 mol $ℓ^{-1}$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 mℓ of 10% sulphamic acid and shake vigorously for 15 seconds before decanting into centrifuge tubes.

7. Rinse barrels with 200 mℓ of 0.01% Tween 80 and shake.

8. Decant and repeat with 100 mℓ 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 mℓ centrifuge tubes.

12. Centrifuge at 3 000g 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 mℓ.

15. Apply the antibody and detect microscopically.

APPENDIX 2

Membrane filtration (Shepherd and Wyn-Jones, 1996)

1. Collect a 10 ℓ water sample and filter through a 1.2 μm cellulose acetate flat membrane placed on a filter manifold (Millipore, 142 mm diameter)

2. Sonicate the membrane in 10 mℓ of 0.1% Tween (40 Hz; 10 minutes).

3. Collect the eluate in plastic centrifuge tubes and centrifuge at 1 800 *g* for 10 minutes.

4. Discard the supernatant and resuspend the pellet.

5. Immunoassay.

APPENDIX 3

Membrane dissolution (Aldom and Chagla, 1995)

1. Filter a 10 ℓ water sample through a 1.2 μm 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 mℓ acetone and wait two minutes.
4. Centrifuge at 650 *g* (15 minutes).
5. Resuspend in 50 mℓ acetone and centrifuge (650 *g*; 15 minutes).
6. Discard supernatant and resuspend in 50 mℓ of 95% ethanol.
7. Centrifuge at 650 *g* (15 minutes) and discard supernatant.
8. Resuspend in 50 mℓ of 70% ethanol and centrifuge (650 *g*; 15 minutes).
9. Resuspend in 10 mℓ eluting fluid.
10. Immunoassay

APPENDIX 4

Immunofluorescence assay (Crypto/Giardia Cel IF, Cellabs)

1. Place 20 $\mu\ell$ 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 $\mu\ell$ 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 5

Table 1. Number of oocysts counted in 5 $\mu\ell$ of a 1:5 dilution pipetted onto each of six microscope wells

WELL	NUMBER OF OOCYSTS
1	90
2	64
3	83
4	90
5	75
6	78

Table 2. Number of cysts counted in 5 $\mu\ell$ of a 1:10 dilution pipetted onto each of six microscope wells

WELL	NUMBER OF CYSTS
1	42
2	53
3	57
4	65
5	56
6	82

APPENDIX 6

Recovery of oocysts and cysts from seeded tap water samples using different concentration methods

Method	Number of oocysts recovered[a]	Percentage oocysts recovered (%)	Number of cysts recovered[b]	Percentage cysts recovered (%)
Calcium carbonate flocculation				
1	700	70.0	780	78.0
2	750	75.0	765	76.5
3	785	78.5	690	69.0
4	675	67.5	730	73.0
5	640	64.0	715	71.5
6	760	76.0	705	70.5
Membrane filtration				
1	350	35.0	420	42.0
2	405	40.5	560	56.0
3	390	39.0	610	61.0
4	335	33.5	555	55.5
5	280	28.0	530	53.0
6	320	32.0	490	49.0
Membrane dissolution				
1	0	0.0	0	0.0
2	150	15.0	230	23.0
3	0	0.0	0	0.0
4	73	7.3	192	19.2
5	117	11.7	340	34.0
6	109	10.9	210	21.0

[a]seeded with 10^3 oocysts
[b]seeded with 10^3 cysts

APPENDIX 7

Recovery of oocysts and cysts from seeded turbid water (10 NTU) using different concentration methods

Method	Number of oocysts recovered[a]	Percentage oocysts recovered (%)	Number of cysts recovered[b]	Percentage cysts recovered (%)
Calcium carbonate flocculation				
1	620	62.0	724	72.4
2	653	65.0	713	71.3
3	756	75.6	680	68.0
4	741	74.1	736	73.6
5	749	74.	742	74.2
6	691	69.1	753	75.3
Membrane filtration				
1	239	23.9	200	20.0
2	140	14.0	316	31.6
3	165	16.5	352	35.2
4	257	25.7	406	40.6
5	290	29.0	374	37.4
6	217	21.7	241	24.1
Membrane dissolution				
1	0	0.0	0	0.0
2	0	0.0	0	0.0
3	12	1.2	49	4.9
4	31	3.1	54	5.4
5	25	2.5	100	10.0
6	18	1.8	72	7.2

[a]seeded with 10^3 oocysts
[b]seeded with 10^3 cysts

APPENDIX 8

Recovery of oocysts and cysts from seeded turbid water (15 NTU) using different concentration methods

Method	Number of oocysts recovered[a]	Percentage oocysts recovered	Number of cysts recovered[b]	Percentage cysts recovered
Calcium carbonate flocculation				
1	716	71.6	715	71.5
2	672	67.2	723	72.3
3	614	61.4	710	71.0
4	693	69.3	650	65.0
5	650	650	712	71.2
6	703	70.3	694	69.4
Membrane filtration				
1	421	42.1	376	37.6
2	376	37.6	459	45.9
3	447	44.7	537	53.7
4	313	31.3	477	47.7
5	415	41.5	518	51.8
6	459	45.9	499.	49.9
Membrane dissolution				
1	22	2.2	51	5.1
2	42	4.2	49	4.9
3	0	0.0	0	0.0
4	37	3.7	63	6.3
5	18	1.8	22	2.2
6	0	0.0	0	0.0

[a]seeded with 10^3 oocysts
[b]seeded with 10^3 cysts

APPENDIX 9

Recovery of oocysts and cysts from seeded turbid water (20 NTU) using different concentration methods

Method	Number of oocysts recovered[a]	Percentage oocysts recovered (%)	Number of cysts recovered[b]	Percentage cysts recovered
Calcium carbonate flocculation				
1	580	58.0	637	63.7
2	635	63.5	593	59.3
3	656	65.6	660	66.0
4	662	66.2	678	67.8
5	693	69.3	659	65.9
6	650	65.0	685	68.5
Membrane filtration				
1	240	24.0	300	30.0
2	295	29.5	351	35.1
3	376	37.6	494	49.4
4	390	39.0	506	50.6
5	323	32.3	432	43.2
6	316	31.6	324	32.4
Membrane dissolution				
1	7	0.7	11	1.1
2	0	0.0	18	1.8
3	10	1.0	0	0.0
4	19	1.9	38	3.8
5	1	0.1	24	2.4
6	3	0.3	7	0.7

[a]seeded with 10^3 oocysts
[b]seeded with 10^3 cysts

APPENDIX 10

Recovery of oocysts and cysts following pre-filtration using a Visser helminth filter and concentration by calcium carbonate flocculation

Turbidity (NTU)	Number of oocysts recovered[a]	Percentage oocysts recovered	Number of cysts recovered[b]	Percentage cysts recovered
10 ± 0.5	631	63.1	682	68.2
	692	69.2	713	71.3
	633	63.3	645	64.5
	710	71.0	696	69.6
	682	68.2	687	68.7
	736	73.6	701	70.1
15 ± 0.5	630	63.0	695	69.5
	650	65.0	683	68.3
	597	59.7	709	70.9
	635	63.5	654	65.4
	618	61.8	630	63.0
	593	59.3	661	66.1
20 ± 0.5	659	65.9	624	62.4
	694	69.4	671	67.1
	615	61.5	663	66.3
	579	57.9	629	62.9
	611	61.1	692	69.2
	624	62.4	651	65.1

[a]seeded with 10^3 oocysts

[b]seeded with 10^3 cysts

APPENDIX 11

Number of oocysts and cysts trapped by the different pore size filter components of the Visser helminth filter (The results for the 100 μm pore size filter were negative and are therefore not depicted)

Filter pore size (μm)	**Turbidity (NTU)**					
	10 ± 0.5		**15 ± 0.5**		**20 ± 0.5**	
	oocysts[a]	**cysts[b]**	**oocysts[a]**	**cysts[b]**	**oocysts[a]**	**cysts[b]**
80	50	20	37	16	71	29
	17	16	24	24	93	26
	41	11	57	17	42	30
	15	18	44	14	100	24
	32	13	51	22	78	27
	12	17	30	21	62	31
35	66	10	49	25	65	24
	58	22	62	21	61	22
	77	16	72	26	70	18
	76	17	69	18	63	23
	62	23	71	16	59	29
	65	27	65	20	54	32

[a]seeded with 10^3 oocysts

[b]seeded with 10^3 cysts

APPENDIX 12

Recovery of oocysts and cysts following adjustment of pH to 6.0 ± 0.5 after calcium carbonate flocculation

Turbidity (NTU)	Number of oocysts recovered[a]	Percentage oocysts recovered (%)	Number of cysts recovered[b]	Percentage cysts recovered (%)
10 ± 0.5	640	64.0	675	67.5
	649	64.9	692	69.2
	756	75.6	742	74.2
	692	69.2	763	76.3
	734	73.4	784	78.4
	760	76.0	750	75.0
15 ± 0.5	631	63.1	719	71.9
	764	76.4	642	64.2
	699	69.9	723	72.3
	776	77.6	751	75.1
	685	68.5	687	68.7
	621	62.1	726	72.6
20 ± 0.5	710	71	621	62.1
	662	66.2	679	67.9
	644	64.4	715	71.5
	673	67.3	691	69.1
	649	64.9	662	66.2
	682	68.2	681	68.1

[a]seeded with 10^3 oocysts

[b]seeded with 10^3 cysts

APPENDIX 13

Recovery of oocysts and cysts following sucrose flotation of the concentrate

Turbidity (NTU)	Number of oocysts recovered[a]	Percentage oocysts recovered (%)	Number of cysts recovered[b]	Percentage cysts recovered (%)
10 ± 0.5	70	7.0	130	13.0
	180	18.0	157	15.7
	166	16.6	160	16.0
	157	15.7	210	21.0
	179	17.9	192	19.2
	148	14.8	173	17.3
15 ± 0.5	115	11.5	133	14.4
	210	21	172	17.2
	117	11.7	201	20.1
	100	10	250	25
	126	12.6	153	15.3
	113	11.3	181	18.1
20 ± 0.5	263	26.3	350	35
	310	31	227	22.7
	208	20.6	231	23.1
	190	19	279	27.9
	213	21.3	170	17
	198	19.8	243	24.3

[a]seeded with 10^3 oocysts

[b]seeded with 10^3 cysts

APPENDIX 14

Recovery of oocysts and cysts remaining in the pellet after sucrose flotation of the concentrate

Turbidity (NTU)	Number of oocysts recovered[a]	Percentage oocysts recovered (%)	Number of cysts recovered[b]	Percentage cysts recovered (%)
10 ± 0.5	140	14.0	153	15.3
	179	17.9	162	16.2
	165	16.5	210	21
	250	25.0	171	17.1
	18.1	18.1	154	15.4
	16.7	16.7	172	17.2
15 ± 0.5	172	17.2	190	19.0
	253	25.3	212	21.2
	242	24.2	270	27.0
	214	21.4	195	19.5
	280	28.0	213	21.3
	231	23.1	216	21.6
20 ± 0.5	240	24.0	190	19.0
	246	24.6	311	31.1
	273	27.3	273	27.3
	252	25.2	330	33.0
	320	32.0	254	25.4
	266	26.6	322	32.2

[a]seeded with 10^3 oocysts

[b]seeded with 10^3 cysts

APPENDIX 15

Slide immunoenzymatic (SIA) costing analysis

EQUIPMENT		
Simple spectrophotometer	R 1- 54	
Micropipette	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	R 1--35	
Giardia antibody	R 5-40	
Complete media	R 0-58	
Secondary antibody	R 0-07	
o-phenyldiamine	R 0-01	
Citric acid	R 1-77	
Peroxide	R 0-30	R 9-48
	———	
TECHNICIAN TIME (R 50 / hour)		
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 16

Sucrose gradient method for *Giardia* purification from faecal specimens (Roberts-Thompson *et al.*, 1976)

1. A pea sized amount of faecal specimen was suspended in 3 mℓ of distilled water in a 10 mℓ centrifuge tube using an orange stick.
2. The suspension was transferred to a clean 10 mℓ centrifuge tube and the volume made up to 10 mℓ with distilled water. The lid of the centrifuge tube was tightened and covered with parafilm.
3. The suspension was centrifuged at 2200 rpm (870*g*) for 3-5 min. The supernatant was removed with a pasteur pipette.
4. The pellet was resuspended in 3 mℓ of distilled water and emulsified. The volume was made up to 10 ml and centrifuged again at 2000 rpm (719*g*) for 3-5 min.
5. Step 4 was repeated twice but the centrifuge speed was reduced to 1800 (582*g*) and 1600 rpm (460*g*) in subsequent spins. After the 1600 rpm spin the pellet was resuspended in 4 mℓ distilled water.
6. The sample was carefully suspended over 4 mℓ 0.85M sucrose and centrifuged at 1800 rpm (582*g*) for 15 min.
7. The water suspension and the interface between the sucrose and the water were removed using a pasteur pipette and placed in a clean centrifuge tube.
8. The sample was centrifuged at 2200 rpm (870*g*) for 4 min.
9. The supernatant was removed, leaving the pellet in approximately 0.5 mℓ of liquid.

APPENDIX 17

Table 1. Occurrence of *Cryptosporidium* and *Giardia* in Midmar Dam main basin from July 1995 to September 1996

MONTH	No. *Cryptosporidium* 10 ℓ^{-1}	No. of *Giardia* 10 ℓ^{-1}
July	30	0
December	800	200
January	500	150
February	350	190
June	0	60
July	0	0
September	90	85

Table 2. Occurrence of *Cryptosporidium* per ten litres in rivers flowing into Midmar Dam

MONTH	uMngeni Midmar inflow	uMthinzima Midmar inflow	iNguklu past Mpophomeni turnoff	iGqishi inflow to Midmar
15	0	0	0	
March	0	n/s	n/s	n/s
April	0	0	0	10
May	n/s	n/s	n/s	n/s
June	n/s	0	n/s	0
July	0	n/s	n/s	n/s
August	0	0	0	22
September	n/s	n/s	n/s	7

n/s = not sampled

Table 3. Occurrence of *Giardia* per ten litres in rivers flowing into Midmar Dam

MONTH	uMngeni Midmar inflow	uMthinzima Midmar inflow	iNguklu past Mpophomeni turnoff	iGqishi inflow to Midmar
February	1000	200	0	0
March	0	n/s	n/s	n/s
April	750	0	500	375
May	n/s	n/s	n/s	n/s
June	0	n/s	n/s	n/s
July	0	n/s	n/s	0
August	0	0	0	0
September	n/s	n/s	n/s	1500

n/s = not sampled

APPENDIX 18

Occurrence of *Cryptosporidium* and *Giardia* in Darvill effluent from July 1995 to December 1996

MONTH	No. *Cryptosporidium* 10 ℓ^{-1}	No. of *Giardia* 10 ℓ^{-1}
July	0	0
August	0	0
September	100	400
October	600	0
November	900	0
December	1500	350
January	n/s	n/s
February	n/s	n/s
March	n/s	n/s
April	n/s	n/s
May	n/s	n/s
June	n/s	n/s
July	300	100
August	n/s	n/s
September	70	675
October	750	450
November	n/s	n/s
December	100	600

n/s = not sampled

APPENDIX 19

Pearson correlation results between *Cryptosporidium* and *Giardia* and various water quality parameters determined using MSExcel

	Crypto	*Giardia*	Temp	pH	Turbidity	E.coli	Coliforms	F strep	Coliphages	Algae
Crypto	1									
Giardia	0.516613	1								
Temp	0.001157	-0.01629	1							
pH	-0.11762	-0.18041	-0.07492	1						
Turbidity	0.47318	0.496536	0.090755	-0.91815	1					
E.coli	0.143713	0.56524	0.284667	-0.13715	0.272104	1				
Coliforms	-0.0333	0.067369	0.236906	-0.01471	0.272104	0.982779	1			
F strep	-0.0315	0.817875	0.272152	-0.00965	0.357245	0.836699	0.791024	1		
Coliphages	-0.00093	0.021408	0.110909	-0.16074	0.0129	0.404239	1	0.113803761	1	
Algae	-0.0301	-0.0308	-0.09585	-0.11056	0.395083	0.007866	-0.00046	0.165969	1	1

Crypto = Cryptosporidium
Temp. = temperature (°C)
F strep = faecal streptococci