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**Methods of Testing
For *Giardia* in
Water**

A thesis presented in partial fulfilment
of the requirements,

for the degree,

Master of Science in Microbiology
Massey University.

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1995

ABSTRACT

Since the 1960's when the first waterborne outbreaks of *Giardia* were reported in America, it has been recognised as a disease causing organism. From these outbreaks the USA Environmental Protection Agency (EPA) developed a method for testing large volumes of water for *Giardia* cysts, this was adapted into the 16th edition of the Standard Methods.

To test the method cultured cysts were required for spiked trials. A published method of encystation by Schupp et al (1988) was investigated as a potential source of cysts. Morphologically correct cysts were gained in the greatest number at 37°C over 72 hours at a bile concentration of 5g/l.

Using cultured cysts and cysts from animals and water, viability and the least number needed to initiate a culture were assessed. When 10 of the cysts produced *in vitro* were excysted it was possible to obtain a culture. For cysts from animal and water origins at levels up to 10,000 cysts, it was not possible to obtain cultures.

Variations of the Standard Method of water testing for *Giardia* had been reported by different laboratories. We investigated the sensitivity of this method using some of the reported variations such as staining on a membrane filter, the use of monoclonal antibody stains and methods of washing cysts free of the sampling core.

We found the method could detect to the 5×10^2 cysts/500l of water, a recovery of 10%. The recoveries obtained over a range of cysts spiked was between 10-40%.

An alternative method to sampling and processing the sample was tangential filtration. Four tangential filtration units were compared to the concentration techniques of centrifugation and sedimentation (these were those used in the Standard Method). The tangential filtration units were found not to be as sensitive as centrifugation and sedimentation. They also

presented difficulties with particulate matter or sediment. When compared to the sampling method, the unit was unable to concentrate the 500l of tap water due to the sediment levels.

Staining methods were evaluated. Slide staining was compared to staining on a filter, the filter method was found to give a better recovery. Comparison between commercially available monoclonal antibody stain, a polyclonal antibody stain and Lugols iodine stain, found that the monoclonal and polyclonal antibody stains lead to easier identification by illuminating the cyst (it still had to be checked for internal morphology) than the iodine stain. The monoclonal antibody stains were found to be more specific than the polyclonal stain.

Methods of inactivating the antigens recognised by the monoclonal antibody stain persist so cross contamination between samples was investigated. Hypochlorite concentrations of 4% and higher over 20 minutes were found to inactivate the antigen recognised. Other chemicals were compared but none were found to inactivate the antigen.

A study of a family infected with *Giardia* was undertaken, to test methods used in the laboratory and study modes of transmission. *Giardia* cysts were found in the river that supplied the farm tank but not in the tank itself. The house tank also tested negative for *Giardia*. The family had young children attending school and playgroup, person to person transmission may also have been involved. Animals on the farm had positive tests for *Giardia*.

ACKNOWLEDGEMENTS

Thank you to Proffesor Tim Brown and all his staff for the help and support that they gave espically the technians and the other post graduate students.

Thank you to Steve for your friendship and help. Your input was great.

Thank you to Cadbury Confectionery Ltd for the use of the computers also the people I work with for there understanding espicially Sarah, Christine, Karen, Jo, Harriet and Grant.

Thanks to my family for their support, also to Tass, Blue, Maggie, Bart and KD. To Nick, Donna, Janeen, David, Wallie, Eileen, Jeremy, Vicky, Geoff and Steve your support has been awesome.

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INTRODUCTION

1.1. THE ORGANISM

1.1.1 Taxonomy and Structure

In 1681, while viewing his own fecal matter, van Leewenhoek was the first to describe the intestinal protozoan *Giardia*. Lambl, in 1859 made morphological descriptions and illustrations leading to the a human strain of *Giardia* being named after him (*Giardia lamblia*). Grassi (Adams 1991) gave a detailed discription of the *Giardia* trophozoites and cyst stages. Despite these early discoveries *Giardia* has only recently (since the 1960's) been regarded as a human pathogen.

Giardia is a binucleate flagellated protozoan parasite taxonomically classed as of the Phylum Sarcomastigophora, Class Zoomastigophorea, Order Diplomonadida and Genus *Giardia*. The human associated species has various specific names *G. lamblia*, *G. intestinalis*, *G. duodinalis*, and *G. enterica*. This work will refer to the human isolated species as *G. intestinalis* (Erlandsen et al 1988a).

Filice (1952) used the shape of the median body of the trophozoite to differienate *Giardia* into three species. His description is the most widely accepted method of differentiation.

G. agilis has a median body that is long, and is like club shaped rods. This species has been isolated from amphibians. *G. muris* has a small rounded pair of median bodies which are seen in the midline, and has been isolated from the tracts of rhodents, birds and reptiles. *G. intestialis*, the species isolated from humans, has a pair of median bodies similar to a claw hammer lying transversely across the body. This species has been isolated from mammals, birds and reptiles (Filice 1952, Erlandsen et al 1988a).

Other researchers have used host specificity as a means of speciation. This practice has led to over forty species of

Giardia being named with no gross morphological differences for differentiation being shown (Meyer 1985, Erlandsen et al 1988a).

Further attempts have been made to differentiate species within the existing genus using biochemical and molecular methods such as DNA-DNA hybridization, agglutination responses, DNA fingerprinting, polymerase chain reaction (PCR), and morphological differences observed using electron microscope technology (Korman et al 1986, Uji et al 1988, Standen et al 1990, Adams 1991).

1.1.2 Life cycle and Multiplication

Giardia has two stages to its lifecycle: the infective cyst and the reproductive trophozoite. The cyst is 5-8µm in size and can be round to elliptical. The wall enclosing the cyst is 0.3µm thick, the outer structure consisting of filaments with the inner (membranous) structure having an inner and outer portion separated by a thin layer of cytoplasm (Shearer and Lapham 1988, Adams 1991).

Most cysts contain four nuclei although some which have not undergone nuclear division have only two. The inside of the cyst contains flagella, axonemes, vacuoles, ribosomes and fragments of the ventral disc.

The pear shaped trophozoites are 12-15µm long and 6-8µm wide dorsally convex and ventrally concave, with bilateral symmetry. The cytoskeleton consists of a funis, a median body, a concave surface (on the anterior two thirds of the ventral surface) known as a sucking disc and four pairs of flagella. The trophozoite survives in the small intestine of the host and is responsible for causing symptoms associated with Giardiasis (Feely et al 1984, Shearer and Lapham 1988, Adams 1991).

Giardia replicate by binary fission (a form of a-sexual reproduction). Cytokinesis occurs both in the cyst and

trophozoite, contrary to what was initially reported. Karyokinesis occurs initially with division of the locomotive apparatus followed by the adhesion disk and then the cytoplasm (Adams 1991).

The infective cyst has no form of propulsion unlike the trophozoite. Using the four sets of flagella the trophozoites swim with a tumbling or skipping movement. Glebski (1967) determined that the flagella did not beat in rhythm, instead oarlike movements of anterior and posterior lateral flagella combined with the dorsal and lateral flexion of the tail acting as a rudder, was thought to be responsible for forward momentum (Filice 1967).

1.1.3 Excystment and Encystment

The entire lifecycle of *Giardia intestinalis* has been duplicated *in vitro*. This involves methods of excystation, culturing the trophozoites and encystation (Meyer and Pope 1965, Meyer 1970, Bhatia and Warhurst 1981, Sauch 1988). Encystation of *G.muris* has been described, although successful axenic culture has yet to be reported (Adam 1991, Feely et al 1991).

Initially excystation was accomplished *in vivo*. Hegner (1927) having induced excystation by injecting cysts into rats, concluded that moisture, temperature and digestive juices, were required for the process to occur. It was not until the 1970's that the requirements were refined.

Bingham and Meyer (1970) were the first to excyst *G. intestinalis* cysts purified from faeces, *in vitro*. This was achieved by exposing the cysts to low pH, then transferring to a nutrient medium. Other researchers have used variations of acid induction, washing and incubation in a nutrient media, to improve rates of excystation (Meyer 1976, Bingham et al 1979, Rice and Schaefer 1981, Bhatia and Warhurst 1981, Schaefer et al 1984, Kasprzak and Majewska 1985, Feely 1986, Sauch 1988, Erlandsen et al 1990, Koudela 1990, Al-Tukhi et al 1991).

Meyer's studies then focussed on optimal time and temperature of storage of the cyst prior to excystation, as well as pH and temperature for optimal excystation. Other factors to improve excystation have been identified, including the use of bicarbonate and reducing agents (Gillin et al 1988).

Recent work by Feely et al (1991), has shown that excystation of *Giardia muris* can be induced by a phosphate-bicarbonate medium. The method avoided exposure to reducing agents, low pH and exogenous enzymes.

With work on excystation *in vitro* being accomplished, descriptions of the process have become available. Within 5-10 minutes of conditions conducive to excystation being obtained, flagella motion in the cyst begins. The cell wall then breaks and the posterior end of the trophozoite exits first. Cytokinesis begins within 30 minutes producing two trophozoites. The trophozoites attach to the substratum available and start to increase in number (Adams 1991).

Cyst detection in water is being implicated in outbreaks of the disease in New Zealand. The ability to ascertain whether the cyst is viable is important. The preferred method of assessing viability is by excystation although other methods such as fluorogenic dyes are being assessed (**introduction 1.5.4**).

Methods of inducing encystation vary, but most involve modifications to the bile content of the medium. *In vivo* encystation has been undertaken, but much interest has focused on *in vitro* encystation as a means of controlling and completing the lifecycle outside of the host (Schupp et al 1988a, Schupp et al 1988b, Faubert et al 1991).

In 1888, Perroncito described *in vivo* encystation followed by Lavier in 1942 who microscopically studied trophozoites and cysts from the intestines of rats and amphibians and stools of humans (reviewed in Adam 1991).

Schupp et al (1988a) monitored axenically grown cultures and recorded the presence of cysts. These were found to be morphologically similar to *Giardia* cysts formed *in vitro*. Viability was demonstrated by the uptake of fluorescent dyes and excystation.

Schupp et al (1988b) described a method which involved increasing the bile concentration in the media as a means of producing viable *Giardia* cysts *in vitro*. Morphology was evaluated using electron microscopy (with fluorescent dyes), animal infectivity and excystation to determine viability. The largest number of viable cysts were found to be produced at 5mg/ml bile concentration.

With the completion of the lifecycle *in vitro* more is understood about the process of encystation. Encystment-specific antigens are found in golgi apparatus in the first five hours of the onset of encystment. These are packed into encystment-specific vesicles which appear within approximately the first 18 hours of contact, and move to form the wall of the cyst. Low molecular mass antigens appear followed by high molecular mass antigens. Within 24-48 hours formation of the wall is complete. The nuclei then divide simultaneously resulting in a cyst with four nuclei (Schupp et al 1988, Adams 1991, Faubert et al 1991).

1.2 Giardiasis

1.2.1 *Giardia* as a Pathogen

With asymptomatic carriers as high as 76% of outbreak populations (Shearer and Lapham 1988), *Giardia* was recognised only in the 60's and 70's as a pathogenic organism. Statistics show it, in recent years, as being one of the most common disease causing parasites in the USA (Flanagan 1991).

Symptoms amongst patients vary dramatically. They range between acute, sub-acute to chronic (Meyer and Jarroll 1980,

Shearer and Lapham 1988, Adam 1991, Flanagan 1992).

Expression of the disease is variable, so that diagnosis on symptoms alone is not possible. Several methods exist for detection, each varying in success due to the intermittent expression of the disease (Shearer and Lapham 1988, Miller 1988, Adam 1991).

Persons more susceptible to infection include people having deficiencies of the immune system, protein energy malnutrition, insufficient stomach acid, or bacterial overgrowth of the small intestine. Factors inherent in the host are thought to be the cause of giardial infections. (Shearer and Lapham 1988, Adam 1991).

Hypogammaglobulinemia patients have an increased risk of severe Giardiasis, with many studies being based around their carriage of the disease as a means of studying the mechanism of infection. Selective IgA deficiency as a risk factor for Giardiasis remains controversial as it is common in the general population (10% prevalence) suggesting people with the deficiency handle infection normally (Flanagan 1992).

Ingestion of as few as 1-10 cysts is thought to cause infection with most researchers finding that ingestion of 100 cysts will cause an infection (Shearer and Lapham 1988). Symptoms usually begin 7-9 days following ingestion. Acute Giardiasis lasts 3-4 days with symptoms ranging in severity from loose stools to explosive diarrhoea with foul smelling stools, flatulence and abdominal bloating. Abdominal cramps (mid epigastric) nausea, loss of appetite, vomiting, chills, low grade fever, headaches, belching and weakness are commonly described symptoms which range in severity depending on the host susceptibility. Blood and mucus are only rarely found in stools (Shearer and Lapham 1988, Flanagan 1992).

Normally the acute phase resolves spontaneously and subacute or chronic stages follow. The sub-acute stage is characterised by intermittent attacks of diaherroa, flatulence, abdominal pain or a burning sensation, belching of

foul smelling gas, fatigue and malaise (Shearer and Lapham 1988, Flanagan 1992).

Chronic stages have periodic brief episodes of loose semi-solid, foul smelling stools with constipation occasionally between attacks, foul smelling flatus, abdominal distention, weight loss and intermittent fatigue (Shearer and Lapham 1988, Flanagan 1992).

There is intermittent expression of symptoms with some people only suffering the acute stage, and with few patients developing symptoms at all stages. Spontaneous recovery normally happens within six weeks, but some cases have been reported as lasting longer (Shearer and Lapham 1988, Flanagan 1992).

Giardiasis can interfere with the body's ability to digest and absorb nutrients such as fats, carbohydrates, vitamins A and B12 and folic acid. Malabsorption can be associated with milk intolerance, meat intolerance, anemia and fat passage with stools. Protein-energy malnutrition appears to predispose to giardial infections (Ament et al 1972, Shearer and Lapham 1988, Adam 1991).

Previous infection may induce a partial immunity to subsequent disease. Evidence comes from endemic areas where residents were less likely than tourists to show symptoms of giardiasis (Shearer and Lapham 1988).

Initially it was thought that the sheer number and physical presence of *Giardia* trophozoites in the jejunal epithelium may cause the malabsorption described. Superficial invasion of intestinal mucosal and epithelial cells is thought to occur in some humans. Bacterial overgrowth is also thought to cause diarrhoea but treatment of this did not relieve the symptoms. It was suggested that malabsorption could result from a direct toxic effect caused by the trophozoites. Animal models are being used to establish why malabsorption occurs (Hoskins et al 1967, Ament and Rubin 1972, Owen et al 1979, Miller 1988, Adams 1991).

1.2.2 Immunology

Healthy humans and animals spontaneously resolve *Giardial* infection. Individuals with immunodeficiency diseases show increased susceptibility to Giardiasis (Hoskin et al 1967, Ament and Rubi 1972, Shearer and Lapham 1988, Adams 1991). Repeated exposure to *Giardia* produces partial resistance to reinfection. These points suggest immunological processes are responsible for recovery (Heyworth 1988).

Information on the immunological response given has come from clinical and epidemiological studies, analysis of experimentally infected human volunteers *in vitro*, and studies involving trophozoites and immune cells from a variety of hosts animal models including *G.intestinalis* infected gerbils and of *G.muris* infected mice (Shearer and Lapham 1988, Gottstein et al 1990, Gottstein and Nash 1991, Adams 1991).

Both the humoral and cell mediated reactions play a part in the host response to *Giardia*. Studies on immunologically normal individuals show anti *Giardia* antibody including IgG trophozoite antibodies occurring in human sera and IgA anti trophozoite antibodies are found in human milk (Adams 1991, Shearer and Lapham 1988). Others have suggested that IgA is the most important component of the humoral response having been demonstrated to be present on the epithelial surface of human jejunal biopsy specimens. The role of the IgA antibody is still not clear(Adams 1991, Heyworth 1992).

G.muris infected mice have shown the antibody response to be dependant on intact cell-mediated immunity. Whether the response is T-cell dependant is unknown but investigations continue(Adams 1991, Heyworth 1992).

The involvement of macrophages in giardiasis is unknown. They may function as antigen presenting cells to phagocytes or may kill opsonised trophozoites. The macrophage and monocyte

reaction is thought to be reduced with a normal IgG, IgA and lymphocyte response (Miller 1988, Adams 1991).

Host protection has come from the intestinal mucos layer and intestinal motility. It is thought the organism protects itself by use of antigenic variation and IgA proteinase. No immune protection has been found in human breast milk. (Adams 1991, Shearer and Lapham 1988).

1.2.3 Diagnosis

With symptoms varing greatly, clinical identification is required for diagnosis of Giardia. Examination of stool samples is generally carried out with three separate samples taken over a period of days, due to the intermittent expression of the presence of cysts. Accuracy of this method ranges between 30-100%. Other methods available for diagnosis are the string test (90% accuracy) and small bowel biuopsy mucosal smear (60-100% accuracy) (Shearer and Lapham 1988). Stool examination is still favoured due to the less invasive nature of the examination and the cost.

It is not fully understood why many of the symptoms occur. Reasons such as bacterial overgrowth, invasion of intestinal mucosa and epithelial cells, shortening or flattening of the intestinal villia, exeterotoxins, have been suggested. The disease mechanism needs to be understood to explain why some cases are asymptomatic (Adams 1991, Hoskins et al 1967, Ament et al 1972, Meyer and Jarroll 1980, Miller 1988).

1.2.4 Treatment

There are four commonly used drugs in the treatment of giardiasis: metronidazole, tinidazole, furazolidone and quinacrine. All were developed for use against other diseases and found empirically to be also active against *Giardia*. Treatment is normally dependant upon the personal preference of the prescribing physician, drug availablity and assessment of possible side effects. (Jay 1991, Boreham et al 1988).

Metronidazole and tinidazole are both nitroimidazoles and may cause nausea, gastrointestinal upsets, lassitude, skin rashes, drowsiness, disulfiram-like reactions with alcohol, and occasionally transient leucopenia and peripgeral neuropathy. They are carcinogenic in rodents and mutagenic in bacteria. Metronidazole has a cure rate of 46-95% and tinidazole 88-100%. Two other nitroimidazoles that are being evaluated for use are ornidazole and satranidazole (Smith et al 1981, Meyer et al 1980 and Boreham et al 1988).

Furazolidone is considered the drug of choice by many physicians, especially in young children. Side effects include headaches, nausea, vomiting, skin rashes, diarrhea and malaise. It can cause agranulocytosis and hemolytic anemia. It has a cure rate of 58-92% (Meyer and Jarroll 1980, Boreham et al 1988).

Quinacrine is the drug of choice in North America. It is an antimalarial compound and may cause dizziness, headaches and gastrointestinal disturbances. Toxic psychoses has been reported in 1-2% of patients with exfoliative dermatitis and aplastic anemia also occurs. The cure rate is 60-100%. (Jay 1991, Boreham et al 1988, Smith et al 1981, Meyer et al 1980)

Paramycin sulfate has been recommended for treatment of pregnant woman because it is not absorbed from the gut. Controlled trials have not been done on this drug and some physicians are cautious about prescribing it.

Other drugs which have been used in treatment are amodiaquine, berine sulfate, sulfasalazine and erythromycin, none of which have been exposed to controlled clinical trials. (Boreham et al 1988, Meyer et al 1980).

1.2.5 Epidemiology

Giardia has been reported in most countries, both developed and underdeveloped. It is considered the most commonly

isolated intestinal parasite worldwide (Shearer and Lapham 1988). Prevalence rates are between <1-50%, with higher prevalence in underdeveloped countries than developed countries, and higher incidence in urban than in rural areas(Flanagan 1992).

Prevalence rates of 8-40% have been reported in South America, the Caribbean, the Middle East and South East Asia. Australia, New Zealand and North America have rates between 2-7%. Even in countries with low prevalence pockets of high and low levels of infection occur (Flanagan 1992, Harley 1988).

High levels of endemic and epidemic *Giardia* infection in the Rocky Mountain States of North America is thought to be caused by contaminated drinking water (Flanagan 1992).

Serovalence studies demonstrated that 40% of Peruvian children by six months of age will have had a *Giardia* infection. Bangladesh and Zimbabwe children show prevalence rates of 80%. In developed countries the highest rates of infection are of children in childcare, leading to secondary infection of families and the community(Shearer and Lapham 1988, Harley 1988).

Suggestions have been made that *Giardia* is not worth treating, mainly due to the high rate of reinfection. Arguments against this have been based on the detrimental effects of malabsorption and the gain in body weight when the individual is treated for *Giardia* (Flanagan 1992).

At-risk groups are discussed in the following person to person modes of transmission: they are children, travellers to endemic areas, immunocompromised patients, homosexuals, people in institutions or homes, and people having undergone gastric surgery (Meyer 1970).

Work on the age and sex of infected individuals has identified two age groups in which infection was most common; 1-4 year olds and 20-39 year olds. Children are seen as an at risk

group due to poor personal hygiene and contact with other children. The 20-39 year olds all seem to have been travel-related infections. Males tended to out number females in the 1-4 year old age group but the reverse was true in the 20-39 year old age group (Flanagan 1992).

1.2.6 Genetics

Study of the genome of *Giardia* has proved to be difficult. The number of chromosomes is estimated at four by the use of light microscopy and fluorescent DAPI-stained nuclei. Pulse field separations have suggested at least five distinct sets of chromosomes with a number of minor bands showing similarity to major bands of varying sizes (Upcroft et al 1988, Adams 1991).

Estimations of chromosome size are between 1×10^6 and 4×10^6 bp, a total of 1.2×10^7 bp for the 5 chromosomes. Investigators have suggested the haploid genome size was 1.1×10^7 bp by densitometric scanning (Upcroft et al 1988, Adams 1991).

It is thought that the remarkable genetic diversity amongst *Giardia* isolates is due to clonal divergence as *Giardia* has no sexual stage in its lifecycle to account for change (Adams 1991).

1.3 Cultivation

1.3.1 Axenic Growth

Cultivation of *Giardia intestinalis* was attempted as early as 1927 using physiological saline solutions, but with limited success. The culture survived up to 19 days, in the simple media (Chatterjee 1927, Penso 1929).

Karapetjan (1963) maintained cultures of *G. intestinalis* symbiotically with *Candida guilliermondii* and chick

fibroblasts for 7 months. Even after the chick fibroblasts had died the *Giardia* culture could still be maintained. In 1962 Karapetjan cultured a rabbit isolate *G. intestinalis* in the presence of the yeast *Saccharomyces cerevisia*. This time no chick fibroblasts were used. Daily replacement of the complex media was required but no more yeast was added. Attempts by Karapetyan to axenise *G. intestinalis* were unsuccessful.

Meyer and Pope (1965) modified the method of Karapetyan to include the daily addition of fresh living yeast to successfully culture rabbit and chinchilla isolates.

Meyer (1976) was the first to axenically culture *G. intestinalis*. Cultures were established in a HSP-2 medium with intestinal fungi present. Using a U tube system and diluting out the yeast, axenic conditions were imposed. The HSP-2 medium contained human serum. Batch variation of the serum gave inconsistent results.

Visvesvara (1980) modified Diamond's TSP-1 *Entamoeba histolytica* medium to successfully support *G. intestinalis* cultures.

Diamond *et al* (1978) developed an alternative TY1-S medium (HSP-1) which again showed inconsistencies in its ability to support growth and depended on trypticase and yeast extract.

Farthing *et al* (1983) found that with the addition of bile, the generation time of *Giardia* cultivated in TYI-S dropped to 7.5 hours compared to Diamond's TYI-S culture medium which had a mean generation time of 12 ± 1.1 hours.

Attempts to cultivate other species of *Giardia*, continue. *G. muris* has now been successfully excysted but has not yet been cultivated axenically (Feely 1991).

1.3.2 Cryopreservation

Phillips *et al* (1982) described a method for the storage of *G. intestinalis* at -80°C , using dimethyl sulphoxide (DMSO). A slow rate of freezing was required (this was obtained by wrapping the vials in polystyrene) so that the destructive effects of quick freezing did not affect the viability of the trophozoites.

A modified method of Phillips *et al* (1982) and Wallis & wallis (1980) (described in Tonks (1991) was used to revive the cultures using a quick thaw process to give optimal recovery.

Papers on the thawing and freezing cycles of cysts suggest that the cysts are comparatively resistant to temperature when not stored in optimal conditions (Erlandsen *et al* 1991).

1.4 Modes of Transmission

Giardia infections are transmitted via the fecal-oral route. Three modes of transmission are normally described; person to person, food-borne and water-borne. Since the first major documented outbreak of Giardiasis in Aspen, Colorado (Moore *et al* 1969), water-borne transmission has most frequently been associated with outbreaks. However recent studies have indicated that other modes of transmission are as frequent (Harley 1988, Adams 1991, Flanagan 1992).

1.4.1 Person to Person Transmission

Person to person contact is thought to play a major part in many outbreaks. Discussed by Harley (1988), the three most obvious risk factors in person to person transmission are population density, hygiene conditions and the proportion of susceptible individuals.

Childcare centres and families with young children are in the high risk group, because of young children's lack of personal hygiene combined with the close physical contact that occurs. In the USA, there have been frequent outbreaks since the first documented daycare centre outbreak in 1977 (Black et al 1977, Bartlett et al 1991, Flanagan 1992). In England and Wales, of the 14 outbreaks which occurred between 1985 and 1989 six occurred in daycare centres (Flanagan 1992).

Studies in Alberta have shown that in 1984 one third of the cases of Giardiasis occurred in under five year olds with 31% between the ages of one and four (Harley 1988).

Secondary spread to family members has been demonstrated. Polis (1986) suggested that 47% of children in his study group transmitted the disease to at least one household contact. Flanagan (1992) discussed the occurrence of secondary spread from children at daycare to family members occurring from 17%-47%.

Evidence exists that between adults with good hygiene practice, the organism does not spread readily. People living in institutions, homes and colonies have been infected for similar reasons as people involved in childcare centres (Harley 1988, Flannigan 1992).

Studies have also suggested *Giardia* infections may be transmitted by sexual activity, particularly among male homosexuals (Meyer and Jarroll 1980).

1.4.2 Animal Transmission

The role of animals in transmission of *Giardia* is still vigorously debated (Erlandsen and Bemrick 1988). There is considerable evidence that the strains infecting humans are also the strains which infect animals (Shearer and Lapham 1988, Lewis 1988, Roach and Wallis 1988, Gasser 1990).

Beavers in North America have been implicated in the transmission of *Giardia* to humans. Several reports suggest that above the beaver line, *Giardia* is undetected, whilst below the beaver line *Giardia* cysts are found (Meyer and Jarroll 1980, Shearer and Lapham 1988, Roach and Wallis 1988, Erlandsen 1988, Erlandsen and Bemrick 1988, McFetter 1989, Erlandsen 1990, Adam 1991).

Using new methods of examining DNA, it is now possible to determine if the strains taken from animals and humans are the same (Standen et al 1990, Wallis and Wenmen 1988, Mahbubani et al 1991).

1.4.3 Food-borne Transmission

Suspected and confirmed cases of *Giardia* occurring after the consumption of contaminated food have been documented. Contaminated water used for cleaning fruits and vegetables, and unsanitary carriers of prepared food, have also been linked to outbreaks (Harley 1988, Jay 1991).

Lopez et al (1978) documented an outbreak on the island of Modestia where 1,400 Americans contracted *Giardia*, Tapwater, icecream, and raw vegetables were seen as the most likely sources of infection.

The first well documented food-borne and communal source outbreak was in a rural Minnesota community in December 1979, where 29 of 60 school employees contracted giardiasis from home-canned salmon prepared by a worker following the changing of the nappy of an asymptomatic infant (Osterholm et al 1981).

Another well documented case, occurring in July 1985, was that of a Connecticut picnic where 13 of 16 individuals met the case definition of giardiasis; with the contamination of noodle salad being implicated. The salad preparer became ill the following day with most of the other victims developing symptoms after 6-20 days (Petersea et al 1988).

Harley (1988) studied an outbreak of *Giardia* at an Alberta Hutterite colony where the laundry and kitchen shared the same area. Hand-washing done in a food preparation sink and inadequate laundry facilities contributed to the spread of infection.

1.4.4 Water-borne Transmission

The first major recorded waterborne outbreak of *Giardia* occurred in Aspen, Colorado in the 1965/6 ski season (Moore et al 1969). Retrospective studies indicated that sewage contamination of the wells and creek supplying the ski area were the cause (Juraneck 1988). Over the following 20 years there have been 90 reported waterborne outbreaks in the US involving a total of 23,776 cases of giardiasis (Flanagan 1992).

In 1974-5 an outbreak of *Giardia* occurred in Rome, New York. During this outbreak, investigators from the Centre for Disease Control detected cysts in the water. This was the first time *Giardia* cysts had actually been detected in water connected to an outbreak of Giardiasis (Juraneck 1988).

In Cannas, Washington (U.S.A.), the outbreak in 1976 also involved cysts detected in the water, but discovery involved using a filtration method developed by the Environmental Protection Agency (EPA).

Waterborne outbreaks have not been confined to the USA alone. The first recognised waterborne outbreak in the United Kingdom occurred in Bristol in 1985. Over 100 cases were identified with contamination of water mains downstream from a supply reservoir being implicated (Flanagan 1992).

In many outbreaks the origin of the cysts unclear. Frequently, infected animals such as muskrats, beaver, rats and other animals have been trapped in the watersheds, but it

is not known if these are the primary source of contamination. Investigators have implicated inadequate water treatment and sewage contamination of water as the main problems (Shearer and Lapham 1988).

Treatment with chlorine is not always adequate as the pH, turbidity, temperature, contact time, and dose has to be strictly controlled. However filtration has been found very effective.

Recreational waters, especially swimming pools, have been found to cause infection. Often pool users have been implicated as opposed to contaminated water fed into the pools (Flanigan 1992).

1.5 Water-borne *Giardia*

1.5.1 Standard Method of Sampling

There are several steps involved in the detection of *Giardia* in water. The sample is first collected, usually by filtration. The cysts then have to be collected from the filter by rinsing. Rinse water is then concentrated by centrifugation. The cysts are separated from other debris by a concentration step. As yet there is no culturing technique which has been used with success, so cysts are enumerated under the microscope (Jakubowski 1990, Standard methods).

Over time, a standard method has evolved. In the Rome, New York 1974-5 outbreak, a swimming pool sand filter technique was used by the Centre for Communicable Disease (U.S.A.). The sand filter was backwashed and particulate matter coagulated and allowed to settle. The sediment was then tested for *Giardia* cysts (Shearer and Lapham 1988).

The Environmental Protection Agency (EPA in the U.S.A.) developed a large volume sampling technique based on a virus sampling apparatus. The method (used in the Vermont outbreak,

in 1976) entailed using a 10um nominal porosity, yarn wound Orlon filter. One cyst was detected (Flannigan 1992).

Standard Methods (16th Edition) evolved (from these earlier attempts) the method now used for detection of *Giardia*. The EPA(U.S.A.) now recommends using a 1 um yarn wound filter is used with a minimal sample size of 380 L.

Initially this was used as a presence/absence method. Jakubowski (1990) has since refined it into a quantitative method. Others have since measured for cyst recovery efficiency, and have been able to estimate the number of cysts present in waterways, sewage and effluent.

There are seasonal variations in the number of cysts detected in waterways. Also the cysts are not evenly distributed in the waterways, with the rate of flow and time of day playing a major role in detection. Animals associated with the area may also influence the number of cysts present, although there is much discussion on the zoonotic aspect of Giardiasis (Gasser 1990).

Basic procedures of sampling and filtration are described by the Standard Method but there is much variation in interpretation between different laboratories. Large differences in results are often seen, and this makes comparison between laboratories, difficult (Jakubowski 1990).

Following sampling the collection cartridges have to be transferred to a lab. Hibler (1987) suggested methods for doing this. The cartridge is placed in two zip lock plastic bags and the correct information written on the outside bag. Information such as name, contact phone number and address, date and time of sampling, volume sampled, water source and type are required. Other information such as turbidity, temperature, pH, chlorination or other treatments, are also considered useful.

Extraction of the material from the cartridge is done by hand. Simple unwinding of the filter is possible although most

technician cut longitudinally through the filter, removing the plastic core. The filter fibres are washed to remove cysts (Jakubowski 1990).

The greatest loss of cysts occurs at this point. Although different solutions are employed (eg, eluting solution) many cysts are caught in fibres are lost through washing.

Other methods have been used for *Giardia* cyst detection, mainly for the evaluation of treatment efficiency. Investigators usually used membrane filters because cyst recovery efficiency can be defined with greater precision (Regli et al 1988).

1.5.2 Concentration and Identification

The fibre wash-water is concentrated by centrifugation. Although other methods, such as tangential filtration, have been trialled, purification techniques are used to isolate cysts and remove contaminating material (Issac-Renton et al 1988).

Zinc sulphate is suggested by Standard Methods for purification. Other methods use sucrose, Peco II, sucrose-Percoll, Sheather's solution and potassium citrate. Rose et al (1988) evaluated all methods except Percoll and sucrose. All were between 66-77% efficient in recovery, except Zinc sulphate which had a recovery of 40%. Cysts separate out between the specific gravity of 1.05-1.10. A variety of other micro-organisms and debris also separate out at this point.

Lugol's iodine is routinely used to stain cysts. They are then viewed microscopically with identification being performed at 400 x magnification. A positive observation is based on objects having the correct size and shape as well as two internal morphological features typical of *Giardia* cysts. This method requires a lot of time and acute concentration. Unless the technician is experienced cysts can go undetected (Jakubowski 1990).

Flourescent antibody stains are now available. Initially they were developed by Riggs *et al* (1983) for stool specimens. They are invaluable in water detection work, due to the low number of cysts often present and the presence of other aquatic organisms crustacerans, algae, etc. It is possible for the cyst to be obscured, or to blend into the background. With antibody staining they are very pronounced. Hibler 1988 found that as the turbidity of the water sample increased, the recovery of cysts declined. The fluorescent antibody method saves time and makes it easier to locate cysts amongst the debris.

Riggs *et al* (1983) initiated studies to determine if it was possible to obtain a monoclonal antibody specific to human strains of *Giardia*. One monoclonal which was produced, when tested against 647 animal samples, only reacted with four of them.

1.5.3 Sensitivity of the Standard Method

Riggs *et al* (1988) experimented with the sensitivity of the Standard Method using spiked samples. Human cysts were collected from feces and purified. When 0.7 to 1.0 cysts/L were used, recovery was 27-41%. When 0.1-0.2 cysts/L, were used, recovery was 27% to 69%.

Testing of environmental samples has detected 0.7 cysts to 198 cysts/L in raw sewage (Arizona), 0-2.6 cysts in chloronated secondary effluent. There are reports of 1.2cysts/L in a river with domestic effluent discharge and cattle pasture run off, 0.35 cysts/L in a lake with domestic effluent discharge and 0.009 cysts/L in a river-port of a protected water shed (Shearer and Lapham 1988).

These reports give very little indication of the sensitivity of the method. Most users in New Zealand want an absence/presence test.

1.5.4 Viability of Cysts

When cysts are detected in waterways, very little can be determined about them. The age and conditions of storage, animal or human isolate, and the viability of the cyst, are all important but as yet cannot be identified from the cyst. The main methods developed to determine viability so far are excystation and animal infectivity (Schupp et al 1988, Feely 1984).

Description and methods of excystation have been successful when examining cysts from fecal matter but as yet there have been no reports of successful excystation of cysts isolated from water (Flannigan 1992).

The main methods investigated to determine viability are dye exclusion, fluoregenic dyes, excystation and animal infectivity (Schupp and Erlandsen 1987, deRegnier et al 1989, Schaefer 1988, Sauch et al 1991, Hudson et al 1988, Labatiuk et al 1991, Schupp et al 1988b, Smith and Smith 1989).

Without knowing the origin of isolated cysts, the problematic replacement into another host will yield unreliable information on viability, due to the suspected specificity of the organism (Schupp and Erlandsen 1987, Schaefer 1988, Labatiuk et al 1991, Adams 1991).

Different methods now exist for *in vitro* excystation. One of the main advantages of this method is that it is more easily quantifiable. Bingham and Meyer (1976) were the first investigators to be successful in this method using low pH incubation followed by a wash step, then incubation to induce excystation. Schaefer et al 1984, Sauch 1988, and Feely 1984, have all based methods on these three steps.

Disadvantages with excystation methods are the requirement of a large amount of cysts and the 2-3 hours required to complete

excystation. Excystation methods for *G.muris* tend to be reliable and are reproductive, but *G.intestinalis* trials have not yielded reliable, reproductive results (Schaefer 1988).

Research has been done into the use of dyes for assessing viability. Eosin dye exclusion has been found to correlate well with excystment. Fluorogenic dyes such as FluoraBora I (3-(dansylamido)-phenylboronic acid) showed good correlation in *G.muris* excystation but for *G.intestinalis* correlation was low (Schaefer 1988).

Work with fluorescein diacetate and propidium iodide by Schupp and Erlandsen (1987) has shown promise, with good correlation when compared to excystation and animal infectivity. However, some variability has been noted with the fluorescence of fluorescein diacetate. It is to be remembered these dyes are not *Giardia* specific, thereby any other macromolecules will also stain leading to possible confusion (Schaefer 1988, Schupp et al 1988, Hudson et al 1988).

Differential interference contrast microscopy, is another method being used to differentiate living from dead cells, however this is an expensive method (Schaefer 1988).

1.5.5 Water Treatment and Sanitation

There are two ways of removing the threat of illness by *Giardia* contamination from waterways by removal of the cysts using effective filtration, or by making the cyst nonviable through disinfection.

Craun (1988) reported that the 1970 cases of *Giardia*, between 1965-1984, were due to deficiencies in the treatment of surface water sources. Effective methods of disinfecting or removing the *Giardia* cysts are therefore required.

Logsdon's (1988) paper compares filtration processes appropriate for cyst removal. Hoff (1986) also discusses

relevant filtration methods in depth. Other papers which discuss filtration methods are Regli et al (1988), van Roodselaar (1988), Gertig et al (1988), Pennell et al (1988), Hibler et al (1989), and Sauch et al (1991).

The main filtration methods which have been evaluated are slow sand filtration, diatomaceous earth (DE) filtration, and coagulase filtration. This includes conventional filtration and in-line filtration. The merits of each are still being debated (Regli et al 1988, van Roodselaar 1988, Sauch et al 1991, Gertig et al 1988, Pennell et al 1988, Hibler et al 1989, Logsdon 1988, and Hoff 1988).

In North America the EPA proposed regulatory changes which would involve the removal/inactivation of *Giardia* cysts by 99.9% in surface water systems (Regli et al 1988, Van Roodselaar 1988).

Methods of disinfection involve chlorine principally, the use and strength of which has been debated in its ability to inactivate *Giardia*. Chemical coagulants are also used prior to some filtration processes (Gertig et al 1988, Hoff 1988).

Combinations of chemical treatment and filtration (ie multiple barrier systems) are the most effective means of removing cysts. The best treatment depends heavily on the type of water, the flow, temperature variations, and chemical treatments (Miller 1988, Regli et al 1988, Hoff 1988, Hibler et al 1989).

1.6 Giardiasis in New Zealand

Giardiasis cases have been identified in New Zealand since the 1940's (Walker et al 1991), but there is little information on the modes of transmission of the disease. The first waterways found to be positive in New Zealand were reported in April 1990, with the Kakanui water supply system (a South Canterbury Town) being implicated. With water testing now occurring

throughout the country other waterways have been found to be positive for *Giardia* cysts (Brown pers. comm.) With a high percentage of New Zealanders using and drinking untreated water, and with high number of septic tanks and private outfall systems in New Zealand, potential for outbreaks of *Giardia* is also high.

Data from the Communicable Disease Centre of New Zealand (CDCNZ) suggests there are approximately 2000 cases annually but this number is probably an underestimate, with a number of cases being asymptomatic (Walker et al 1991).

The testing of animals as possible carriers of *Giardia*, and able to infect humans, has been undertaken. Results show that animals such as dogs, cats, (Tonks et al 1991) sheep, cattle, birds, pigs and feral rodents (Marino et al 1992, Marino pers. comm.) have had positive *Giardia* contaminated fecal specimens, but whether these are capable of causing human disease is unknown. Reports by other researchers would suggest animals are capable of being reservoirs of human disease, but reliable methods of distinguishing human from animal infecting strains are not yet available (Georgi et al 1986).

The study by Okell et al (1990) of an Eastern Bay of Plenty town over a three year period concluded that person to person transfer occurs in young children, and that parenting adults were the main mode of transmission. This is consistent with American results (Harley 1988).

The purpose of this study was to investigate the modes of transmission that may have affected its spread, and to investigate the environment in which it occurs.

MATERIALS

2.1 Strains of axenic cultures

The axenic cultures used in this thesis were;

1. Bris/83/HEPU/106
2. Hast/87/MUGU/68

For information on the nomenclature of strains see Boreham et al 1984.

2.2 Culture Media

2.2.1 TY1-S-33

The most commonly used media by researchers is modified TY1-S-33 (Diamond et al 1978, Diamond 1968). This was the media used in this study for culturing the strains examined and with further modifications, as subsequently noted for encystation and excystation procedures. The media listed here are as taken from Tonks (1988) thesis.

Trypticase Soy broth (BBL No.11768 or Oxoid No.CM129)	20.0g
Yeast extract(Difco 0127-01)	10.0g
Glucose	10.0g
NaCl	2.0g
K_2HPO_4	1.0g
KH_2PO_4	0.6g
L-Cysteine Monohydrochloride (Sigma 7880)	1.5g
Ferric Ammonium Citrate (Brown Pearls)	0.023g
L-ascorbic acid	0.2g
NCTC 135 or NCTC 109 ^a	100.0ml
Bile bacteriological	0.8g
Benzyl penicillin	0.06g
Gentamycin sulphate	0.05g
Vancomycin	0.02g
Bovine serum ^b	100.0ml
R.O. water to	1000ml

The above components were mixed with 500ml of R.O. water, using a magnetic stirrer. When dissolved the volume was adjusted to make 1L using R.O. water. Following mixing, the pH was adjusted to 7.1 +/- 0.1 using $1 \text{ mol}^{-1} \text{ NaOH}$.

The medium was sterilized by positive pressure filtration through a 0.2um pore membrane filter with a 0.45um pore membrane prefilter (the filtration unit was first sterilized by autoclaving it at 121°C for 15 minutes at 15 psi). The resulting medium was stored for a maximum of 10 days at 4°C .

Some of the components differed in their ability to support growth, and where this occurred the source and/or treatment of the component is listed.

NCTC media and bovine serum had some preparation prior to use, They were prepared as follows.

a. Preparation of NCTC

NCTC 135 or NCTC 109

9.7g

R.O. water was added and made up to 1L

This was combined into a medium prior to membrane filtration.

b. Bovine serum

Bovine serum was obtained by centrifuging and purifying blood obtained from the Waitaki freezing works, Fielding.

2.2.2 Bile modified TY1-S-33

TY1-S-33 used for normal culturing procedures contained 0.8g/l bacteriological bile, which was added prior to filter sterilization. Higher levels of bile were required in the encystation work (Schupp et al 1988b), so a TY1-S-33 medium was

prepared which contained no bile. This was sterilized by filtration.

A solution containing 30g/50ml bile bacteriological to R.O. water was mixed and autoclaved, this was added to the medium before use as required.

2.2.3 Antibiotic control of overgrowth

During excystation, bacterial contamination sometimes occurred where this had not been already controlled by the antibiotics already used. In the modified TY1-S-33 media (Feely 1988), the following were added:

Tetracycline	10ug/ml
Streptomycin sulphate	50ug/ml
Chloramphenicol	50ug/ml

For fungal and yeast contamination control the following was added;

Amphotericin B	10ug/ml
----------------	---------

2.2.4 Hanks Balanced Salt Solution (HBSS)

This media (Feely 1988), was used for excystation and pH adjusted as required. It was made of two stock solutions:

Stock solution A:

I) NaCl	16.0g
KCl	0.8g
MgSO ₂ .7H ₂ O	0.2g
MgCl ₂ .6H ₂ O	0.2g
R.O. water to	80 mls.

II) CaCl_2 0.28g
 R.O. water to 10mls.

Solutions I and II were combined slowly. R.O. water was added to bring the final volume of stock solution A to 100mls. It was stored at 4°C with 2mls of chloroform added.

Stock solution B:

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	0.304g
KH_2PO_4	0.12g
Glucose	2.0g
R.O. water to	80ml

10mls 0.4% phenol red in NaOH was added to the solution. R.O. water was added to adjust the solution to 100mls. This was stored at 4°C with 0.2mls of chloroform added.

Working strength HBSS.

Solution A	100ml
Solution B	100ml
R.O. water made to	1000ml

Sterilization was by autoclaving at 121°C for 15 minutes at 15psi.

2.3 Giardia Cyst Origin

2.3.1 Human cysts

These were provided from the Palmerston North Medical Laboratory, in human fecal samples which had tested positive for *Giardia*.

2.3.2 Animal cysts

These were obtained from the Massey University *Giardia* Unit (MUGU) from research into animal carriers and these were in fecal samples which had tested positive for *Giardia*.

2.3.3 Cysts produced *in vitro*

These were obtained by the method in 3.1.2. (Schupp et al 1988b)

All cysts were purified on a sucrose gradient (Methods 3.3.2) and stored in sterile R.O. water or fixed in 10% formalin when required.

2.4 Water testing equipment

The following filtration apparatus and solutions were used for filtration and cyst recovery work.

2.4.1 Pump and Filter Unit

For sampling of water a rechargeable battery ran a pump which pushed water through the cartridge housing containing a 1um nominal porosity filter cartridge. The amount of water put through the cartridge was measured by a water meter. The following are the specific equipment used (Standard Methods, Jakubowski 1990).

The battery was a Portalac^R Rechargeable sealed lead acid battery, Model number PE 12 volt 15 Ah.

The pump was a submersible marine pump.

The water meter was a Kent^R PSM water meter model 15mm PN16

The cartridge housing used was Aqua pure^R APIIT housing from Cuno, Pacific Pty Limited

The cartridge was a Micro-wynd II^R 52 DPPP Polycarbonate/cotton filter wound 1 micron nominal porosity filter.

The hosing used was Nylex^R 2 x 3 metre lengths of 20 mm diameter

The clips to secure hosing were Gardina^R snap on clips

2.4.2 Eluting solution

The following stock solutions were combined to make an eluting solution for washing the filter fibers to remove any cysts (Jakubowski 1990).

I) Phosphate Buffered Saline 10x Stock Solution

NaCl	80.0g
Potassium dihydrogen phosphaphate	2.0g
Hydrated disodium hydrogen phosphate	29.0g
Potassium chloride	2.0g
R.O. water to 1000ml	

This solution was diluted 1/10 with R.O. water and pH adjusted to 7.4 before it was used.

Sucrose 34.2g
Milli Q water to 100ml

2.4.5 Mercury Lamp Microscope

The microscope used for illuminating the monoclonal antibody stains was a Reichert-Jung^R Microstar 110 with a filter of Mercury Vapour.

2.4.6 Manifold and Filters

The manifold used for the staining of samples on filters was a Red-Evac^R Model No PV100 obtained from Hoefer Scientific Instruments (Jakubowski 1990).

2.4.7 Centrifuges

The centrifuge used for concentration of wash water from the filters was a Sorval RC-3 centrifuge with a HG-4L rotor.

2.5 Tangential Units

Unit dimensions: the following tangential filtration units, pumps and membranes were used in methods 3.4.

1) Millipore Pellican System :

using a polyvinylidene difluoride membrane 0.1m^2 , pore size $0.45\mu\text{m}$, with a pump that was a Master Flex high capacity 13l min^{-1} .

- 2) Filtron Minisette Ultrafiltration system:
using a polyethersulfanemembrane 0.07m^2 , Pore size $0.8\mu\text{m}$, with a pump that was a Master Flex high capacity 131 min^{-1} .
- 3) Sartocoon Mini Cross Flow System:
using a cellulose acetate membrane 0.1m^2 , pore size $0.2\mu\text{m}$ with a pump that was an Air diaphragm PTFE 4-7, 5001 min^{-1} .
- 4) Sartocoon II:
using a polyolefin membrane 0.6m^2 pore size $0.2\mu\text{m}$ with a pump that was an Air diaphragm PTFE 4-7, 50016 min^{-1} .

2.6 Staining Materials

2.6.1 Types of stain

The following stains were used for staining and identifying cysts methods 3.5.2., 'a', 'b' and 'c' are all commercially available monoclonal antibody stains, d is a polyclonal antibody stain 'e' is Lugols Iodine.

- a) MerifluorTM *Giardia* - Meridian Diagnostics Inc
Indirect Immunofluorescent Detection Procedure
Catalog # 230025
- b) HydrofluorTM Combo - Meridian Diagnostics Inc
Indirect Immunofluorescent Detection Procedure for *Giardia* cysts and *Cryptosporidium* oocysts in Environmental samples
Catalog # 240025
- c) *Giardia* - Cell IF test - Cellabs - Direct Immunofluorescent detection procedure for *Giardia* cysts.
- d) Polyclonal antibody - produced by J. Chan and Dr J. Clarke Massey University Chan Thesis (1991).

e) Lugols Iodine

Potassium Iodide 10g

Iodine crystals 5g

Dissolve Potassium iodide in 100mls distilled water add 5g of iodine crystals slowly. Filter and store in brown bottle for no longer than one month.

2.6.2 Chemicals involved with staining

Chemicals were studied to determine if they inhibited the antibody stain from staining the cyst (methods 3.5.3). The following chemicals were assessed,

75% Ethanol

3% Hypochlorite

4% hypochlorite

5% Hypochlorite

5M NaOH

5M HCl

All the chemicals were made at double concentration so when the cyst solution was added it brought them to the quoted concentration.

METHODS

3.1 *In Vitro* Encystment of *Giardia intestinalis* Trophozoites.

3.1.1 The effect of bile concentration, time and temperature on encystment induced *in vitro*.

The strains, Bris/83/HEPU/106 and Hast/87/MUGU/68 were used to determine the effect of bile concentration, time and temperature on encystment *in vitro* (Schupp et al 1988b).

- (i) The strains were grown to log phase in glass 18 ml Kimax tubes using modified TY1-S-33 medium. The presence of a complete monolayer of growth was assessed using a stereoscopic microscope.
- (ii) Bile modified TYI-S-33 media, with differing bile concentrations (0.08, 4, 5, 6, 10, 15, 20 g/l) was added to the cultures. Medium without bile, and medium containing the normal level of bile (0.8 g/l), were used as controls.
- (iii) Duplicates at each concentration were placed at differing temperatures (4, 26, 37°C) for differing time periods (24, 48, 72, 96 hours). At the end of each time-period a duplicate set of cultures at each bile concentration and temperature were removed.
- (iv) The cultures were each centrifuged at 1000 x g for 10 minutes with washings in two changes of sterile R.O. water. Cultures were placed at 4°C overnight, to allow for remaining trophozoites to be destroyed.

- (v) The tubes were mixed in a vortex mixer for 5 minutes and drops mounted on a haemocytometer grid.
- (iv) Identification and the number of cysts present were determined using the haemocytometer grid with phase contrast microscopy.

3.1.2 In Vitro Produced Cysts Used for Experiments.

Due to the unavailability of large numbers of cysts from water sources, the Schupp *et al* (1988b) method of producing cysts *in vitro* was used.

- (i) Strains of Bris/83/HEPU/106 and Hast/87/MUGU/68 were cultured to log phase in 18 ml Kimax tubes using TYI-S-33 medium.
- (ii) Once log phase was reached the medium was replaced by TYI-S-33 with a bile concentration of 5.5 g/l. (This had been found to be the optimum bile concentration from 3.1.1 *in vitro* encystment of *Giardia intestinalis* trophozoites based on Schupp *et al*s 1988b work).
- (iii) The same protocol was followed as in 3.1.1 (steps (iv)-(vi)) for the concentration and identification of cysts.

3.2 Excystment of *Giardia Intestinalis* Cysts

3.2.1 *In vitro* excystment.

The following was based on the Feely (1986) method of excystment which was: exposure to low pH, wash, and incubation (Tonks 1991).

- (i) *In vitro* produced cysts, obtained by using the protocol described in 3.1.2, were enumerated using a haemocytometer grid and phase contrast microscopy. They were diluted in sterile water to the appropriate concentrations.
- (ii) HBSS medium, adjusted to pH 2 using HCl, was placed in an 18 ml Kimax tube containing 1 ml of the cyst concentration. The tube was filled to the top. It was incubated on its side for 20 minutes at 37°C.
- (iii) Following incubation, the tube was spun at 600 x g for 2 minutes, and the supernatant discarded. The cysts were washed twice in TY1-S-33 media before being incubated at 37°C in TY1-S-33 media.
- (iv) The cultures were checked daily for trophozoites with media being replaced every 3 days.
- (v) Trophozoite presence was checked using a stereoscopic microscope.

3.2.2 *In Vitro* Excystation from Cysts of Water & Animal Origin

A similar method of excystation was used as in 3.2.1 but the cysts first had to be purified (Feely 1986, Tonks 1991).

- (i) Cysts were obtained as described in materials 2.3.1 and 2.3.2
- (ii) These cysts were all purified using a sucrose gradient. 1 ml of sample containing cysts was increased to 30 ml using eluting solution. This was mixed thoroughly for 5 minutes using a vortex mixer.
- (iii) The sample was then underlaid with 15 ml 1M sucrose solution taking care not to disturb the interface.
- (iv) Centrifugation at 700 x g for 10 minutes followed, with the spin being taken slowly up to speed and no brake used when slowing.
- (v) The supernatant was carefully removed to within 10 ml of the interface and discarded.
- (vi) The interface was removed and placed with eluting solution to 50 ml. To reform the pellet, this was centrifuged at 1000 x g for 10 minutes.
- (vii) The supernatant fluid was removed to within 5 mls of the pellet and discarded.
- (viii) When the sample contained a large amount of particulate matter the process was repeated.

- (ix) The protocol of excystment described in 3.2.1 steps (ii)-(iv) was then followed.

3.3 Standard Method of Water Testing

The Standard Method (Standard Methods, Jakubowski 1990) was followed: any modifications are mentioned below.

3.3.1 Water Testing & Filter Extraction

- (i) In flowing water, the pump was placed upstream of the effluent hose. With still water, the effluent hosing and pump were placed as far apart as practical.
- (ii) As much water as possible was pumped through the filter to give as great a chance as possible of detecting *Giardia* cysts (detection is dependent on the particulate nature of the water). The standard method requires, for statistical reasons, at least 380 l of water be filtered.
- (iii) The cartridge and any trapped water was removed from the housing and placed in a plastic bag. The bag was labelled with all information (Jakubowski 1990) and taken to the laboratory where it was processed within 24/48 hours of sampling.
- (iv) Two plastic lined buckets each containing approximately 1.5 l of eluting solution were readied. The cartridge, and the water with it, were placed in the first bucket.
- (v) The filter fibres were separated from the support core by a longitudinal cut down the

length of the filter cartridge using a scalpel blade.

- (vi) The string holding the fibres was removed and rinsed in the second bucket, then discarded. The fibres were rinsed, starting with the fibres closest to the inner core (least dirty) and moving through to the fibres of the outer core. They were rinsed in the first and the second buckets before being discarded.
- (vii) Using the Sorvall centrifuge (**materials 2.4.7**), the sample in the eluting solution was concentrated at 1000 x g for 10 minutes.
- (viii) The supernatant was removed and the pellet re-suspended in eluting solution. If the sample was not to be used for excystment work it was preserved in 3.7% formalin.

3.3.2 Purification On a Sucrose Gradient

The samples often contained a lot of particulate matter and had to be purified before staining to allow the cysts to be seen.

- (i) The pellet volume was increased to 30 ml using eluting solution and mixed using a vortex mixer.
- (ii) The same protocol was used as in 3.2.2 (steps (ii)-(ix)).
- (iii) If a large amount of particulate matter was present, the sucrose gradient was repeated. A drop of the sample was then placed on a

slide and viewed at 400 x magnification. If the particulate matter appeared very dense, the sample was diluted until the particles appeared discrete across the slide.

3.3.3 Staining on a Filtration Manifold

The filtration manifold allowed for the staining of up to 12 filters at one time (Jakubowski 1990).

- (i) The filtration manifold was prepared as follows before the diluted sample (methods 3.3.2) was added. The vacuum through each of the wells was shut off. Two 25 mm diameter filters were used for each sample. Both were soaked in phosphate-buffered saline prior to use.
- (ii) The larger porosity filter (variable porosity depending on availability normally 45µm) was placed on the well. A 1 µm filter was placed on top of this. The vacuum was turned on to suck the filters onto the manifold, ensuring there were no wrinkles. The vacuum was then closed off.
- (iii) With the vacuum off, 1 ml of sterile water was added to each well, followed by the diluted sample. The vacuum was turned on to drain each well then shut off again. The wells were rinsed thoroughly with 2 ml of bovine serum albumin. The vacuum then drained this off. The filters were then ready for staining.
- (iv) A positive and negative control were run with each set of samples.

- (v) Commercial staining kits (**materials 2.7.1**) were used to stain the samples. The instructions with these were followed in each case (**appendix 2**). Except where noted the MerifluorTM *Giardia* monoclonal antibody stain was used.
- (vi) Slides were labelled and mounting fluid added to each. The corresponding filter was then placed on the slide, a drop of mounting fluid was added and a cover slip.
- (vii) Each slide was viewed using epifluorescence. It was scanned at 200 x for apple green fluorescent cysts. Presumptive cysts were viewed at 400 x magnification.
- (viii) For confirmation of *Giardia* the presumptive cysts were viewed at 1000 x using phase contrast microscopy. If two or more internal features were observed the object was confirmed as *Giardia*.
- (ix) Initially the test was done as a positive/negative test until a method from Jakubowski (1990) allowed for the calculation of the number of cysts per 100 l.

3.3.4 Calculation of the Number of *Giardia* Cysts Present

The number of cysts were calculated using the formula from Jakubowski (1990).

- (i) The formula was:

$$x/100 \text{ l} = \frac{(\text{PRG or CG}) (100)}{\text{FVR}}$$

where:

- V(1) = volume of the original water sample
 P (ml) = eluate packed pellet volume

$$F = \frac{P \text{ subjected to flotation}}{P}$$

 R = percentage (as a decimal) of floated sediment examined
 PRG = total number of presumptive *Giardia* cysts detected
 CG = total number of confirmed *Giardia* cysts detected.

- (ii) To calculate the detection limits the following formula was used:

$$x/100 \text{ l} = \frac{(1) (100)}{\text{FVR}}$$

3.3.5 Sensitivity of the Standard Method of Water Testing

The following was aimed at determining the lower detection limits of the standard method were (Standard Methods, Jakubowski 1990).

- (i) Cysts produced *in vitro* (method 3.2.1) were diluted from 10^6 down to 10^0 .
- (ii) 500 l of water containing a cyst dilution was pumped through the filter unit.
- (iii) The cartridge was removed and processed (methods 3.3.1). The sample was then purified on a sucrose gradient (methods 3.3.2).
- (iv) 10% of the sample obtained was stained (methods 3.3.3) and the cysts were counted.

- (v) Calculation of the number of cysts was then done using method 3.3.4.

3.4 Use of Tangential Filtration Units

3.4.1 Comparison of concentration techniques using tangential filtration units and centrifugation.

The normal method of concentrating the fibre wash water is by centrifugation. This method was compared to the use of tangential filtration (Jakubowski 1990, Standard Methods, Millipore catalogues, Filtron catalogues, Sartorius catalogues).

- i) *In vitro* produced cysts (methods 3.2.1) were diluted. Dilutions were added to 4.1 RO water (this being the approximate volume obtained after washing the filter).

- iii) Each sample in duplicate was subjected to concentration.

Concentration Methods

A) Tangential Filtration Units

- (i) Each tangential filtration unit was prepared according to the manufacturer's instruction: this procedure entailed assembly, putting the membrane in, and testing the membrane.

- (ii) The sample was then circulated through the tangential unit with the filtrate outlet closed for two minutes.
- (iii) The filtrate valve was opened and the sample was concentrated to 250-500 ml. This was the least concentration possible taking into consideration the length of the hoses, etc.
- (iii) A final volume concentration of 400-500 ml was obtained once the unit was flushed out.

B) Centrifugation

- (i) The 4 l sample was placed in 4 x 1 l (litre) buckets (a 4 l rotor was used).
- (ii) The buckets were centrifuged at 1,000 x g for 10 minutes.
- (iii) The supernatant was aspirated from the top to within 2 cm of the pellet.
- (iv) Each bucket was rinsed into a beaker giving approximately 300 ml of end sample.

Final Concentration

- (i) The end sample from each method was centrifuged at 1,000 g for 10 minutes to concentrate into a pellet.
- (ii) The protocol of staining and enumeration (**methods 3.3.3 and 3.3.4** respectively) were followed.

3.4.2 The use of tangential filtration units as compared to the standard method of water testing for *Giardia*.

- (i) Cysts were prepared and counted (method 3.1.2). Dilutions were made from 10^6 to 10^0 (dilutions were prepared in 4 ml, ie, $10^6/4$ ml).
- (ii) The standard method of water testing (method 3.3) was used. Cysts from the appropriate dilutions were added in 1 ml lots at 100, 200, 300 and 400 l stages.
- (iii) Tangential filtration units were assembled using the manufacturer's instructions.
- (iv) A flow meter was placed at the intake of each unit and 500 l was pumped through the tangential filtration unit. Cysts (at the various dilutions) were added in 1 ml amounts at 100, 200, 300 and 400 l stages.
- (v) The retentate was passed into two 200 l drums.
- (vi) When 500 l had passed through the tangential filtration unit, each drum was individually concentrated to 2.5 l using the filter unit.
- (vii) The two lots of 2.5 l were rinsed out of the drum and added together forming 5-6 l. This was concentrated using the tangential filter system to 500 ml.
- (viii) The 500 ml was concentrated to a pellet at 1,000 x g for 10 minutes.

- (ix) The sample was stained and viewed using method 3.3.3. *Giardia* cysts per 100 l were calculated using method 3.3.4.

3.5 Comparison of Staining Methods

3.5.1 Comparison of slide staining to staining using filters on a manifold (Jakubowski 1990, Sauch 1988).

- (i) Dilutions of 10^2 /ml and 10^4 /ml were made using cysts produced *in vitro* (method 3.1.2).
- (ii) Duplicate slides were made using 0.1 ml of the dilution. These were stained using MerifluorTM *Giardia* monoclonal stain, following manufacturer's instructions.
- (iii) The staining on filters used the manifold followed method 3.3.3. A volume of 0.1 ml of the dilution was again mounted on the filter and the same monoclonal staining kit was used.
- (iv) The cysts were viewed (following manufacturer's instructions) using epifluorescence, and counted, with the number on each slide being calculated.

3.5.2 Comparison of commercially available monoclonal antibody stains for detection of *Giardia*.

There are many monoclonal antibody stains for staining cysts available, some of the commercial kits were evaluated (Jakubowski 1990, Rose et al 1989, Quinones et al 1988, Stibbs et al 1988, Sterling et al 1988).

- (i) Three types of commercially available monoclonal antibody stain were assessed. These are listed in **materials 2.6.1**.
- (ii) The commercially available stains were compared to polyclonal antibody indirect stain and Lugols iodine stain (**materials 2.6.1**).
- (iii) The samples used for staining were from three sources:
 - Dannevirke study
 - Mt Stewart study
 - commercial water testing lab at Massey University, Palmerston North.
- (iv) Control samples (both positive and negative) were available with the commercial manufacturer's product. In addition cysts produced *in vitro* and stored in RO water, were also used.

Commercial Monoclonal Antibody Stains

For the three listed commercially available monoclonal antibody stains the manufacturer's instructions were followed.
Appendix 1.

Iodine Stain

- (i) For this procedure 10 ul of sample was placed on a clean labelled slide with 10 ul of Lugols iodine (**materials 2.7.1**) added and mixed with the sample.

- (ii) The stain was left for 20 minutes to allow penetration to take place before a coverslip was placed on the sample.
- (iii) The sample was viewed using 400x and 1,000x magnification under phase contrast microscopy.

Polyclonal Stain

- (i) Initially different dilutions of the polyclonal antibody were used on a cyst control sample, to identify the best dilution for detecting rabbit antibody. The best dilutions found were 1/1,000 for polyclonal antibody and 1/50 for rabbit antibody.
- (ii) The same directions for the Meridian Combo Kit were followed, substituting the diluted polyclonal for the primary antibody and the labelled rabbit antibody for the secondary antibody solution.
- (iii) The samples were viewed and counted using an epifluorescent microscope.

3.5.3 Effect of chemicals on the staining ability of the cysts with monoclonal antibody.

- (i) Cysts produced *in vitro* (method 3.2.1) were diluted to a 10^6 cysts/ml concentration. A volume of 0.1 mls was then stained using the Merifluor Giardia Kit to check cyst fluorescence.
- (ii) Double strength solutions of 75% ethanol, 3%, 4% and 5% hypochlorite, 5M NaOH and 5M HCl were made (**materials 2.6.2**)

Water was used as a negative control.

- (iii) A volume of 6 ml of the cyst solution was placed in separate universal bottles.
- (iv) To 6 ml of cyst solution was added 6 ml of double strength of the chemical. This was done in duplicate for each chemical.
- (v) At intervals of 0, 1, 3, 10, 13, 21, 31, 100 and 300 minutes, one ml of sample was removed.
- (vi) This one ml was placed directly into 50 ml of distilled water and mixed using a vortex mixer. It was then spun in a centrifuge at 1,000x g for 10 minutes. This wash was repeated twice.
- (vii) The supernatant was removed and the sample pellet re-suspended in 0.5 mls of R.O. water.
- (viii) This sample was stained using the Merifluor Fluorescence Kit(materials 2.6.1), by following the manufacturer's instructions.
- (ix) Fluorescence of the cysts was noted.

3.6 New Zealand Testing

3.6.1 Dannivirke Testing

The farm used in the study is approximately 20 km from Dannevirke (shown in **results 4.6** Figure 14).

- (i) The Dannivirke family was contacted through health professionals concerning its recent

diagnosis of *Giardia*. Permission was gained to do a study.

- (ii) The farm was visited and mapped out, a sampling plan concerning the waterways was devised.
- (iii) The standard method of sampling for *Giardia* in water was used (**methods 3.3**).
- (iv) The farm was visited eight times in the following year.
- (v) Animal testing was done by an independant researcher.

RESULTS

4.1 *In vitro* encystment of *Giardia intestinalis*

4.1.1 The effect of bile concentration, time and temperature on encystment induced *in vitro*.

Axenic culture of *G. intestinalis* was first achieved by Meyer (1976). Research has since centred around methods of *in vitro* excystation and encystation to complete the lifecycle *in vitro*.

Completion of the lifecycle *in vitro* would allow studies into the biochemical analysis of the cyst and cyst wall, investigation of giardial agents for blocking formation of the cell wall, and infectivity of the cysts (Schupp et al 1988, Adam 1991).

G. intestinalis colonises the bile-rich small intestine of the host. Passage through the small intestine exposes them to slightly alkaline pH and bile whilst through the large intestine they are exposed to lactic acid. Before being passed into the environment outside the host they encyst to enable survival (Adams 1991, Ament et al 1972, Hoskins et al 1967).

Farthing (1983) reported the effect of bile and bile salts on accelerating the growth *in vitro* of *Giardia* cysts. Schupp et al (1988) reported a method involving the increase of mammalian bile which lead to encystation *in vitro*.

Schupp et al (1988b) studied the relationship of increased bile to production of cysts. This work and that of Miller (1988) were used to find conditions in which cysts would be produced. Other investigators have since reported successful *in vitro* encystation most using bile salts (Gillin et al 1988).

Two strains of *G. intestinalis* were used (Bris/83/HEPU/106 and Hast/87/MUGU/68) in this work. Information on these was

obtained from Miller's (1988) work. Culture growth occurred between 30-40°C with a slow decline in trophozoite numbers occurring at 25°C. Optimum growth for both cultures was achieved at 37°C (Miller 1988).

Growth in TY1-S-33 medium was dependent upon pH of that medium, the optimum being 6.75 to 7.25. At pH levels above this, culture growth rates rapidly declined with the most marked effect being with the Hast/87/MUGU/68 strain (Miller 1988).

The cultures used were grown to log phase (approximately 2-3 days) before adding the different bile amounts (0, 0.8, 4, 5, 6, 10, 15, 20g/L) to each culture. The strains were routinely cultured in 0.8g/L bile within the TY1-S-33 media.

The results of cysts counted are shown in Tables 1-6. Table 1 shows the results for the Bris/83/HEPU/106 and Hast/87/MUGU/68 strain at 4°C over the time periods 24, 48, 72 and 96 hours at various bile concentrations.

Table 2 shows the results for the Hast/87/MUGU/68 strain under the same conditions as Table 1. Tables 3 and 4 show the Bris/83/HEPU/106 and Hast/87/MUGU/68 strains respectively at 26°C over various times and bile concentrations. Tables 5 and 6 show Bris/83/HEPU/106 and Hast/87/MUGU/68 strains at 37°C under the various conditions.

Figure 1 indicates that few cysts are produced after 24 hrs incubation at any temperature. Few cysts were produced in all the cultures grown at less than 37°C. Bris/83/HEPU/106 and Hast/87/MUGU/68 strains show an increase in the number of cysts produced at 37°C.

The number of cysts produced after 48 hours in (Figure 2) increased only in strains incubated at 37°C. There was little change in the number of cysts in the cultures stored below this temperature. A dramatic decrease in the cover of the monolayer was also observed in those incubated at less than 37°C.

As the bile concentration increased, the monolayer became less dense as a result of encystment. This was observed for the entire experiment: ie, at all temperatures.

The Hast/87/MUGU/68 strain showed a peak number of cysts at a bile concentration of 5g/L (Figure 2). The Bris/83/HEPU/106 strain showed a slightly lower peak at this bile concentration.

At 37⁰C the Bris/83/HEPU/106 strain showed a higher peak number of cysts than the Hast/87/MUGU/68 strain following 72 hrs incubation (Figure 3). In all the graphs it can be seen that the cultures incubated at less than 37⁰C showed little change in the number of cysts.

After 96 hrs (Figure 4) both strains had 16.7×10^3 cysts present. The Bris/83/HEPU/106 strain remained higher in cyst numbers than the Hast/87/MUGU/68 strain which decreased overall. The peak was still evident in the 37⁰C Bris/83/HEPU/106 strain at a bile concentration of 5g/l.

In all the Figures (1-4) two peaks in the number of cysts can be seen as the bile increased. The first occurred around 5g/L with a decrease to about 10g/L. This was followed by a second peak which was generally smaller. A higher second peak was seen for the Bris/83/HEPU/106 strain at a bile concentration of 15g/l(Figure 1).

Figure 5 showed encystment of Bris/83/HEPU/106 at 4⁰C. The highest number of cysts recorded was 2. Figure 6 shows much the same for the Hast/87/MUGU/68 strain, the highest count being 2.5 with a steady climb occurring with the increase in bile.

Figures 7 and 8 are similar to 5 and 6 showing there is little increase in the cyst counts at 26⁰C.

Encystment of Bris/83/HEPU/106 and Hast/87/MUGU/68 strains at 37⁰C is shown in Figures 9 and 10. For the Bris/83/HEPU/106

strain (Figure 9) the highest number of cysts occurred at 72 hrs at 5g/l bile concentration with a count of 23.3×10^3 . The number of cysts at 24 hrs were the lowest. For the Hast/87/MUGU/68 strain the maximum number of cysts were recorded after 48 hrs at 37°C in 5g/l bile. This decreased to less than 6.7×10^3 cysts at 20g/l after 96 hrs incubation.

Figures 5-10 show a general increase in cyst numbers over the incubation periods up to 96 hrs.

All the results obtained are on an average of 2 duplicate samples with each experiment run twice.

Table 1. Encystment of the Bris/83/HEPU/106 strain incubated at 4 degrees C

Time hr	Bile Concentration (g/L)							
	0.0	0.8	4	5	6	10	15	20
24	0.8	0.0	0.8	0.0	0.0	0.0	0.0	1.7
48	0.0	0.8	0.0	0.8	0.8	0.8	0.8	1.7
72	0.0	1.7	1.7	0.8	0.8	0.8	2.5	3.3
96	0.8	0.8	0.0	0.8	1.7	0.0	1.7	0.8

Cyst formation (x1000) at Bile Concentrations

Table 2. Encystment of the Hast/87/MUGU/106 strain incubated at 4 degrees C

Time hr	Bile Concentration (g/L)							
	0.0	0.8	4	5	6	10	15	20
24	0.8	0.8	2.5	0.8	2.5	2.5	2.5	0.8
48	0.8	0.8	2.5	0.8	0.8	0.0	2.5	1.7
72	0.8	1.7	0.8	1.7	0.8	1.7	3.3	4.2
96	0.0	0.8	1.7	0.8	0.0	1.7	1.7	0.8

Cyst formation (x1000) at Bile Concentrations

Table 3. Encystment of the Bris/83/HEPU/106 strain incubated at 26 degrees C

	Bile Concentration (g/L)							
Time hr	0.0	0.8	4	5	6	10	15	20
24	0.0	0.8	1.7	1.7	0.8	2.5	2.5	1.7
48	0.8	1.7	0.8	1.7	0.0	1.7	4.2	2.5
72	0.8	0.0	1.7	1.7	4.2	2.5	0.8	0.8
96	0.0	0.8	0.8	1.7	0.8	0.8	2.5	4.2

Cyst formation (x1000) at Bile Concentrations

Table 4. Encystment of the Hast/87/MUGU/106 strain incubated at 26 degrees C

	Bile Concentration (g/L)							
Time hr	0.0	0.8	4	5	6	10	15	20
24	0.8	0.0	0.8	1.7	1.7	2.5	1.7	0.8
48	2.5	0.8	1.7	3.3	2.5	0.8	0.8	2.5
72	0.0	2.5	3.3	3.3	2.5	3.3	0.8	0.8
96	0.8	0.8	2.5	2.5	0.0	0.0	0.8	0.8

Cyst formation (x1000) at Bile Concentrations

Table 5. Encystment of the Bris/83/HEPU/106 strain incubated at 37 degrees C

	Bile Concentration (g/L)							
Time hr	0.0	0.8	4	5	6	10	15	20
24	2.5	1.7	6.6	4.2	5.8	5.8	7.5	3.3
48	0.0	3.3	6.6	16.7	16.7	7.5	18.3	15
72	0.8	4.2	7.5	22.5	10.8	17.5	11.7	11.7
96	2.5	2.5	5.8	11.8	15.8	8.3	10.8	12.5

Cyst formation (x1000) at Bile Concentrations

Table 6. Encystment of the Hast/87/MUGU/106 strain incubated at 37 degrees C

	Bile Concentration (g/L)							
Time hr	0.0	0.8	4	5	6	10	15	20
24	1.7	2.5	5.0	0.8	0.0	3.3	4.2	4.2
48	0.8	4.2	5.8	22.5	15.8	7.5	5.8	4.2
72	1.7	2.5	7.5	15.0	14.2	6.6	8.3	2.5
96	0.0	2.5	2.5	5.8	4.2	5.8	5.0	6.6

Cyst formation (x1000) at Bile Concentrations

Fig 1: Cysts produced following 24 hours incubation

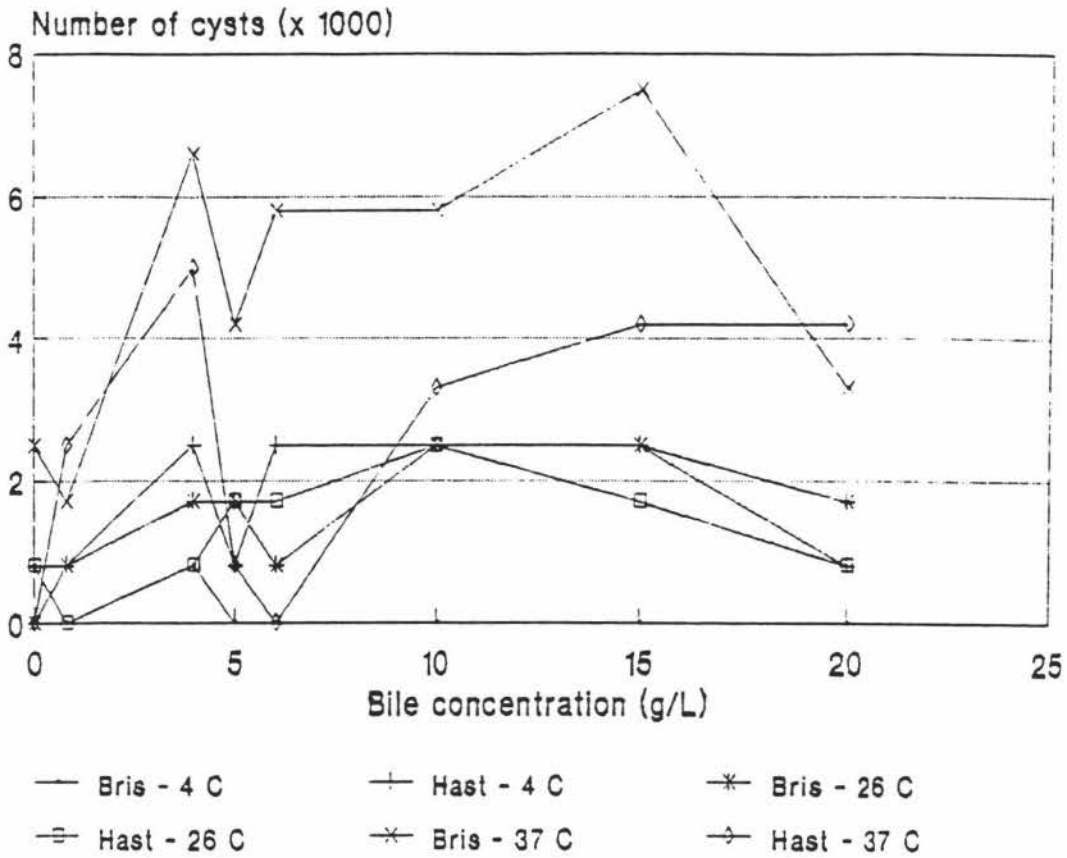


Fig 2: Cysts produced following 48 hours incubation

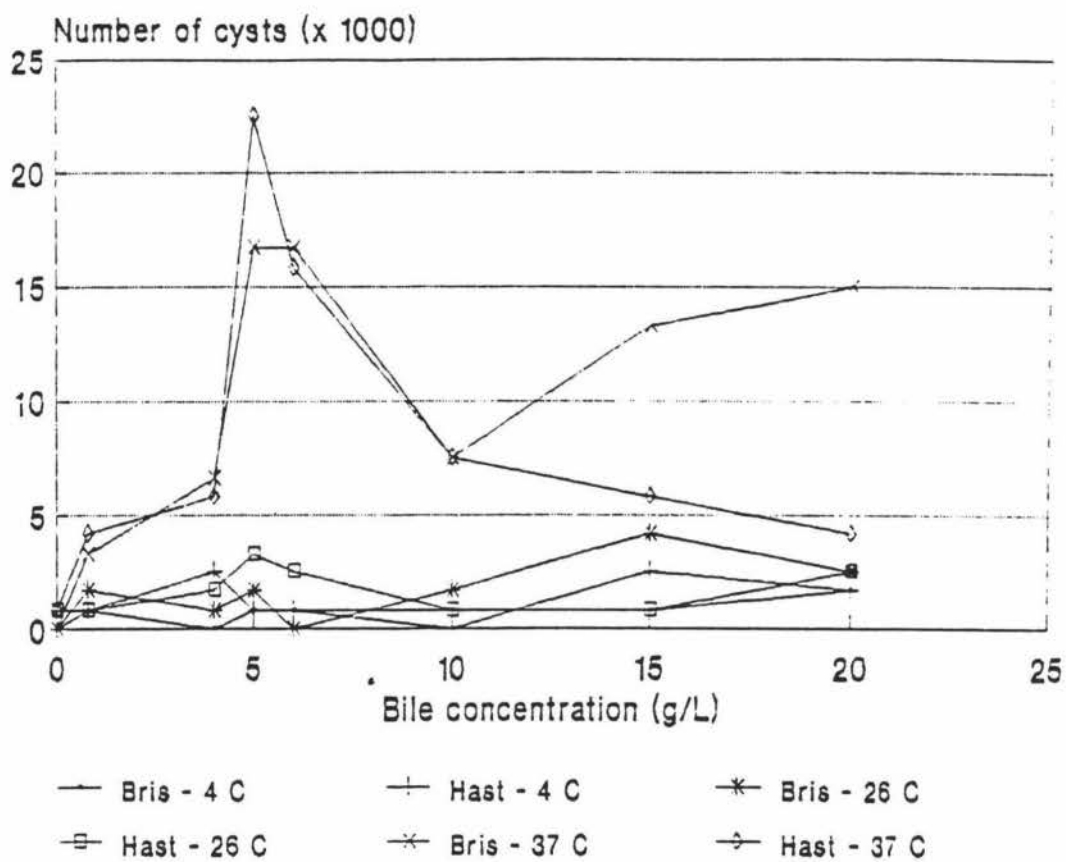


Fig 3: Cysts produced following 72 hours incubation

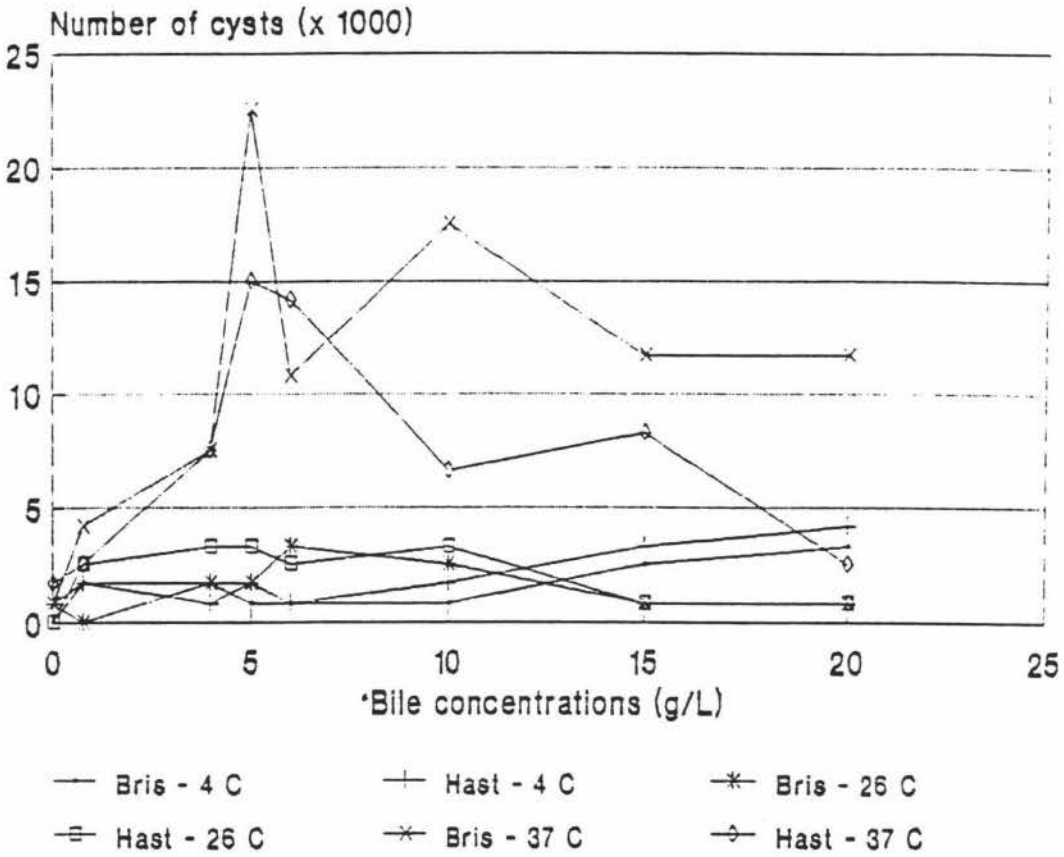


Fig 4: Cysts produced following 96 hours incubation

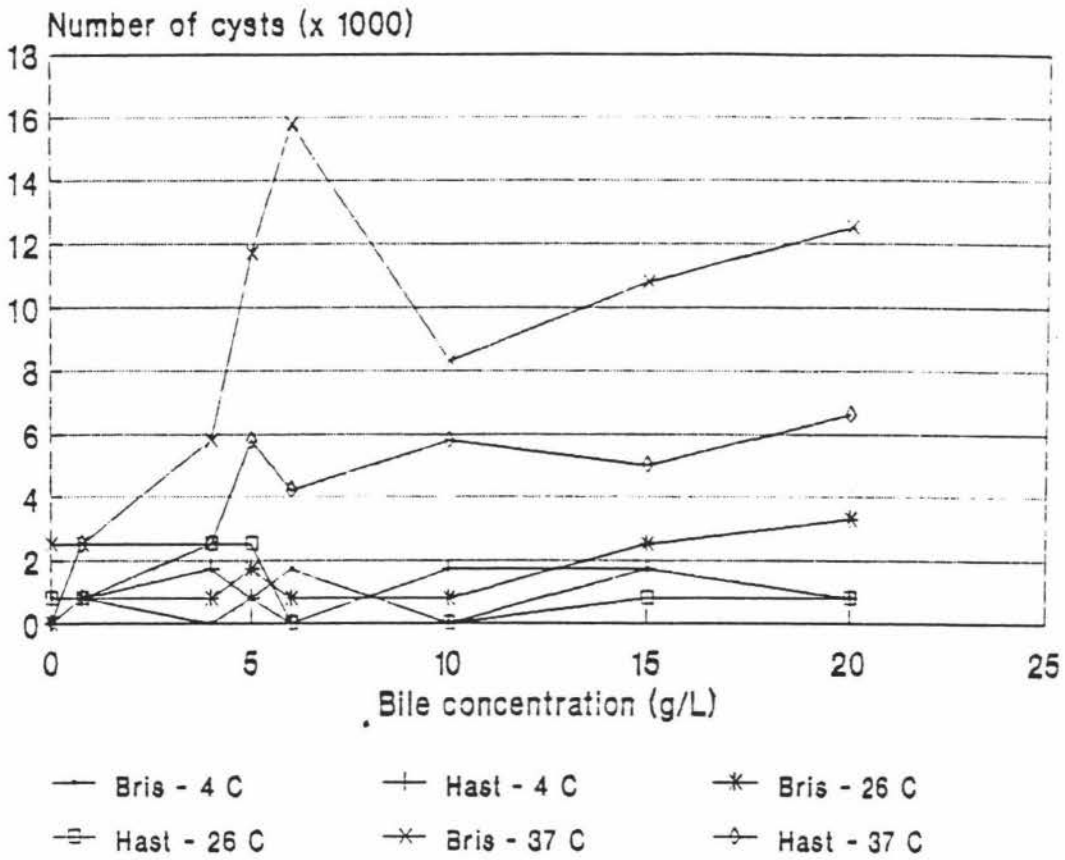


Fig 5: Encystment of Bris/83/HEPU/106 cultivated at 4°C

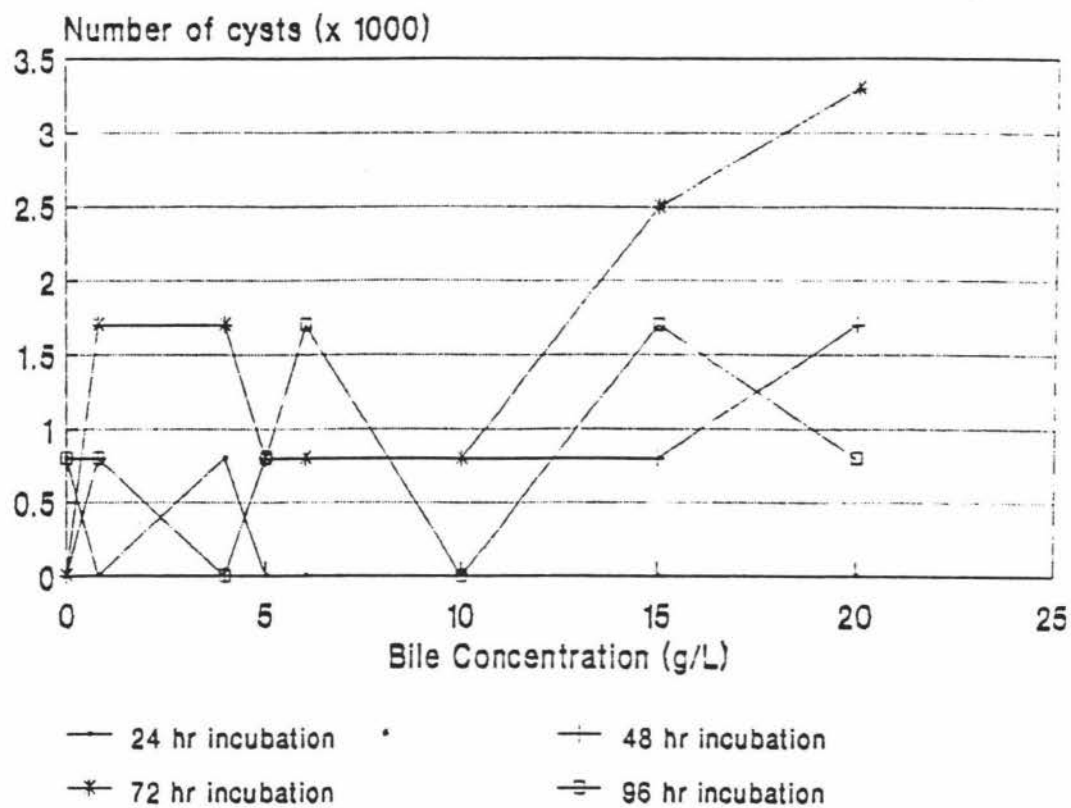


Fig 6: Encystment of HAST/87/MUGU/68 cultivated at 4°C

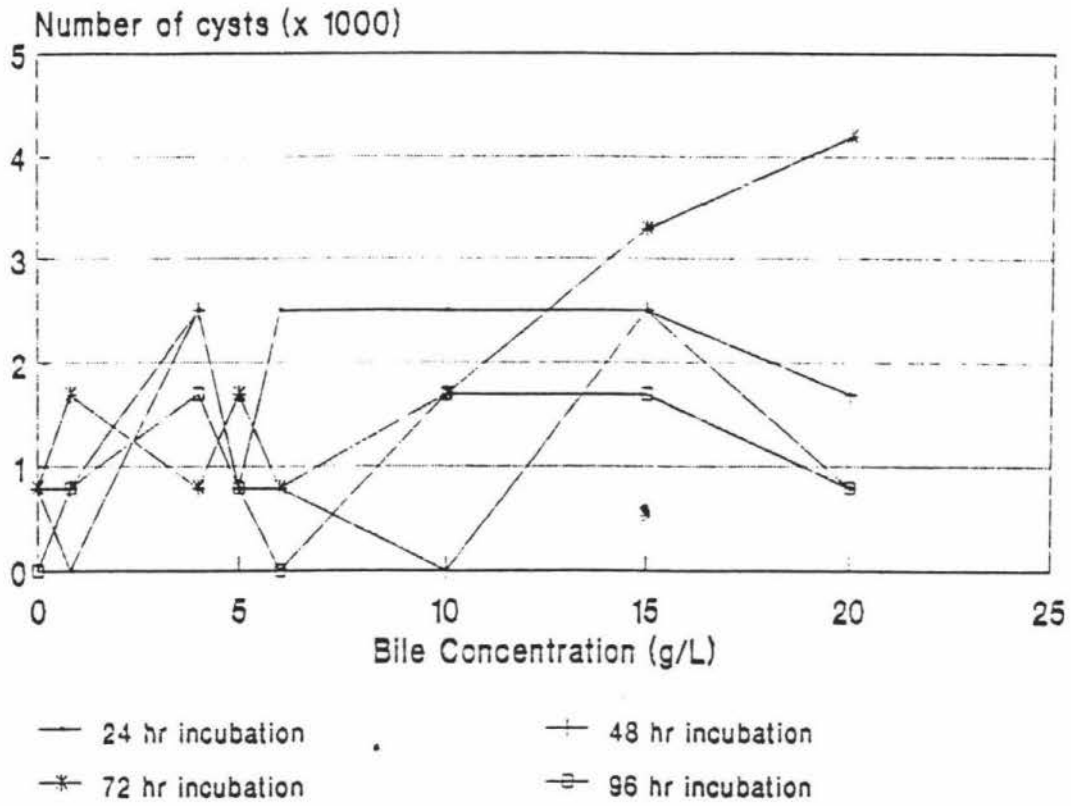


Fig 7: Encystment of Bris/83/HEPU/106 cultivated at 26°C

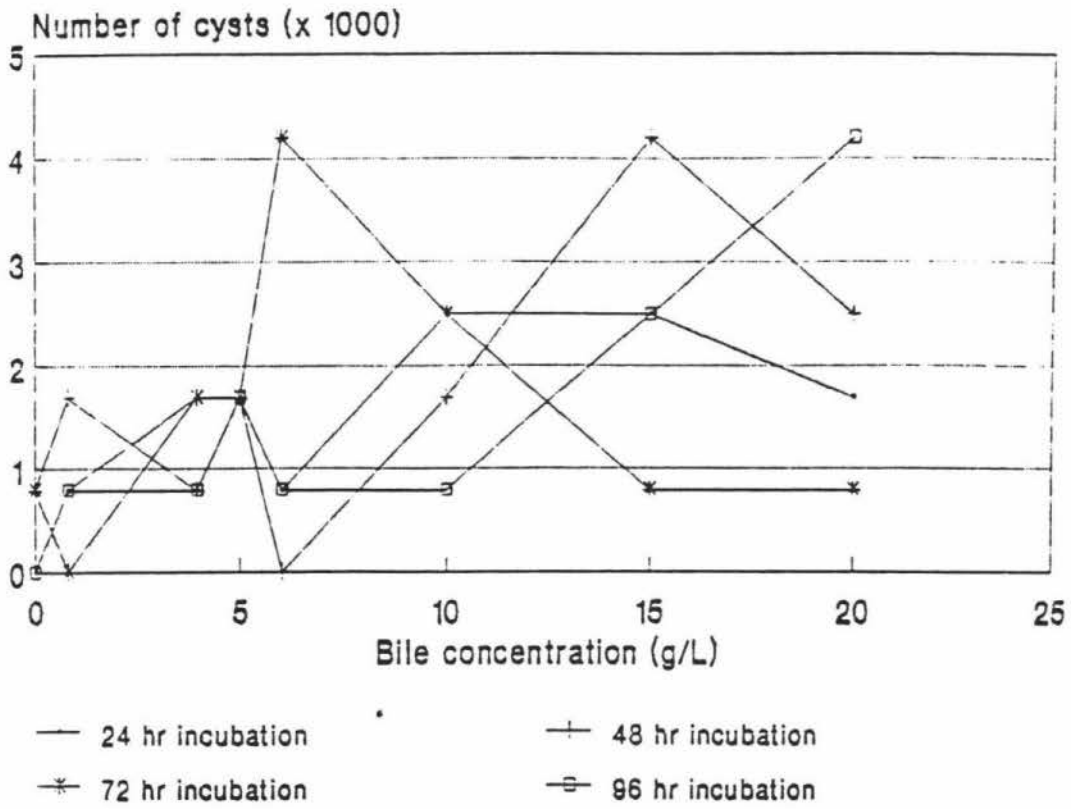


Fig 8: Encystment of HAST/87/HEPU/68 cultivated at 26°C

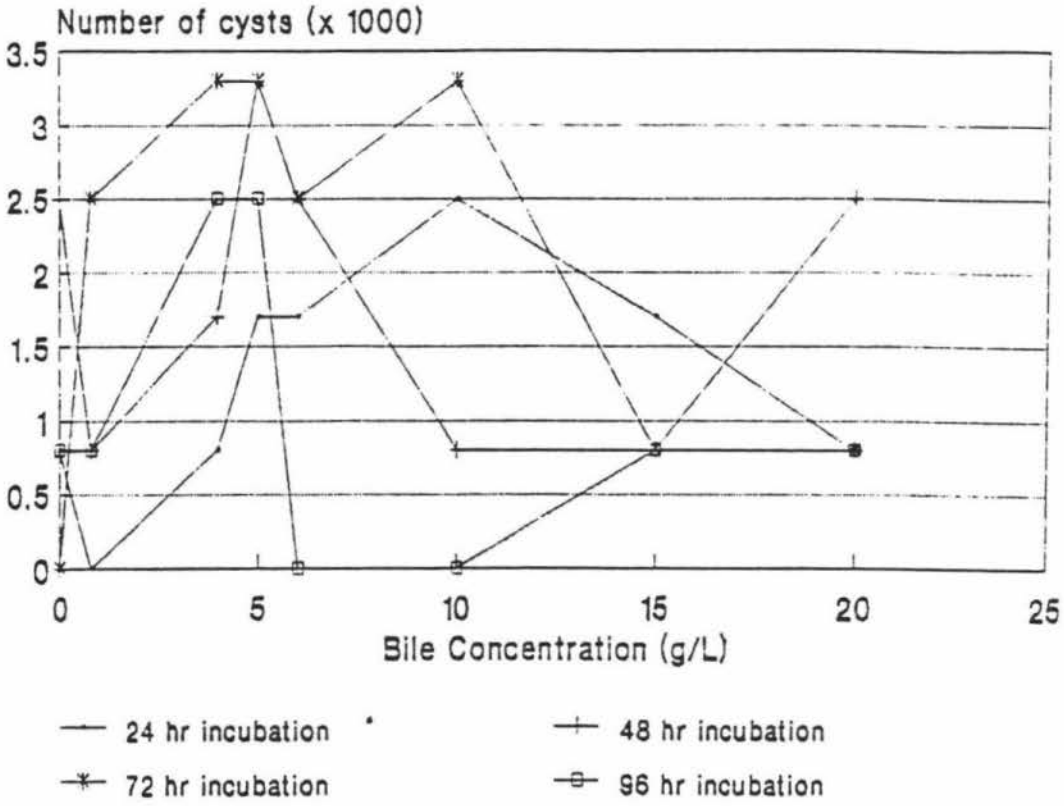


Fig 9: Encystment of Bris/83/HEPU/106 cultivated at 37°C

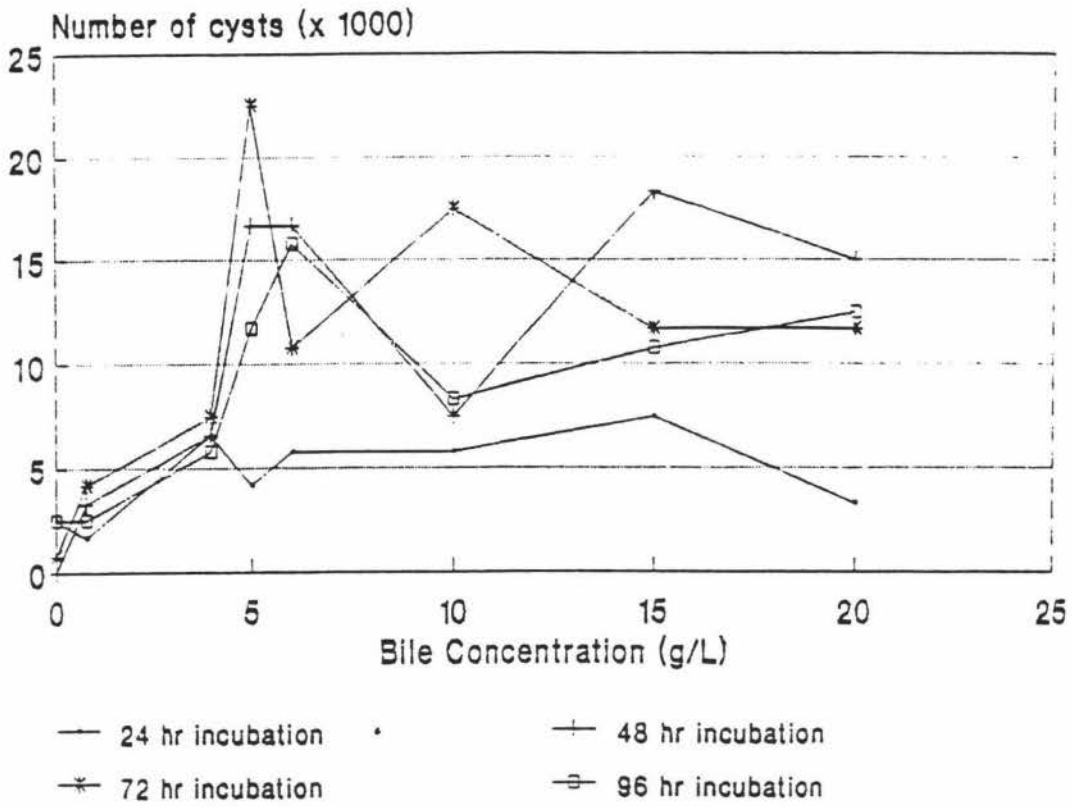
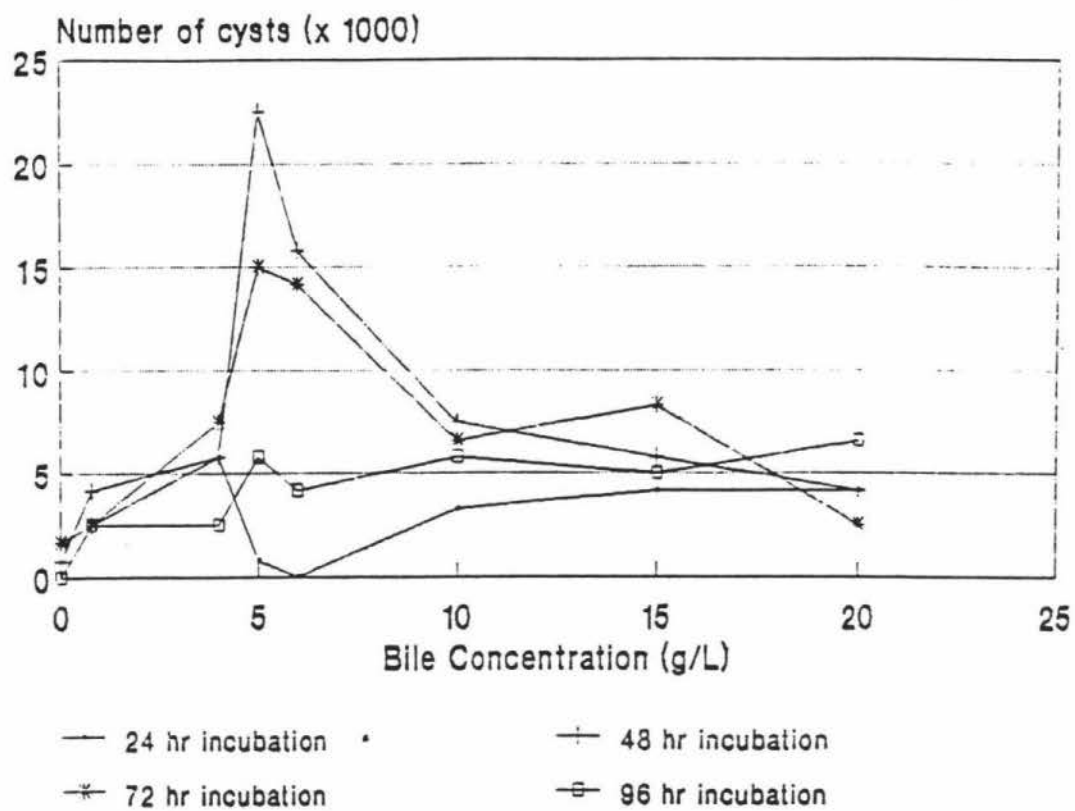


Fig 10: Encystment of HAST/87/MUGU/68 cultivated at 37°C



4.2 Excystment of *Giardia intestinalis*

4.2.1 *In vitro* excystment of cysts produced *in vitro*

Meyer (1976) was the first to excyst and axenically culture *G. intestinalis*, he used a low pH solution to induce excystation followed by incubation in a nutrient medium. Other physiological factors of importance in excystation, have now been identified. These include bicarbonate and reducing agents which have led to an improvement in the process of *in vitro* excystation.

Excystment efficiencies have been very low and are found to be extremely variable with cysts isolated from different sources and in comparisons between different laboratories (Gillin et al 1988). Strains vary in their ability to excyst. Comparisons between *G. intestinalis* and *G. muris* have shown *G. muris* cysts are able to excyst immediately following excretion, whilst *G. intestinalis* requires a maturation period at a low temperature over several days (Adams 1992).

Many methods of excystment have been used. Generally they all use a three step process: low pH inducement, washing and incubation in nutrient media. Tonks (1988) used Feelys (1984) method of excystation, with several adjustments, to successfully excyst *Giardia* cysts.

Feelys (1984) method used HBSS adjusted to pH 7.2 with sodium bicarbonate, then adjusted to pH 2 using 1M HCl. Cysts were incubated in this solution for 27 minutes at 37°C. Washing and incubation in TY1-S-33 followed. Tonks modified the TY1-S-33 medium with 0.2 L-cysteine HCl (as described in Kasprzak and Majewska 1983,1985) and 50/50 bovine foetal calf serum was used.

The following experimental work was designed to investigate the lowest number of cysts which could be excysted to give a

sustainable culture. It has been suggested that ingestion of 10 cysts led to an infection *in vivo* in humans (Shearer and Lapham 1988). Schaefer III et al (1991) demonstrated that 1/10 mongolian gerbils became infected following the ingestion of 10 cysts. Following ingestion of 100 cysts 4/10 became infected with 100% infection being reached at 10,000 cysts.

This work compared two strains of *G. intestinalis* cysts (Bris/83/HEPU/68 and Hast/87/MUGU/68), which had been produced *in vitro* and stored at 4°C for 6-10 days to allow the cysts to mature, before dilution and excystation.

Results of excystation and the establishment of a culture are shown in Tables 7 and 8. Table 7 shows Bris/83/HEPU/106 strain and Table 8 shows Hast/87/MUGU/68 strain.

The Bris/83/HEPU/106 strain showed 100% ability to form a culture from a cyst concentration of 5×10^1 to 10^6 . The Hast/87/MUGU/68 strain showed 100% ability to form a culture, from the 10^2 - 10^6 concentration of cysts.

The Bris/83/HEPU/106 strain produced 2/6 cultures with a concentration of 10 cysts, this was the same result as for the Hast/87/MUGU/68 strain. Neither of the strains produced cultures with a lower concentration of cysts.

The Hast/87/MUGU/68 produced 4/6 cultures using concentrations of 50 cysts but Bris/83/HEPU/106 produced 6/6 cultures at the same concentration.

Table 7. Attempted excystment of *in vitro* produced cysts of the Bris/83/HEPU/106 strain.

Cyst Conc	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
10 ⁶	+	+	+	+		
10 ⁵	+	+	+	+		
10 ⁴	+	+	+	+		
10 ³	+	+	+	+	+	+
500	+	+	+	+	+	+
100	+	+	+	+	+	+
50	+	+	+	+	+	+
10	-	+	-	+	-	-
5	-	-	-	-	-	-
1	-	*	-	-	-	-
0	-	-	-	-	-	-

KEY

- + Growth
- No growth
- * Contamination

Table 8. Attempted excystment of *in vitro* produced cysts of the Hast/87/MUGU/106 strain.

Cyst Conc	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
10^6	+	+	+	+		
10^5	+	+	+	+		
10^4	+	+	+	+	+	+
10^3	+	+	+	+	+	+
500	+	+	+	+	+	+
100	+	+	+	+	+	+
50	+	-	+	-	+	+
10	+	-	-	-	-	+
5	-	-	-	-	-	-
1	-	-	-	-	-	-
0	-	-	-	-	-	-

KEY

- + Growth
- No growth
- * Contamination

4.2.2 *In vitro* excystment of cysts of water and animal origin

Excystment of *Giardia* cysts being able to establish axenic culture has been possible from animal and human fecal samples since the first report in 1976 (Meyer). The excystation of *Giardia* cysts from water, has never been reported

Methods to determine viability are being established. Research into the establishment of reliable staining methods has been undertaken with concentrated interest being shown in propidium iodide and fluorescein diacetate staining methods. Molecularbiological methods using polymerase chain reaction (PCR) are also being studied.

At present the most respected method of viability testing is still by the excystment of cysts and, as has been shown there are several methods available. Many of the methods are able to demonstrate excystment, but are unable to establish culture (Gillin et al 1988).

When *Giardia* cysts are detected in waterways very little can be learned about them other than their morphological state. Unlike cysts in fecal samples, animal origins cannot be identified unless they are either *G. muris* or *G. agilis*. The age of the cyst (how long the cyst has been in the water) cannot, as yet be established.

The cysts used in this study came from three sources as discussed in materials (2.3.1). The fecal samples all were small amounts <5g. This lead to a maximum trial of approximately 15 attempts, and a minimum of ten attempts at excystment on each.

The water samples were consistently 5ml with all having less than 100 cysts/ml present. Where possible, at least five attempts at excystment were made with each sample. The samples were labelled river 1-10. They came from the commercial water testing

laboratory at Massey University, Palmerston North and it was not possible to obtain names of the waterways.

The results of all attempts to excyst and establish axenic culture of the animal samples are shown in table 9. Only the sample labelled Dog 2 (from a dog in Auckland) had a small number of trophozoites, which were viewed 24 hours following excystment. These decreased in number over the three days following excystment with none found on the fourth day.

Water sample excystment attempts are shown in Table 10. The river samples had shown positive for *Giardia*, with cysts being present at <100 cysts/ml; but excystment attempts failed. No trophozoites were viewed following attempts at excystment.

Contamination was a major problem during the trails, and attempts were made to control it (materials 2.2.3). Damage to the culture by contamination was never clear but in the case of the Dog 2 sample prior to contamination, trophozoites were observed. These disappeared following contamination by an unidentified yeast.

Table 9. Attempted excystment of animal origin cysts to give continuous culture

Source	Estimated Cysts	Stain Result	Trophozoite Present	Culture
Dog 1	1000	p	-	-
Dog 2	10000	p	+	-
Cat	1000	p	-	-
Opposum 1	1000	p	-	-
Cow	1000	p	-	-
Sheep	10000	p	-	-
Opposum 2	1000	p	-	-
Sheep	10000	p	-	-
Opposum 3	1000	p	-	-
Mouse	10000	p	-	-
Cysts	10000	p	+	+

KEY

+ growth

- no growth

p a positive cyst sample by staining with a monoclonal antibody stain

cysts cysts produced *in vitro*

Table 10. Attempted excystment of cysts from natural waterways

Source	Stain test	Trophozoites	Culture
River 1	p	-	-
River 2	p	-	-
River 3	p	-	-
River 4	p	-	-
River 5	p	-	-
River 6	p	-	-
River 7	p	-	-
River 8	p	-	-
River 9	p	-	-
River 10	p	-	-
Spiked cysts 1	p	+	+
Spiked cysts 2	p	+	+
+ Control	p	+	+
- Control	n	-	-

Key

+ Growth

- No Growth

p Positive cyst sample by staining with a monoclonal antibody stain

n Negative cyst sample at staining with a monoclonal antibody stain

4.3 The Standard Method of water testing

4.3.1 Sensitivity of the Standard Method of water testing

The sampling apparatus developed by the Environmental Protection Agency (EPA) for testing waters for *Giardia* has changed little since it was first used in 1976. However, the processing of the cartridge, and methods for the concentration and identification of *Giardia* cysts have progressed. Although most investigators follow the 16th edition of the Standard Methods outline for the procedure, there is variation between laboratories in the fine detail of the procedure and, therefore variation in the results obtained (Jakubowski 1990).

The following experiment was undertaken following the Standard Method, with modifications from the Jakubowski (1990) method, to evaluate the sensitivity of the Standard Method. Results are shown in Table 11 and displayed in Figure 11.

At initial inoculations higher than $10^4/5001$, the cysts were present in numbers too high to count. At $10^4/5001$ initial inoculation, an average of 2450 cysts/5001 were recovered. This gave an efficiency of 24.5%. With $5 \times 10^3/5001$ initially inoculated, 2000 cysts were recovered an efficiency of 40%. The 10^3 cysts/5001 initial inoculations had a cyst recovery of 120 cysts/5001 a 24% efficiency.

The lowest number of cysts detectable in an initial inoculation was 5×10^2 cysts/5001, an efficiency of 10%.

Tap water was used to avoid the need to control sediment (problem in collecting natural samples) which obscures the cysts. Unfortunately the sediment in the tap water was greater than expected (20-30g), and even after concentration on a sucrose gradient, approximately 5g of sediment remained.

The following results are averages of duplicates. Each sample was run and processed separately, with counts averaged out. .

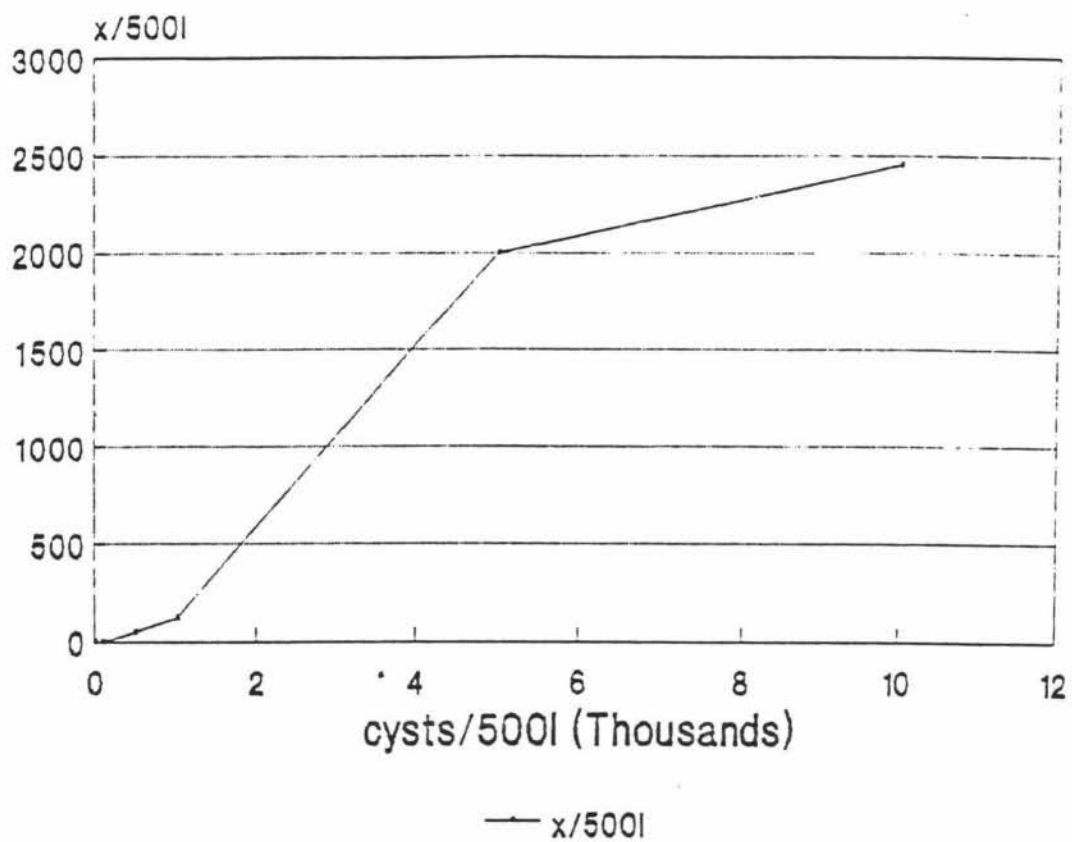
Table 11. Sensitivity of the Standard Method used for the detection of *Giardia* in water.

Cysts/500l	x /500l	% Recovery
10^6	>2500	-
5×10^5	>2500	-
10^5	>2500	-
5×10^4	>2500	-
10^4	2450	24.5%
5×10^3	2000	40%
10^3	120*	24%
5×10^2	50	10%
10^2	0	0%
5×10^1	0	0%
10^1	0	0%
5	0	0%
0	0	0%

Key

- * Only one sample obtained
- Unable to calculate recovery

Fig 11: Graph of sensitivity of the Standard Method.



4.4 Use of Tangential Filtration Units

4.4.1 Comparison of concentration techniques using tangential filtration and centrifugation

Tangential filters work on the principle that liquid is circulated across a membrane at a constant rate with a filtrate drawn off at a much slower rate. This enables the membrane to remain unblocked as liquid passes through it, whilst at the same time the solution becomes concentrated. This method of filtration has a variety of uses from concentrating antibodies to concentrating cells or organisms in culture. In these experiments it was used to concentrate *Giardia* cysts in water samples (Issac-Renton et al 1988, Tangential Filtration Catalogues).

The Standard Method for detecting *Giardia* in large bodies of water is by using a cartridge filtering system with an efficiency of between 15 to 69%. Other methods have been investigated such as the use of polycarbonate membrane filters (Shearer and Lapham 1988).

The aim of this study was to concentrate the 4-5l of spiked eluting solution that represented the wash solution normally concentrated by centrifugation. The aim was to establish whether the tangential filtration units had the ability to run under these conditions before comparison with the full standard method (Hastie et al 1991).

The tangential filtration units were being compared to centrifugation as described by Standard Methods, and also with the method of sedimentation described in Standard Methods. Sedimentation involves leaving the filter extract to stand overnight, allowing the cysts to settle out before the supernatant is aspirated off.

Each of the tangential units was assembled to the manufactures instructions, and the membranes were tested prior to use. When a fluid of 10^6 cysts/4l was run through the unit, the filtrate was concentrated by centrifugation to ensure the membrane integrity. Each of the samples was run in duplicate, collected, processed, stained and before the results were averaged.

Results of the comparison between the methods: tangential filtration, centrifugation and sedimentation are in Tables 12,13,14 and illustrated in Figure 12. Using centrifugation, cysts were detectable to 50 cysts/4l with a 10% recovery. With sedimentation it was possible to detect 100 cysts/4l with a 35% recovery. The recovery efficiency for centrifugation at the lower levels of cysts ranged between 10-34%. Sedimentation ranged between 34-35%.

The larger surface area membranes were the least sensitive. The Pellecon unit detected 5 of 5×10^3 cysts/4l with a recovery of 0.01%. At 10^4 cysts/4l, 70 cysts were detected with a recovery of 0.7%. The Sarticon II unit, with a similar surface area, unit detected 35 cysts from 5×10^3 cysts (a recovery of 0.07%). At 10^4 cysts/4l it detected 50 cysts, a recovery of 0.5%.

The smaller surface area units had lower detection levels than the larger units. The Filtron Minisette was the most sensitive of all the units, detecting at 500 cysts/4l with 5 cysts detected; a recovery rate of 1%. The Sarticon minisette unit detected 10^3 cysts/4l with a recovery rate of 2%.

Overall, centrifugation recovered to the lowest level of cysts spiked at 50 cysts/4l and sedimentation detected to 10^2 cysts/4l of spiked cysts. The Filtron minisette was the most sensitive of the tangential filters at 500 cysts/4l. The Sarticon minisette detected to 10^3 cysts/4l, with the two larger surface area filters, the Pellecon unit and the Sarticon II unit detecting cysts at 5×10^4 cysts/4l.

When challenged with the sediment samples (shown in Table 13) the smaller surface area units Filtron miniset and the Sarticon miniset, were unable to function with 5g of particulate matter before the membrane pressure became too high (>100psi). The Sarticon II's, small pump was unable to function at the pressures demanded, and so this unit could not be fully assessed. The Millipore Pellicon unit was the only tangential filtration unit that was able to function with particulate matter in the sample. Centrifugation and sedimentation were not affected by the amount of particulate matter in the sample.

In this assessment of efficiency, the other consideration was turnaround time. Timing included processing the sample, the process itself, decontamination and to the start of the next sample. These times, with explanations, are summarized in Table 14. The manufactures cleaning instructions for each specific membrane in the tangential filtration units were followed.

The smaller units took longer to rinse (flushing of 50l of water) than the larger units (80 min compared to 65 min total). Centrifugation was the quickest method at 55 minutes and sedimentation, because it had to be left overnight to allow settling, was the slowest (>8 hours).

Table 12. Comparison of tangential filtration with conventional methods of concentrating water for the detection of *Giardia* cysts.

Cyst Conc. in 4l	Millipore Pellicon Unit	Filtron Minisette Unit	Sarticon Minisette Unit	Sarticon II unit	Centrifugation	Sedimentation
10^6	>250	>250	>250	>250	>250	>250
5×10^5	>250	>250	>250	>250	>250	>250
10^5	>250	>250	>250	265 (0.27%)	>250	>250
5×10^4	215* (0.43%)	190* (0.38%)	275* (0.55%)	75 (0.15%)	>250	>250
10^4	70 (0.7%)	155 (1.55%)	180 (1.8%)	50 (0.5%)	>250	>250
5×10^3	5 (0.1%)	95 (1.9%)	60 (1.2%)	35 (0.7%)	>250	>250
10^3	0	50 (5%)	20 (2%)	0	>250	>250
5×10^2	0	5 (1%)	0	0	170* (34%)	170* (34%)
10^2	0	0	0	0	10 (10%)	35 (35%)
5×10^1	0	0	0	0	5 (10%)	0
10^1	0	0	0	0	0	0
5	0	0	0	0	0	0
1	0	0	0	0	0	0
0	0	0	0	0	0	0

KEY

* only one result obtained

> greater than that number presented

(x%) percentage recovery

Table 13. Ability of tangential filtration units compared to conventional methods in functioning in the presence of sediment.

	Millipore Pellicon Unit	Filtron Minisetete Unit	Sarticon Minisetete Unit	Sarticon II unit	Centrifugation	Sedimentation
5g soil	+	-	-	*	+	+
10g soil	+	-	-	*	+	+

KEY

- unable to function
- + able to function
- * no result obtained

Table 14. Time taken for the tangential filtration units compared to the conventional methods in the concentration of water samples.

	Millipore Pellicon Unit	Filtron Minisetete Unit	Sarticon Minisetete Unit	Sarticon II unit	Centrifugation	Sedimentation
Actual run (min)	2-5	5-10	5-10	2-5	20*	Overnight
Back flush (min)	5-10	5-10	5-10	5-10	5-10	N/A
Cleaning method	Chemical	Chemical	Chemical	A/C or Chemical	A/C	A/C
Time taken (min)	15-20	15-20	15-20	15-20	15	15
Flushing (litres)	>50	>50	>50	>50	N/A	N/A
Time (min)	20-30	30-40	30-40	20-30	N/A	N/A
Max time (min)	65	80	80	65	>8 hrs	55

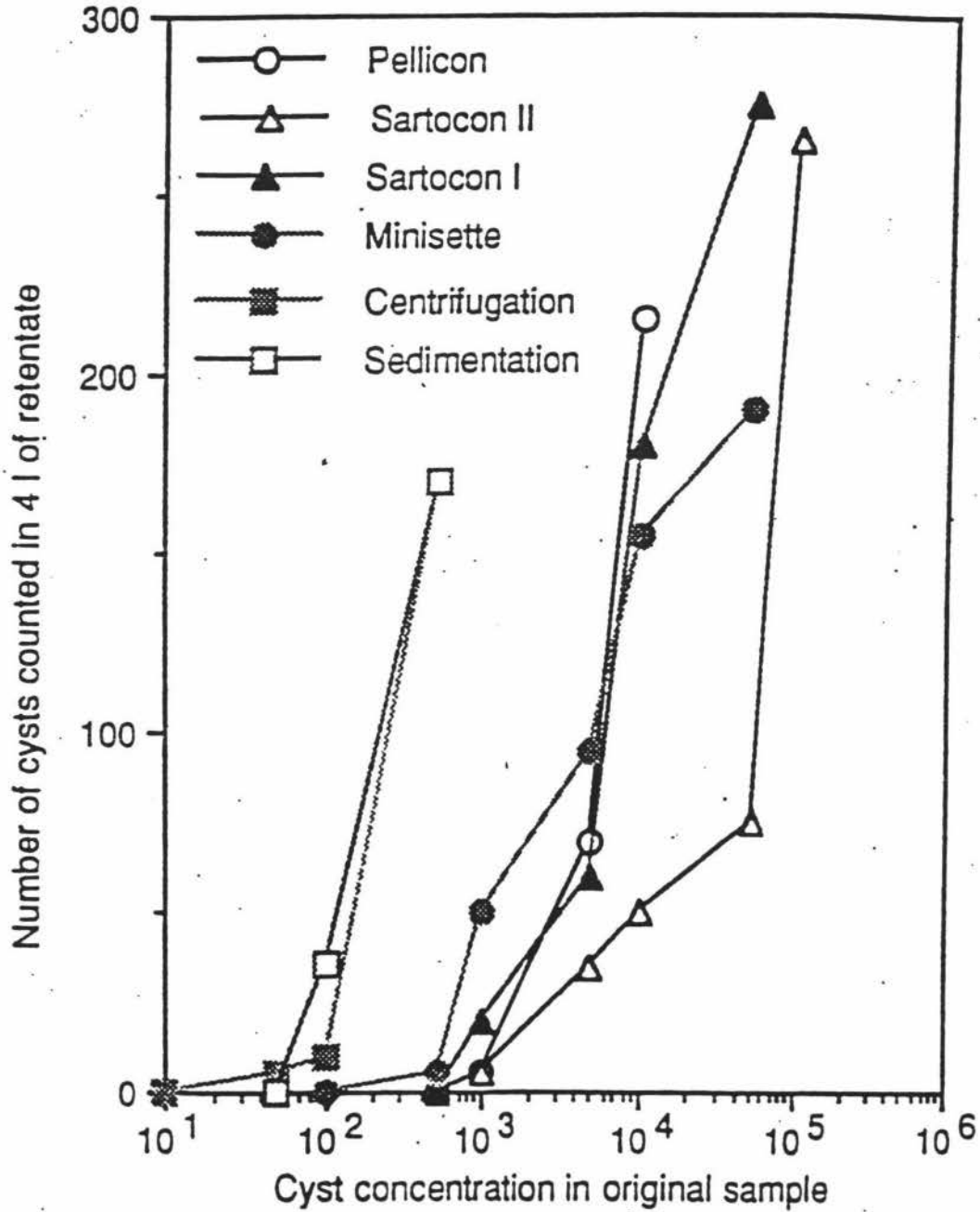
Key

A/C Autoclave

> Greater than the time shown

N/A Not applicable

Fig 12: Average number of *Giardia* cysts counted after concentration



4.4.2 The use of tangential filtration units compared to the standard method of water testing for *Giardia*.

Issac-Renton *et al* (1988) used a tangential filter unit for detection of *Giardia* cysts in water. A four litre sample spiked with cysts representing an environmental sample was used. This experiment was designed to use a large sample of tap water (>380l) to assess the possibilities of using a tangential filter in the field for water testing.

Initially, in these trials a concentration of 10^6 cysts/500l was used following the protocol set out in **Methods** (3.4.2). Concentration of the first barrel was accomplished with a slight build up in pressure across the membrane. This resulted in the second barrel taking longer with a build up across the membrane.

The normal operating pressure was approximately a feed inlet of 20 psi and the retentate outlet pressure of 10 psi. As soon as there was deviance from these, measures were taken to investigate the reason. The feed inlet pressure increased as the experiment progressed. When it started to build up dramatically the pump was stopped to avoid damage to the membrane (the pressure across the membrane cannot exceeded 100 psi).

The retentate valve was closed and the sample was allowed to recirculate over the membrane. Another attempt was made to run the unit. When the pressure approached the maximum that the membrane and pump could withstand, the unit was closed down and the sample flushed out.

Manufacture's cleaning instructions for the membrane were followed and the apparatus reassembled. Concentration of the remaining sample (approximately 100l) was re-attempted. The pressure built up rapidly across the membrane, even though the unit was run with a low retentate and a high transmembrane flow. Approximately 30l of the sample remained. The sediment content

of the water was found to be very high (approximately 10g/100l) so the experiment was ended.

It was resolved that the tangential filtration unit was unable to function under the conditions imposed by this experiment.

4.5 Comparison of staining techniques

4.5.1 Comparison of staining on slides to staining filters on a manifold

When staining directly on to a slide only a very small amount (10-20um) of the sample can be stained. The Jakubowski (1990) method of testing for *Giardia*, recommends the use of a manifold filtration unit allowing staining to occur on a membrane filter. With this unit, up to several millilitres of a sample can be stained. This is advantageous when cysts are present in a concentrated purified water sample in a low numbers.

A trial was undertaken to compare direct slide staining to filter staining . Results are given in Table 15. The slide count used 10um of sample, and the filter method used 100um, so the slide count was calculated for the equivalent amount of sample (by multiplying by 10).

Test numbers 1 to 10, spiked with 100 cysts , for the slide count gave only 2/10 counts. In the other eight tests no cysts were detected. Staining on membrane filters gave a result for each test, and with higher counts than those obtained from the slide.

The manifold filter method gives a much more accurate result and in some cases the manifold filter method detected cysts which were not detected in the slide test.

Table 15. Result of staining on a slide compared to a membrane

Test Number	Slide Count	Membrane Count
1	10	68
2	0	92
3	0	73
4	0	84
5	0	61
6	0	94
7	0	99
8	0	105
9	0	110
10	10	95
11	10	>250
12	0	>250
13	0	>250
14	10	>250
15	0	>250
16	50	>250
17	0	>250
18	0	>250
19	130	>250
20	60	>250

KEY

Test numbers 1-10 were counts from 100 cyst dilution
 Test numbers 11-20 were counts from 100 cyst dilution

4.5.2 Comparison of commercially available monoclonal antibody stains, a polyclonal stain and an iodine stain for *Giardia* identification.

The primary method in identifying *Giardia* from a sample is microscopy, although the use of techniques such as P.C.R. and ELISA, are becoming commercially available. To enhance the viewing of cysts, different stains are available. Many diagnostic laboratories use permanent stains such as trichrome, iodine etc. The main difficulty with these is the length of time necessary for locating and the difficulty in identifying, *Giardia* cysts.

Immunofluorescence techniques using monoclonal and polyclonal antibodies to illuminate the cysts allow for identification because of their enhanced specificity. Although the internal structures still have to be checked, these techniques allow for the quick scanning of slides to identify the fluorescent green cysts.

Another difference between these methods is the amount of sample able to be stained. The trichrome, iodine stains are mainly performed on slides. This allows approximately 10ul of sample to be stained. The use of immunofluorescent techniques, allows staining on a membrane through which the quantity tested depends on the amount of particulate matter present.

Initially, a study of the number of cysts to be identifiable on a slide, as compared to a membrane, was undertaken (4.5.1).

Immunofluorescence techniques using commercially available monoclonal antibody, a polyclonal antibody, and an iodide stain were compared. All but one of the monoclonal antibody stains was designed for use on fecal samples with the one designed for environmental samples also able to detect *Cryptosporidium*.

Results are shown in Table 16. The conventional method of using Lugols iodine yeilded a 1/15 positive results with much of the surrounding material also taking up the stain.

The indirect monoclonal antibody designed for staining directly on a slide detected 4/15 samples positive. Nonspecific staining occured with all the immunofluorescent techniques but was worse with the indirect and polyclonal antibody tests (the iodine was not considered).

The direct monoclonal stained 1/15 as positive with the polyclonal antibody (produced at Massey) staining 3/15 samples positive. Both of these types of stain were performed on a slide sample.

The 'combo kit' monoclonal antibody used for detecting *Cryptosporidium* as well as *Giardia*, stained 12/15 samples positive. This kit is normally used for staining on membranes but in this instance a slide was used.

Table 16. Comparison of staining technique.

#	Source	Lugols Iodine	Indirect Mono	Direct Mono	Indirect Poly	Combo Kit
1	Waiouru	0	1	0	0	3
2	Waiouru	0	0	0	0	0
3	Waiouru	1	8	0	2	10
4	Mt Stewart	0	0	2	1	5
5	Dannivirke	0	0	0	0	0
6	Dannivirke	0	0	0	0	0
7	Dannivirke	0	0	0	0	0
8	Tauranga	0	0	0	0	0
9	Tauranga	0	1	0	0	4
10	River	0	0	0	0	3
11	River	0	0	0	1	1
12	River	0	0	0	0	1
13	River	0	2	0	0	6
14	River	0	0	0	0	3
15	River	0	0	0	0	6
16	Pond + Cysts	2	15	12	8	92
17	Pond	0	0	0	0	0
18	R.O. + Cysts	1	11	8	6	54
19	R.O. water	0	0	0	0	0
20	Commercial +	+	+	+	+	+
21	Commercial -	-	-	-	-	-

Key

Commercial + Positive sample from a commercial kit (same positive used throughout).

Commercial - Negative sample from a commercial kit (same negative used throughout).

Cysts Cysts produced by *in vitro* encystation

+ Cysts were observed

- Cysts were not observed

4.5.3 Monoclonal antibody staining of chemically treated *Giardia* cysts.

When testing in remote areas, normal laboratory cleaning/sterilizing facilities are unavailable. A simple method of inactivating cysts, so that the monoclonal antibody stain would be unable to both recognise, and attach, to the cysts was sought. Success in this search would allow for the flushing of equipment in remote areas (between the sampling) and prevent contamination of further samples. Flushing with chemicals in order to physically remove (or chemically inactivate) the antigen that the monoclonal antibody recognises are two possible ways of achieving the inactivation of *Giardia* cysts.

Six types of chemical were assessed for their use in field trials. The results are detailed in Table 17. Water was also used as a non-treated control, in order to ascertain whether cysts were losing their fluorescent capacity by means other than the chemicals introduced.

All other parameters were kept constant. The same strain of cysts produced previously *in vitro* were used, and all trials were run concurrently. Each concentration step was performed in duplicate for three runs and the results assessed.

A 5M HCl solution had no effect on the ability of cysts to fluoresce, although some cysts appeared merely as shells, indicating that the HCl had had some effect. This occurred after about ten minutes, with more shells becoming apparent as time progressed.

A 5M NaOH solution appeared to have no effect on the ability of the cysts to fluoresce. No effect was observed from either HCL, 75% ethanol, or 3% hypochlorite.

Hypochlorite at 4% and 5% did have an effect on the ability of cysts to fluoresce. Exposure to hypochlorite at 4% for 15

minutes produced a visible dimming of the cysts tested with fluorescent antibodies, there also appeared to be fewer cysts fluorescing. After twenty minutes exposure there was no fluorescence noted.

A solution of 5% hypochlorite caused a change within ten minutes, again with a visible lack in the intensity of fluorescence and a reduction in the number of cysts fluorescing. Following 15 minutes exposure to 5% hypochlorite no fluorescence was seen.

Table 17. Effects of chemicals on the monoclonal staining ability of cysts

Time	3% H	4%H	5% H	75 % ETOH	5M NaOH	5M Hcl	H ₂ O
0	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+
10	+	+	+/-	+	+	+	+
15	+	+/-	-	+	+	+	+
20	+	-	-	+	+	+	+
40	+	-	-	+	+	+	+
60	+	-	-	+	+	+	+
120	+	-	-	+	+	+	+

KEY

- + Had fluorescence
- Had no fluorescence
- +/- Dimming fluorescence

4.6 The Dannivirke Study

The modes of transmission of *Giardia* have been the basis of many studies (Adams 1992). *Giardia* spread has also been documented in New Zealand (Walker et al 1991). The three modes of transmission have been discussed in the **Introduction**. The disease is spread by the fecal/oral route. This may occur through fecal contamination of water (waterborne transmission), between individuals and/or animals (person to person and animal to person transmission) and fecal contamination of foods (foodborne transmission).

The farm involved in this study was 22kms from Dannivirke in the central area of the North Island of New Zealand. Figure 13 shows the farm in relation to Dannivirke with Figure 14 showing the paddocks and general layout of the farm.

In February 1990 the Dannivirke family was diagnosed as having giardiasis (positive fecal samples were confirmed at the pathology laboratory, Palmerston North Hospital). At the end of that year (1990), the family were again diagnosed as suffering Giardiasis.

The family consisted of seven individuals, mother, father and five sons the eldest being 10 years old at the time of infection. The farm ran mainly sheep, with a herd of beef cattle, and also used farm dogs. Other animals included a house dog and cat, hens, and feral animals such as opossums, rabbits, and wild mice. Three of the children went to school with two attending a play group on occasional mornings. The mother also attended and helped at the playgroups.

There were two main tanks on the property, marked on Figure 14, the house tank and farm tank. The house tank held water collected from the roof. When the house tank got low it was

filled from the farm tank. This water was used as drinking water and also in the bathroom, shower and toilet. Before the positive diagnosis this water was not used for the laundry but following the family infections this practice was ammended.

The farm tank water came from the creek which ran along the boundry of the property (marked on Figure 14). It was pumped to a half way tank were it was stored until required at the farm tank. Farm tank water was used from outside hoses and from the laundry at the beginning of this study.

The river was 0.5-1m deep, slow moving, and prone to flooding. It had a steep banks on the opposite side to the pumphouse and was surrounded by brush and scrub. Upstream (approximately 100m) from the pumphouse on the same side was the Willow stream outfall, flowing in over a steep drop. The water of the river was very turbid. The river itself was located right on the boundry of the property running on the neighbours side, the pumphouse was located on the family's property (marked on Figure 14).

Initially, only the farm and house tanks were tested for *Giardia*. Throughout the study these tanks remained test negative (Table 18). At a later stage once the farm supply layout was understood, more extensive water testing was carried out. This included Willow stream and the river which were both found to be positive for *Giardia*.

The sewage from the house went through a septic tank system. Outfall from the tank drained directly on to the cow paddock surface (Figure 14). Situated on a very steep hill the cow paddock drained into the stream. Following rainfall the stream would flow freely to the river. In the dryer months (summer) it the stream did not have a continual flow.

Soil samples were taken at various places from around the sewage outfall and from the flow in to the creek, test results (Table 18) were negative for *Giardia*.

The stream extends approximately 2km before it joins the river. Sheep and cattle grazed the surrounding steep hills, allowing runoff all the way along, except at the point close to where it joined the river. Here it flattened out before a steep drop into the river. Stock yards were also situated beside the stream.

Animal fecal samples were taken and tested for *Giardia*, from around the farm by an independent researcher. Positive samples were found from the dogs, hens, sheep and cattle at different stages throughout this study (Appendix A, Table 30). Opossums and mice were also found positive for *Giardia* in the farm area (Michelle Marino Pers. Comm).

Table 18. Results of the Dannivirke testing.

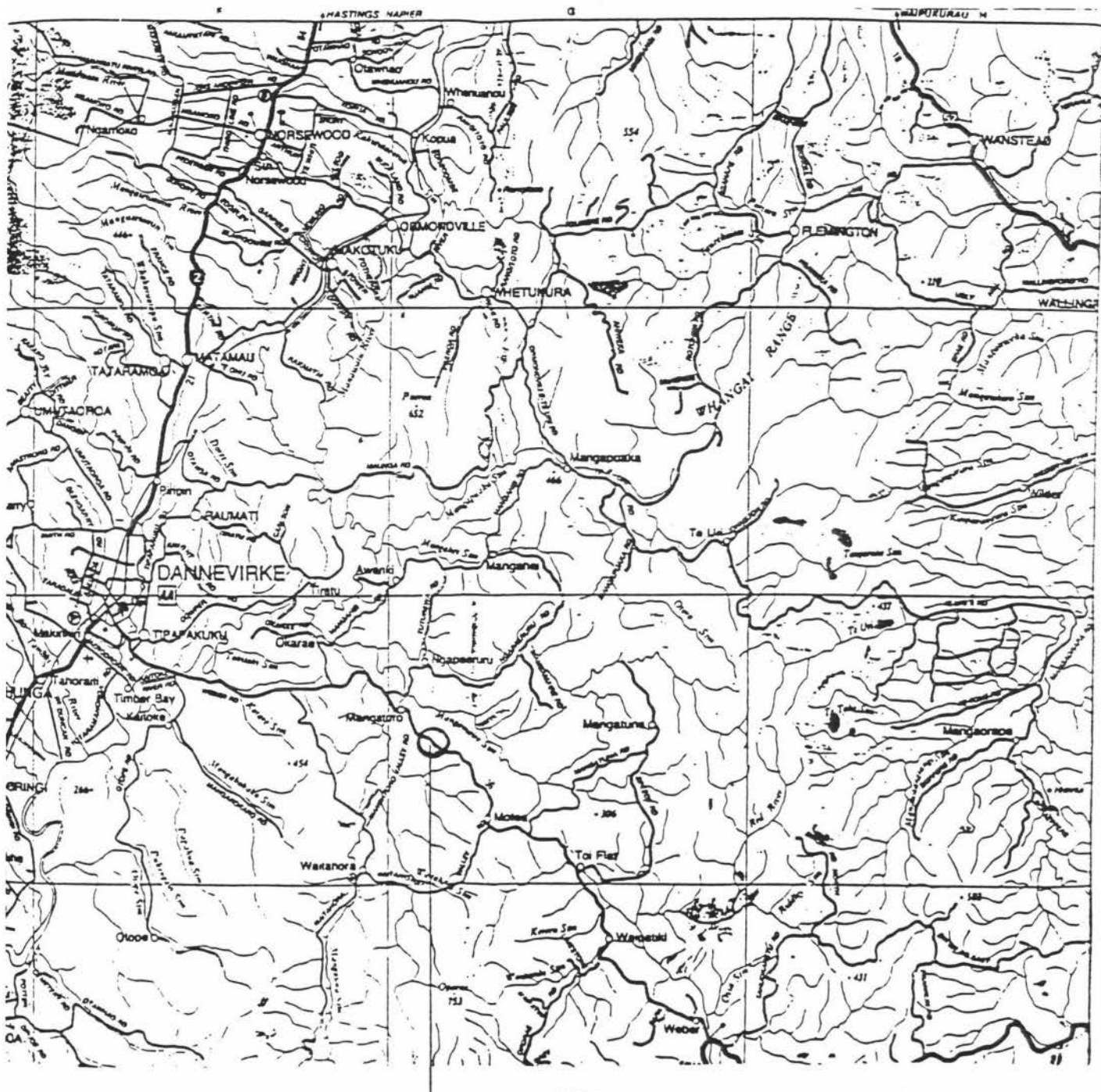
Date	Test Sight	Result	Comment
12/90	House Tank Farm Tank	- -	Rain
7/3/91	House Tank Farm Tank	- -	
11/4/91	House Tank Farm Tank	- -	
10/5/91	House Tank Farm Tank	- -	
13/6/91	House Tank Farm Tank	- -	Heavy Rain
10/9/91	House Tank Farm Tank	- -	
10/10/91	House Tank Farm Tank River Soil - River Soil - Sewerage	- - + - -	Rain
8/11/91	House Tank Farm Tank Willow Creek Soil - Creek Soil - Sewerage	- - + - -	Ocassional Showers

Key

+ Positive for *Giardia*- Negative for *Giardia*

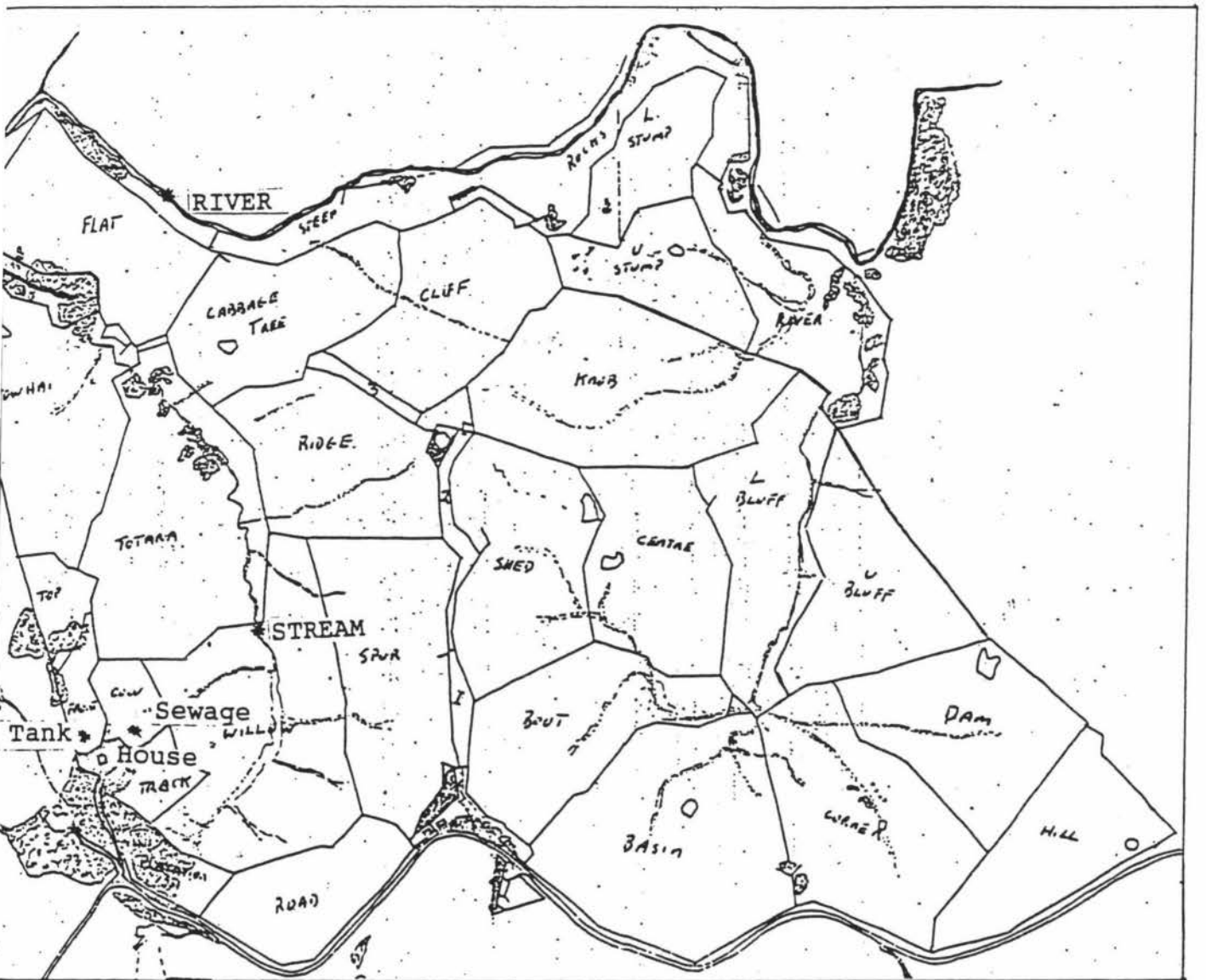
Figure 13. Dannivirke Farm used for field study.

O Farm used in study



O Farm used in study

Figure 14. Lay out of the farm used in the Dannivirke Study.



CONCLUSIONS

5.1 *In vitro* encystment of *Giardia intestinalis*

5.1.1 The effect of bile concentration, time and temperature on encystment induced *in vitro*.

Methods of encystation *in vitro* vary but generally involve the use of increased levels of bile in the medium. Farthing *et al* (1983) discussed how the addition of a low concentration (0.8g/l) of bovine bile or porcine bile to Diamonds TY1-S culture media greatly decreased the generation time from 12 +/- 1.1 hrs to 7.5 hrs. It was found that up to 1g/l promoted growth, but higher levels than this (2g/l) retarded growth.

Farthing *et al* (1985), discussed possible mechanisms by which mammalian bile promoted parasite growth and concluded that biliary lecithin is a likely source of phospholipid for growth since *Giardia* has no way of synthesising membrane lipid.

Monitoring of axenically grown strains of *Giardia* Schupp *et al* (1988) recorded the production of *Giardia* cysts *in vitro*. The amount of bile in the TY1-33 medium was not described but addition of bile to the medium was stated. The cysts were found to be morphologically and immunologically similar to cysts formed *in vivo*.

Further work (Schupp *et al* 1988b) has described that maximum production of cysts *in vitro* was gained when 5g/l of bile was added to the culture medium. The original medium for this culture contained 2.5g/l of bile. Times and temperatures used for this work were not stated.

A supply of cysts was needed to spike samples in order to investigate the sampling and processing techniques connected with detecting waterborne giardiasis. These were initially unavailable through the usual method of concentrating cysts from positive fecal samples. Accordingly, *in vitro* encystation was researched as an alternative.

At the outset we were unaware of optimum times and temperatures, and also unsure of the level of bile that would be required (TY1-S-33 used for culturing had a bile concentration of 0.8g/l). Therefore an experiment was designed to find the optimum conditions needed for the culture strains used.

A bile range of 0-20g/l was used. The temperatures the cultures were kept at whilst incubating in encystation media were 4, 24, 37°C. Length of culturing times was 24-96 hours with cultures being removed every 24 hours.

Our results 4.4.1 showed that the largest amount of morphologically correct cysts were produced at 37°C over 72 hours with 5.0g/l of bile.

The cysts were confirmed on morphological characteristics using phase contrast microscopy. Partially formed cysts, and cysts where the interior was empty, were not counted. Trophozoites were also observed, although they appeared non viable.

Schupp et al (1988) had conducted morphological studies on the *Giardia* cysts they obtained. They found the cysts had two distinct morphological types, based on the cytoplasmic appearance of non viable as well as viable cysts. Nonviable cysts contained granular-looking cytoplasm and incorrect morphology. They had an intact cell wall, but the cytoplasmic contents were shrunken. They were also seen to have an enlarged peritrophic space.

Viable *Giardia* cysts had clearly defined cyst walls, a normal peritrophic space, and the presence of 2-4 nuclei and profiles of the cytoplasmic organelles including flagella, axonemes and elements of the adhesion disk.

Similar differences were noted in the cysts used in this study when viewed under the microscope. At the 4,5 and 6g/l bile

concentration at 37°C for 72 hours, approximately equal numbers of morphologically correct cysts and incorrect cysts were seen. At the same bile concentration and temperature, but 48 hours later, (incomplete cysts having a cyst wall but not being morphologically correct) predominated, with more trophozoites also present.

At the lower temperatures the monolayer of trophozoite growth was destroyed within 48 hours. This was particularly noticeable at 4°C. Few cysts but many unattached trophozoites were observed.

As the bile concentration increased to 20g/l the monolayer decreased with few trophozoites attached. Even at the optimum temperature of 37°C the monolayer in the cultures disappeared and the cysts became morphologically incorrect.

Faubert et al 1991 found that when they induced encystment, most encysting trophozoites remained attached until they started to round up (>24 hours following induction). The researchers forced cysts to detach as early as 12 hours following induction, and found they would not re-attach.

Although a high concentration (2.5g/l) of bile was used for growth in the methods of Schupp et al (1988) when compared to the amount used by this study (0.8g/l), the amount of bile actually needed to induce encystment was found to be the same, 5g/l for 72 hours at 37°C.

5.2 Excystment of *Giardia intestinalis*

5.2.1 *In vitro* excystment of cysts produced *in vitro*, and in cysts of water and animal origin

Cysts produced using the Schupp et al (1988) method of encystment (methods 3.1.1) demonstrated that cysts could excyst, and form an

axenic culture, when as few as 10 cysts were used. The cysts were first purified on a sucrose gradient then observed under a phase contrast microscope at 1000x magnification to be morphologically correct. They had been stored for seven days at 4°C prior to excystation to allow maturation of the cyst to occur as suggested by Gillin et al (1988).

Feelys (1986) method of excystation with modification from Tonks (1988) was used to excyst Bris/83/HEPU/106 and Hast/87/MUGU/68 strains of cysts, both being originally isolated from humans. Brown et al (1992) had reported successful excystation using this method.

The least number of cysts required to produce an axenic culture was ten cysts. Cultures resulted in two out of six attempts for both strains. When 50 cysts were used all attempts at culturing, for the Bris/83/HEPU/106 strain were successful. For the Hast/87/MUGU/68 strain four (from six) attempts produced cultures. When 100 cysts were used all six attempts led to establishment of cultures.

Infectivity studies by Schaefer et al (1991) using Mongolian Gerbils (*Meriones unguiculatus*) demonstrated that when ten cysts were inoculated 1/10 gerbils developed an infection. When 80 cysts were inoculated, 4/10 were infected, when 100 cysts were inoculated the number infected was again 4/10. This trend continued until at 10,000 cysts all ten of the gerbils became infected. These results indicated the low number of cysts produced *in vivo* that are required for an infection to occur.

When Schupp et al (1988) inoculated the 2.75×10^5 cysts produced *in vitro* into gerbils and mice, infections resulted in 30% of mice and 37% of gerbils. Fecal cysts were never seen, but infection was demonstrated by the presence of intestinal trophozoites at necropsy.

Meyer (1976) Kasprzak and Majewski (1985) reported difficulties in establishing an axenic culture. Kasprzak and Majewski (1985) reported taking several weeks for cultures to become established following excystation, Brown et al (1992) reported the same results.

This experiment observed trophozoites in the cultures several days following excystation. Approximately 8-10 days later a complete monolayer had formed in the tube. The ability of each of the strains to form a monolayer so rapidly was interesting. It seems that having been cultured axenically prior to encystation *in vitro*, the cyst was able to adapt more rapidly to axenic culture than a wild strain.

Lower numbers of cysts were required to form a culture in our work when compared to Schupp et al (1988). We believe that establishment of a culture *in vitro*, before encystment *in vitro* adapted the *Giardia* strain more readily to *in vitro* culture than to *in vivo* culture. Schupp et al (1988) suggested that keeping cysts at 37°C for up to three days had affected their viability, however we did not find this to be the case.

Using the same method of excystment as in 4.2.1 it was not possible to establish any cultures from animal and water samples other than the positive controls (results 4.2.2). Only one of the samples had been viewed with adhered trophozoites. The Dog 1 cultures were lost following contamination. Cysts produced *in vitro* did, however produce cultures using this method.

Researchers have reported successful attempts to excyst animal strains found in fecal matter. Erlandsen (1990) reported on attempts at culturing *Giardia* cysts from the great Blue Herron. They had been successful but few cysts had lasted beyond four weeks. Brown et al (1991) reported that following excystment it took several weeks for the cultures they excysted to establish and form a monolayer.

No reports of axenic culturing of waterborne cysts has been reported. Methods of staining to assess viability are now available, but normally so few cysts are detected in water that it is hard to obtain a reasonable number (>1000 cysts) in order to attempt excystment.

In our work (4.2.2), 10^6 cysts produced *in vitro* were put through the sampling procedure (methods 3.3) and once recovered, excystment was attempted, leading to the formation of axenic cultures, with monolayers occurring in 8-10 days. This demonstrates that the sampling procedure used enables the recovery of viable cysts.

5.3 The Standard Method of Water Testing

5.3.1 Sensitivity of the Standard Method of Water testing

There are many factors which influence the recovery of *Giardia* cysts from water. These include the method of sampling and processing used, methods of staining, the amount of additional suspended material, aquatic organisms and the actual distribution of cysts in the water sampled.

Sampling and processing methods have already been discussed, as well as with the variations between laboratories due to factors like the difference in 'set up' and equipment, even though the Standard Method is followed.

Methods of staining and counting cysts also vary. Most laboratories now use fluorescent monoclonal antibody stains to allow easy recognition, counting, and less obscuring by surrounding particles. Fatigue of the microscope operator is now reduced due to the reduction of time spent trying to locate and identify the cysts. Some laboratories still use alternative stains such as iodine, but such methods are more difficult to use, requiring greater experience from the operator.

The amount of suspended solids in the stained and concentrated sample can obscure the cysts. However the use of Monoclonal antibodies make the cyst more easily visible. In these trials tap water was used and spiked with cysts but still a lot of particulate material was recovered (20g in each pellet).

Riggs et al (1988) conducted recovery work using membrane filter techniques. They found that when 0.7-1 cyst/l was seeded, recovery ranged from 27-41% with an average of 35%. When 0.1-0.2 cysts/l were seeded recovery varied from a low of 27% to 69% with an average of 47%.

When 1 cyst/l was seeded this study achieved a 10% recovery rate compared with 35% average recovery by Riggs et al (1988). The membrane method involved backflushing to recover cysts. This procedure loses fewer cysts than the fibre cartridge washing method (which is part of the standard method) as cysts get caught in the discarded filter fibers and are lost.

Membranes are not routinely used in the sampling of environmental water, due to the amount of solids present which then block up the filter.

Issac-Renton et al (1986), when evaluating the Standard Method gave an average recovery rate of between 5-15%. The recovery in our trials (1992) was between 10-40%.

5.4 Use of Tangential Filter Units

5.4.1 Evaluation of tangential filtration compared to centrifugation and the standard method of water testing.

The Standard Method of testing for *Giardia* in water involves sampling using a cartridge filter (1µm nominal porosity); processing the sample by breaking down the fibre filter; and washing in 4-5l of wash solution to remove the trapped cysts. This wash solution is normally concentrated by centrifugation

(or sedimentation) with the sample then purified on a sucrose gradient before staining and enumeration (Standard methods, Jakubowski 1990).

In this trial, investigating the use of tangential filtration was hoped to shorten the process by combining the sampling and concentration steps. The tangential filtration unit would sample the water (>380l) and concentrate it down into a small volume (250-300ml).

Evaluation of tangential filtration was done in two stages. The first compared it to centrifugation. This method reduces the 4-5l of wash water from the fibres to a pellet. This would give information on the efficiency of the method in terms of cyst recovery, and also whether it could function in spite of the sediment found in natural water samples.

The second stage was to compare tangential filtration with the complete Standard Method, and assess performance and recovery rate. Results for the comparison to centrifugation are seen in 4.4.1. Results for the comparison to the Standard Method are seen in results 4.4.2.

The only reported work in this area is by Issac-Renton et al (1986). The Millipore Pellicon Tangential Filtration Unit was compared to the Standard Method of sampling testing for *Giardia*. Six different strains of *Giardia lamblia* were tested. Inocula containing approximately 10^2 to 10^3 cysts were used for each strain in each run. The overall recovery of inoculated cysts was 31% with similar recovery rates for four of the six strains.

In this experiment (1992) we used a range of dilutions. When the recovery rate for the 10^2 and 10^3 cysts is compared to Issac-Renton et al (1986), recoveries recorded here are much lower.

With the Millipore Pellicon unit (the same one Issac-Renton et al (1986) used) no cysts were detected at this level giving a

recovery of 0%. At 5×10^3 cysts/4l only five cysts were detected giving a recovery of 0.01%, at 10^4 cysts/4l, 70 cysts were detected with a recovery of 0.7%.

The Sarticon II (with the same size membrane) gave similar results to the Millipore unit. At 10^2 and 10^3 cysts/4l none were recovered, at 5×10^3 , 35 cysts were detected when 5×10^3 cysts, a recovery of 0.7%.

Units with smaller membranes gave an improved recovery rate. The Filtron minisetete detected 5 cysts at 5×10^2 a recovery of 1%, whilst at 10^3 , 50 cysts were detected, a recovery of 5%. These are still lower percentage recoveries than those reported by Issac-Renton et al (1986).

Centrifugation and sedimentation yielded better recovery rates. Centrifugation detected 5 cysts at 50 cysts/4l (10% recovery). At 10^2 cysts it was possible to detect 10 cysts, a 10% recovery; whilst at 5×10^2 cysts/4l, 170 cysts were detected a recovery of 34%. Sedimentation revealed 35 cysts at the 10^2 cysts/4l level, a recovery of 35%.

Issac-Renton et al (1988) tried to establish the loss of cysts within their system by rinsing 10% buffered citrate-polysorbate detergent through the system to recover trapped cysts. Cysts were recovered in the sediment from the membranes and from the silicon tubing.

Environmental waters contain sediments of varying amounts depending on the water source and flow. Issac-Renton et al (1986) used distilled water in their trials, which would have contained no sediment. Evaluation of the tangential units ability to function using water containing sediment was assessed. Ground up soil was added in 5g and 10g amounts to 4l of water. These samples were then put through the different methods of concentration. The Sarticon II filter was not evaluated due to the pump (with the unit) not being able to function at the higher pressures required by the presence of sediment.

The smaller surface area units (Filtron and Sarticon minisettes) built up pressure until they were about to exceed the membrane limits, even though the sample was being circulated with a very high retentate. Only the Millipore Pellicon Unit was able to process the sample. The pressure also built up in this unit but the unit was still able to function. Sedimentation and centrifugation were unaffected by the sediment due to the processes not using filters.

Results of concentrating tap water had shown that it contained between 1-5g/100l of sediment. River water and other environmental waters contain higher levels than this, and would cause problems for tangential filtration.

The time taken to process samples was evaluated for commercial reasons. Sedimentation took the longest time, more than eight hours. The smaller surface area filters were next, taking longer to flush through the 50l of rinse water than the larger units. Centrifugation took the shortest time overall.

We concluded that the tangential unit's sensitivity and inability to function would prevent its use in the field. Also the unit's weight and the need for a power source to drive it, were disadvantages compared to the sampling apparatus presently in use.

5.5 Comparison of staining techniques

5.5.1 Comparison of staining on slides compared to staining filters on a manifold

Chang and Kabler (1956) used a membrane filtration method for concentrating and detecting the cysts of *Entamoeba histolytica* by membrane filtration. This method has also been used for quantifying bacteria and algae in water (Issac-Renton et al 1986).

Several researches have used membrane filtration to concentrate *Giardia* but they rinsed or backflushed the membrane to remove the cysts prior to staining (Shearer and Lapham 1988). Spaulding *et al* (1983) reported on a method of quantifying *Giardia* cysts by staining on a membrane filter. They found the procedure to be reliable for the detection and recovery of cysts, and also in determining cyst densities. In the Spaulding research (Spaulding *et al* 1983) xylene was used to clear the filters following staining.

The method (Spaulding *et al* 1983) also allowed for a greater amount of sample to be stained, although this is also dependent on the particulate nature of the sample. Using the slide method only <20ul can be stained compared to several millilitres using a filter. The efficiency of the membrane filter technique (Results 4.5.1) is reflected in more accurate results.

Therefore cyst counts were a lot lower when estimated from a slide count as opposed to counting off a filter. Other researchers have reported this. Issac-Renton *et al* (1986) used a membrane filter and staining method for samples during trials with a tangential filter unit and recommended its use for accuracy. Jakubowski (1990) in the EPA method describes a manifold method of staining filters allowing for samples to be stained concurrently.

Drinking water samples usually contain a very low level of cysts. The filtration method allows a larger volume of water to be tested, increasing the chance that the cysts will be detected. This is also now enhanced with the use of monoclonal antibody stains which fluoresce the cyst making it easier to detect.

5.5.2 Comparison of commercially available monoclonal antibody stains, a polyclonal stain, and an iodine stain for *Giardia*.

The use of monoclonal and polyclonal antibody immunofluorescence techniques have enhanced methods for detecting *Giardia* in environmental samples. Unlike fecal samples environmental water samples usually have a low concentration of cysts. Iodine and other similar types of stain give no real contrast to the background material in suspension. However as with immunofluorescent techniques the stained cyst contrasts with the background (Sauch 1985, Rose et al 1989)

This contrast means that the researcher has the ability to scan samples more quickly, and at a lower magnification, until a cyst is found and identified (Sauch 1985, Rose et al 1989, Quinones et al 1988).

Work is now being undertaken to see if the monoclonal antibodies can be made more specific, in order to differentiate between animal and human cysts. Riggs et al (1984) reported that one monoclonal antibody tested would only react with human derived *G.intestinalis* cysts. When it was challenged with 647 animal derived cysts it reacted with only 4 of the specimens.

Another advantage of monoclonal antibodies is the ability to stain on membranes as opposed to slides. Membranes allow a larger volume of sample to be tested depending on how much particulate matter is present. Staining on a slide only allows a small volume of material to be stained. Never the less, slide staining is ideal for fecal samples, were the volume of cysts present is large and therefore easily detectable. Membrane staining is preferred method for environmental samples. This conclusion is verified by the results obtained from slides compared to the manifold filters. Other researchers report that detection in environmental

samples was much greater using the manifold filters (Rose et al 1989).

These results were observed when cysts were added RO water and pond water. In most cases where monoclonals were used on a slide the number revealed was ten times less than the actual concentration in the sample. This was demonstrated in the results where the combo kit detected cysts in many samples in which the other kits had failed.

Lugol's iodine stained very few cysts. This stain also tends to be taken up in the background material. An experienced eye is needed to be able to routinely identify cysts when there are few present (Rose et al 1989).

The indirect monoclonal antibodies allowed relatively good detection taking into account the amount of sample stained on a slide. Nonspecific staining occurred in most samples. It was thought more likely to occur with the indirect monoclonal antibodies, but was found to occur just as often with the direct monoclonal antibodies. Crustaceans and algae seemed to be most regularly stained.

The direct monoclonal antibodies stained fewer samples positive (4/15) compared to 10/15 with the combo kit. The indirect polyclonal antibody stained 3/15 which was less than that of the indirect monoclonal antibody(4/15).

5.5.3 Monoclonal antibody staining of chemically treated cysts

With the testing of water at remote sites for *Giardia*, it is difficult to ensure that no cross contamination occurs between tests. Standard Methods suggests flushing out with hot water (60⁰C or higher) before sampling at another site. This is not always practicable due to the amount of water required and the limited means of heating it.

A method was needed to prevent the monoclonal antibody from recognising cysts from another sample. The only way this could be achieved was by destroying the antigen which the monoclonal recognised. One factor that had to be considered was that different companies develop monoclonal antibodies to react to different antigens on the surface of the cyst.

It was hoped that by destroying the antigen recognised by the antibody (using a chemical), when the next sample containing that antigen, was put through the filter unit only cysts from that water sample would be recognised.

The 3% hypochlorite solution did not destroy the antigen which the monoclonal recognised and after 120 minutes exposure to the chemical no fluorescence was lost. Exposure to the 4% hypochlorite solution for 15 minutes showed increased dimming, and after 20 minutes no fluorescence was noted. At a concentration of 5% hypochlorite, dimming occurred at 10 minutes exposure with no fluorescence remaining after 15 minutes.

Rose *et al* (1989) found that cysts could not be detected using their monoclonal antibodies after exposure to approximately 5000mg/L of sodium hypochlorite. The tests used a range of concentrations of sodium hypochlorite (0,5,50,500,2500,5000 and 10000mg/L) with results showing the number of cysts diminishing as the sodium hypochlorite concentration was increased. There was a large drop in the number of cysts at 500mg/L hypochlorite with no cysts detected at 5000mg/L hypochlorite after 20 minutes exposure.

In our study 75% ethanol had no effect on the ability to stain the cyst with a monoclonal antibody. Ethanol is used in the EPA staining method for dehydrating the cyst following staining using a monoclonal antibody and Evans Blue counterstain (Jakubowski 1990).

Sodium hydroxide at 5M concentration had no effect on the staining ability of the monoclonal antibody indicating that the antigen recognised by the antibody was not affected.

Low pH is used to excyst *Giardia* cysts. As part of its normal lifecycle, the cyst must pass through the stomach which contains hydrochloric acid which may in turn help in excystment. However, hydrochloric acid had no effect on the antigens on the cysts recognised by the monoclonal stain.

Water was used as a control test. Over short periods (up to 120 minutes) water did not affect the cyst. This is supported by the fact that a number of outbreaks occur through waterborne transmission. Rose et al (1989) found that cysts stored in water for 20-22 weeks declined by 67% when stained with monoclonal antibodies.

Other chemicals have been assessed for their ability to inactivate the cyst. Much research has gone in to the use of chlorine, especially bleaches (Sauch and Berman 1991). Quaternary ammonium compounds and monochloramine have also been assessed (Hoff 1988, van Roodselaar 1988)

The resources were not available to explore whether or not the cysts were still viable following chlorine bleach treatment, but this is a promising area for further research.

For instance, if a 5% sodium hypochlorite solution were used as a cleaning method for approximately 20 minutes (to ensure cyst antigens were destroyed), followed by a rinsing step, with some of the water to be tested, it may ensure no cross-contamination between samples.

5.6 The Dannivirke Study

The cause of the initial *Giardia* outbreak and subsequent reinfection, of the Dannivirke family could not be established but several points were noted concerning these infections. Transmission could have been by any of the previously described modes, waterborne, person to person, animal to person or foodborne.

Both of the watertanks on the property were consistently negative when they were tested. However when the river was tested it was found to be positive. This water was pumped up to a holding tank halfway on its journey to the farm tank.

These tanks were deep and the only access to the water was through an opening in the roof of the tank. During summer it is possible for the house tank to get very low. Water is then pumped across from the farm tank to restore the level. During the current study the tank never became low enough to warrant supplementing from the farm tank; nor was it possible for the pump on the sampling apparatus to reach the bottom. When the pump was pushed down as far as it could go sediment blocked the filter rapidly. The resulting sample then tested negative for *Giardia*.

Sewage from the property was treated through a sewage tank system, and drained onto the cow paddock. This led to two potential threats, the paddock drained to a stream in the bottom of a steep gully which flowed into the river upstream from the intake to the pump, which was used to fill the farm tank. The stream tested positive for *Giardia*.

Samples collected in the cow paddock from the faeces of sheep and cattle had tested positive for *Giardia*. Had cross transmission of *Giardia* occurred to infect the animals or had the animals been infected initially and contaminated the stream and in turn the river? The Standard Method of sampling and testing cannot identify animal from human strains of *Giardia*.

The house tank is filled from rain water off the roof. Trapping and testing of wild animals in the area, and also mice and opossums in the area, had found *Giardia*. Had these droppings contaminated the house tank?

Harley (1988) in her study of an outbreak of Giardiasis in an Alberta Hutterite colony ruled out waterborne transmission and

implicated person to person transmission as being the most likely cause of 37% (76 persons) in the colony becoming infected.

Since the first reported outbreak in 1977 (Flanagan 1992) childcare centres have frequently been implicated in outbreaks. This has been linked to close physical contact and poor hygiene naturally exhibited by children (Bartlett et al 1991). This is also seen as important because of its spread into the community, with secondary spread to family members occurring in 17-47% of these cases from children (Flanagan 1992).

The Dannevirke family consisted of five young children. Two went to school three went to child care. The infection could have been contracted either of these locations, especially the playgroup, and passed to other siblings and the parents.

There were no reports of other children in the area being infected although this could be due to the asymptomatic nature of the infection, or to the infections not being reported. (Walker et al 1991)

Some of the domestic animals on the farm had tested positive for *Giardia*. Reports from America indicate cross species transmission does occur. Erlandsen et al (1988) studied the transmission of cysts of human origin to beavers and muskrats resulting in infections. Roach and Wallis (1988) studied the transmission of *Giardia intestinalis* from human and animal sources in wild mice.

Foodborne contamination may occur. The farm tank water was used to water vegetables in the garden. If the vegetables were uncooked this may have contaminated the consumer.

APPENDIX A: DATA FOR RESULTS

Table 19. Data for encystment of the Bris/83/HEPU/106 strain incubated at 4 degrees C

Time hr	Bile Concentration (g/L)							
	0.0	0.8	4	5	6	10	15	20
24	0.0	0.0	0.0	1.7	0.0	0.0	0.0	1.7
	1.7	0.0	1.7	0.0	0.0	0.0	0.0	1.7
48	0.0	0.0	0.0	1.7	0.0	1.7	0.0	1.7
	0.0	1.7	0.0	0.0	1.7	0.0	1.7	1.7
72	0.0	1.7	1.7	1.7	1.7	1.7	1.7	1.7
	0.0	1.7	1.7	0.0	0.0	0.0	3.3	5.0
96	1.7	1.7	0.0	1.7	0.0	0.0	0.0	1.7
	0.0	0.0	0.0	0.0	3.3	0.0	3.3	0.0

Cyst formation (x1000) at Bile Concentrations

Table 20. Data for encystment of the Hast/87/MUGU/106 strain incubated at 4 degrees C

Time hr	Bile Concentration (g/L)							
	0.0	0.8	4	5	6	10	15	20
24	1.7	1.7	1.7	0.0	5.0	1.7	5.0	1.7
	0.0	0.0	3.3	1.7	0.0	3.3	0.0	0.0
48	0.0	1.7	1.7	1.7	1.7	0.0	1.7	1.7
	1.7	0.0	3.3	0.0	0.0	0.0	3.3	1.7
72	1.7	3.3	0.0	0.0	0.0	3.3	1.7	5.0
	0.0	0.0	1.7	3.3	1.7	0.0	5.0	3.3
96	0.0	1.7	1.7	0.0	0.0	0.0	1.7	0.0
	0.0	0.0	1.7	1.7	0.0	3.3	1.7	1.7

Cyst formation (x1000) at Bile Concentrations

Table 21. Data for encystment of the Bris/83/HEPU/106 strain incubated at 26 degrees C

Time hr	Bile Concentration (g/L)							
	0.0	0.8	4	5	6	10	15	20
24	0.0	1.7	0.0	1.7	1.7	3.3	3.3	1.7
	0.0	0.0	3.3	1.7	0.0	1.7	1.7	1.7
48	1.7	3.3	0.0	3.3	0.0	1.7	6.7	3.3
	0.0	0.0	1.7	0.0	0.0	1.7	1.7	1.7
72	0.0	0.0	0.0	3.3	5.0	5.0	0.0	0.0
	1.7	0.0	3.3	0.0	3.3	0.0	1.7	1.7
96	0.0	0.0	1.7	3.3	0.0	1.7	0.0	1.7
	0.0	1.7	0.0	0.0	1.7	0.0	5.0	6.7

Cyst formation (x1000) at Bile Concentrations.

Table 22. Data for encystment of the Hast/87/MUGU/106 strain incubated at 26 degrees C

Time hr	Bile Concentration (g/L)							
	0.0	0.8	4	5	6	10	15	20
24	0.0	0.0	0.0	3.3	1.7	5.0	1.7	1.7
	1.7	0.0	1.7	0.0	1.7	0.0	1.7	0.0
48	0.0	0.0	3.3	3.3	3.3	1.7	0.0	0.0
	5.0	1.7	0.0	3.3	1.7	0.0	1.7	5.0
72	0.0	3.3	5.0	1.7	1.7	1.7	1.7	0.0
	0.0	1.7	1.7	5.0	3.3	5.0	0.0	1.7
96	1.7	0.0	3.3	1.7	0.0	0.0	0.0	1.7
	0.0	1.7	1.7	3.3	0.0	0.0	1.7	0.0

Cyst formation (x1000) at Bile Concentrations

Table 23. Data for encystment of the Bris/83/HEPU/106 strain incubated at 37 degrees C

Time hr	Bile Concentration (g/L)							
	0.0	0.8	4	5	6	10	15	20
24	1.7	0.0	5.0	5.0	8.3	1.7	5.0	5.0
	3.3	3.3	8.3	3.3	3.3	10.0	10.0	1.7
48	0.0	5.0	10.0	13.3	18.3	5.0	20.0	21.7
	0.0	1.7	3.3	20.0	15.0	10.0	16.7	8.3
72	1.7	1.7	10.0	26.7	8.3	20.0	16.7	5.0
	0.0	6.7	5.0	18.3	13.3	15.0	6.7	18.3
96	1.7	3.3	5.0	10.0	20.0	5.0	13.3	18.3
	3.3	1.7	6.7	13.3	11.7	11.7	8.3	10.0

Cyst formation (x1000) at Bile Concentrations

Table 24. Data for encystment of the Hast/87/MUGU/106 strain incubated at 37 degrees C

Time hr	Bile Concentration (g/L)							
	0.0	0.8	4	5	6	10	15	20
24	0.0	3.3	3.3	1.7	0.0	0.0	3.3	5.0
	3.3	1.7	6.7	0.0	0.0	6.7	5.0	3.3
48	1.7	3.3	5.0	26.7	21.7	6.7	6.7	0.0
	0.0	5.0	6.7	18.3	10.0	8.3	5.0	8.3
72	1.0	1.7	6.7	16.7	10.0	10.0	6.7	3.3
	1.7	3.3	8.3	13.3	18.3	3.3	10.0	1.7
96	0.0	0.0	0.0	6.7	3.3	1.7	3.3	5.0
	0.0	5.0	5.0	5.0	5.0	10.0	6.7	8.3

Cyst formation (x1000) at Bile Concentrations

Table 25. Data for attempted excystment of animal origin cysts to give continuous culture.

Source	Est Cysts	Stain	State	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15																																																																																																																																																																																																																																																																																																																																																																																																															
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			C	-	-	-	-	-	-	-	-	-	-						Dog 2	10000	P	T	+	+	+	-	+	-	+	-	+	+	-	+	+	+	-				C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Cat	1000	P	T	-	-	-	-	-	-	-	-	-	-									C	-	-	-	-	-	-	-	-	-	-						Opposum 1	1000	P	T	-	-	-	-	-	-	-	-	-	-									C	-	-	-	-	-	-	-	-	-	-						Cow	1000	P	T	-	-	-	-	-	-	-	-	-	-									C	-	-	-	-	-	-	-	-	-	-						Sheep 1	10000	P	T	-	-	-	-	-	-	-	-	-	-									C	-	-	-	-	-	-	-	-	-	-						Opposum 2	1000	P	T	-	-	-	-	-	-	-	-	-	-									C	-	-	-	-	-	-	-	-	-	-						Sheep 2	1000	P	T	-	-	-	-	-	-	-	-	-	-									C	-	-	-	-	-	-	-	-	-	-						Opposum 3	1000	P	T	-	-	-	-	-	-	-	-	-	-									C	-	-	-	-	-	-	-	-	-	-						Mouse	10000	P	T	-	-	-	-	-	-	-	-	-	-									C	-	-	-	-	-	-	-	-	-	-						Cysts	10000	P	T	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+																			
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Key

- + Growth seen
- No growth
- T Trophozoites
- C Cultures

Table 26. Data for attempted excystment of water origin cysts to give continuous culture.

Source	Stain	State	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
River 1	P	T C	-	-	-	-	-	-	-								
River 2	P	T C	-	-	-	-	-	-	-	-	-						
River 3	P	T C	-	-	-	-	-										
River 4	P	T C	-	-	-	-	-	-	-								
River 5	P	T C	-	-	-	-	-	-									
River 6	P	T C	-	-	-	-	-	-	-	-	-						
River 7	P	T C		-	-	-	-	-	-								
River 8	P	T C	-	-	-	-	-	-	-								
River 9	P	T C	-	-	-	-	-	-									
River 10	P	T C	-	-	-	-	-	-									
S-Cysts 1	P	T C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S-Cysts 2	P	T C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+ Control	P	T C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
- Control	P	T C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Key

- + Growth seen
- No growth
- T Trophozoites
- C Cultures

APPENDIX B: STAINS

The following stains and instructions were used;

Lugols Iodine

Potassium Iodide	10g
Powdered Iodine	5g
RO water	100ml

The potassium iodide was dissolved in the RO water. Powdered iodine was slowly added while stirring. This was then filtered through filter paper and placed in a brown stoppered bottle. Due to breakdown of the iodine it had to be used within three weeks of being prepared.

Polyclonal Antibody Stain

Refer to Chan (1992)

For information on the commercial monoclonal stains see attached copies of instructions.



MERIFLUOR™ Giardia
 Indirect Immunofluorescent Detection Procedure
 Catalog # 230025

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OTHER MERIDIAN PRODUCTS	MERIDIAN CATALOG #
Merifluor™ - Cryptosporidium	230012
Para Pak 1V PVA Formalin	960412
Para Pak 10% Formalin Vials	400101
Para Pak Wettable's Trichrome Slides	970120
Para Pak Macro-Con™	360212

For assistance please call our Technical Service Department toll free at 1-800-543-1010 between the hours of 9 A.M. and 5 P.M. Eastern Standard Time. In Ohio call collect (513) 271-3700.

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LIMITATIONS OF THE PROCEDURE

The MERIFLUOR-Giardia is an in vitro diagnostic procedure to aid in the diagnosis of Giardiasis infection. While the presence of Giardia is often associated with diarrhea and vomiting, asymptomatic cyst shedding has been documented in adults. The presence of cysts in a stool specimen does not preclude the existence of other microorganisms or another underlying condition as the causative agent of a patient's symptoms. For these reasons, appropriate concurrent testing for other etiologic agents should be considered (1, 9, 10, 11, 12, 13, 14).

EXPECTED VALUES

Patients presenting with clinically active Giardia infections are expected to yield a positive test result when cysts are present at detectable levels. Typically, patients in an active disease state shed cysts in moderate to large numbers. Because the number of cysts shed on a given day may vary greatly, every specimen is collected from a patient on alternate days. Some patients may continue to excrete cysts in small numbers for greater than two weeks after complete clinical recovery (4, 8, 9).

Variances in the rate of positivity may be expected due to age, location, and general health conditions of the patient population under study (6).

PERFORMANCE CHARACTERISTICS

A total of 508 stool samples were submitted for evaluation of ova and parasites to a County Health Department located in a large southern city. Of these, 113 samples were positive for Giardia. The evaluation compared to the stain procedures for Merifluor-Giardia, all positive results by both or either method were tested by Para Pak Wettable's Trichrome stain technique. The trichrome stain acted to resolve any discrepancies between home or Merifluor Giardia staining. The Merifluor-Giardia test demonstrated a clinical sensitivity of 97.4% and a specificity of 100%. The positive values of a Merifluor-Giardia Positive and Negative test were 100% and 99.2% respectively; the efficiency was 99.4%. In contrast, the clinical sensitivity of the trichrome method was 88.7% (15).

Other organisms found in stool specimens or other environments did not demonstrate fluorescence using the Merifluor-Giardia test (15). When evaluated by Merifluor-Giardia the following organisms did not fluoresce:

1. **Flagellates:**
 Entamoeba histolytica
 Entamoeba coli
 Entamoeba histolytica
 Isospora belli
 Isospora nana
 Cryptosporidium sp.
 Cryptosporidium parvum
 Entamoeba histolytica
2. **Helminths:**
 Ascaris lumbricoides
 Trichuris trichiura
 Strongyloides stercoraria
 Hymenolepis nana
3. **Bacteria, Yeast and Fungi:**
 Bacillus anthracis
 Campylobacter jejuni
 Rhodospirillum rubrum
 Candida albicans

Place the slide in a Coplin jar (or equivalent) filled with PBS for 5 minutes. Gently agitate several times during the wash & rehydrations.

Remove the slide and add several buffer rinses (the amount is within limits). Gently wash 30 sec. in distilled (deionized) water. Repeat to each of the 3 wells on the slide (use the same formula as in Step #4) incubate in a humidified chamber shielded from incident light for 30 minutes at RT. Protect the stained Labware from incident light or presence of direct sunlight (see PRECAUTIONS #11).

10. Rinse as in Step #4.
11. Remove the slide, add a drop of fluorescent mounting fluid, apply a coverslip and examine each slide wet with a fluorescent microscope (Protect the prepared slide from incident light).
12. Scan each well thoroughly using 10-20X magnification.
13. Procedure for Fresh Fecal Specimens
 The following method permits Giardia cyst detection to be performed using an antibody culture, staining of hook-worm larvae, bacterial culture, occult blood, or stool-10 tests.
1. Add 15 ml of 10% trypsin to 3-5g of stool specimen in a Para-Pak Clean vial.
2. Mix the contents thoroughly.
3. Allow to stand at RT for 30 minutes to ensure adequate digestion.
4. Follow the Para-Pak Macro-Con procedure for specimen concentration.
5. Begin specimen processing as outlined in PROCEDURE - Section B.

QUALITY CONTROL

Inclusion of a positive and negative control determination with each patient specimen provides quality assurance of the reagents contained in the kit in addition to the Patient Activity Reagent and Laboratory Negative Control that are included for just inclusion in the kit. In addition, a water control cysts of unknown nature.

INTERPRETATION OF RESULTS

1. Giardia cysts are oval shaped organisms, 8-12 µm long. The cysts will stain bright apple green. Two to four nuclei may also be visible in the cyst. Internal bodies may be visible in the cyst. The positive control will stain a similar fluorescent staining pattern. Fluorescence of characteristic cyst morphology.
2. The negative control will not exhibit apple green fluorescence of characteristic cyst morphology.
3. Some background confusion may sometimes result as an artifact of slide preparation. The background fluorescence is easily identifiable by its lack of vibrant, apple green color or characteristic morphology of the cyst.

Prepared Specimens

1. Positive test result: Any stool specimen exhibiting one or more cysts with an apple green color and characteristic morphology should be considered positive for the presence of Giardia. If no cysts are found and symptoms persist another specimen should be examined.
2. Negative test result: A stool specimen negative for the presence of Giardia cysts should be considered negative for the presence of Giardia cysts. Background fluorescence which does not exhibit vibrant apple green color or characteristic morphology should also be considered negative for the presence of Giardia.

contents of a PBS Buffer Packet should be dissolved completely before use.

4. The Positive Control Reagent contains viable Giardia cysts; however, it should be handled as a potentially infectious specimen.

5. Patient specimens can contain infectious agents and should be handled and disposed of as potential biohazards. Wear disposable gloves while performing the test procedure and handling patient specimens.

6. Replace the reagent vial caps on their respective vials.

7. Wash hands thoroughly after completion of the test procedure.

8. Sodium azide is a skin irritant. Avoid skin contact with the kit components. Do not mix with acid as this may result in the formation of hydrazoic acid, an extremely toxic gas.

9. Never pipet by mouth.

10. Do not use kit components beyond the indicated expiration date on the kit box.

11. Protect the Labeling Reagent from exposure to incident light.

12. When delivering reagents onto the slide wells use care not to scratch the treated surface with the applicator stick or the pipet tip.

13. When using the diluted Primary Antibody or the Labeling Reagents avoid any foaming; this gently.

14. Fecal or environmental specimens preserved in PVA or MF MIF are not suitable for use with the MERIFLUOR-Giardia Immunofluorescent Detection Procedure Form-in-Kit and SAF preserved specimens may be used (see PROCEDURE).

15. Once the primary antibody procedure has been initiated do not allow the treated microscope slide to completely dry.

16. If normal stool material is not seen upon scanning the slide wells for the presence of Giardia cysts, loss of sample from the slide well may have occurred. This is usually due to an overly vigorous wash procedure or insufficient specimen drying time.

WARNING: Disposal of reagents containing sodium azide (in kit) or copper phthalocyanine can result in the formation of explosive metal azides. This can be avoided by flushing with a large volume of water during such disposal.

SELF LIFE AND STORAGE

The expiration date of the MERIFLUOR-Giardia reagents is indicated on each kit. Store the kit at 2°-8° C and return it to the refrigerator after each use. The Labeling Reagent should be protected from incident light (see PRECAUTIONS #11). The components of the kit should not be frozen.

Stability and reactivity of the reagents should be tested using the provided positive and negative control preparations. If the expected results are not obtained and the reagents are still within their expiration date, please contact Meridian Diagnostics, Inc. Technical Services at 1-800-543-1980. In Ohio call collect (513) 271-3700. Expired reagents should be discarded.

PROCEDURE

Any specimen acquired from a stool, an environmental sample, or other source and preserved in 10% formalin or sodium acetate-acetic acid formalin (SAF) may be used. A procedure for the use of fresh stool is also described.

A. Specimen Collection and Concentration

1. The use of an appropriate specimen is of utmost importance in the diagnostic process (5.13). The MERIFLUOR-

4. Purified water
5. One liter flask or beaker
6. Lint free tissues or cotton swabs
7. Applicator sticks
8. Humidity chamber
9. Wash bottle
10. Caplin jar (or equivalent)
11. Microscope slide coverslips (22 x 50 mm)
12. Fluorescent microscope
13. Fluorescent mounting media - recommended preparation following:

Makes up 0.1M Glycine Buffer:

Glycine 14.0g
NaOH 0.7g
NaCl 17.0g
NaN₃ 1.0g

Add sufficient purified water to 1 liter and adjust the pH to 8.6 as appropriate. Mix one part Glycine buffer (0.1M) with 2 parts glycerol (e.g. 100ml GB + 200ml glycerol). Store at room temperature (RT).

TRANSMITTED LIGHT

The following laser systems or their equivalent have been found to be satisfactory for routine use with transmitted or incident light darkfield assemblies.

Excitation Filter	Barrier Filter	Red Emission Filter
MP 490	K310 or K530	BG 38
BG 12	K310 or K530	BG 38
FITC	K530	BG 38
MP 490	Tungsten Halogen 100W	BG 38
	K310 or K530	BG 38

INCIDENT LIGHT

Light Source - Mercury Vapor 200W, 100W or 50W

well as bloating, flatulence, anorexia and nausea (3,4,6,7,8,10,11,12). Though some cases present a self limiting condition, symptoms of an acute or subacute infection may persist for several days to several months (4,8,9). Therapeutic drug treatment using quinolone hydrochloride, metronidazole, and furazolidone has been recommended for asymptomatic and symptomatic patients (3,4,9,10,11).

Currently, the primary method of clinical diagnosis for giardiasis is by direct microscopic examination of stool or stool concentrates for the presence of cysts. Trophozoites may also be seen. Using conventional staining methods of detection, a sensitivity rate of 50-70% or lower has been observed, especially when cysts were present at low levels (3,5,6,7,8,11).

Examination of duodenal and jejunal fluid aspirates and small intestine biopsy specimens have been used to improve conventional sensitivity rates but they are invasive and costly. Other methods include serology by immunofluorescence (IF) and enzyme immuno assay (ELISA) procedures however, neither technique indicates an acute Giardia infection (5,6,7,11).

BIOLOGICAL PRINCIPLES

The MERIFLUOR-Giardia Immunofluorescent Detection Procedure utilizes the principle of indirect immunofluorescence. A prepared fecal or environmental specimen is incubated with a murine monoclonal antibody directed against a carbohydrate component of this cyst. The specimen is rinsed and incubated a second time with FITC-conjugated anti-murine antibody. The conjugate will detect any murine globulin which has remained on the slide as a result of a specific reaction with Giardia cysts. Any residual conjugate is rinsed away and the slide is examined for apple green color and characteristic morphology of Giardia cysts using a fluorescent microscope.

MATERIALS PROVIDED

Description:

1. Primary Antibody Reagent. Anti-giardia monoclonal antibody containing 1.0% BSA and preserved with sodium azide.
2. Labeling Reagent. Fluorescent conjugated anti-murine globulin containing 1.0% BSA and preserved with sodium azide.
3. Positive Control Reagent. Formulated stool preparation of viable Giardia 40 cysts.
4. Negative Control Reagent. Formulated stool.
5. PBS Buffer Packets. Phosphate and sodium chloride salt. Final volume of 500ml will be pH 7.5 ± 0.5.

Component Volumes:

Component #	Volume
1. Primary Antibody Reagent	250005 0.50 ml
2. Labeling Reagent	0.50 ml
3. Positive Control Reagent	0.25 ml
4. Negative Control Reagent	0.25 ml
5. PBS Buffer Packets	5 each
6. Microscope Slides	25 each

Catalog #220025 provides enough reagent to use reagents to perform 25 tests.

Materials Not Provided:

1. Microser pipet (10-100 µl variable, 10 µl fixed volume or equivalent).
2. Petriol pans.
3. Test tubes and rack.

Giardia Indirect Immunofluorescent Detection Procedure is designed for use with the Para Pak™ line of parasitology products to provide ease and safety of collection. Collect the specimen as directed by the appropriate Para Pak package insert supplied with the corresponding system. Consult the Para Pak Form-in-Kit or SAF package insert for information that can be supplied by Meridian Diagnostics, Inc. Technical Services Department. Phone: call 1-800-543-1980. In Ohio call collect (513) 271-3700.

2. The specimen must be mixed accurately in the preservative. The preservative stock mixture must be allowed to stand for a minimum of 30 minutes at RT between the time of specimen collection and processing to ensure adequate fixation.
3. If a preliminary stained smear for identification of other parasites is desired from Para Pak in SAF preserved material, the procedure can be found in the Para Pak SAF package insert.
4. The specimen may be concentrated prior to processing. Several methodologies have been reported to obtain cyst-enriched samples (5,6,8). The Para Pak Macro-Cut™ is recommended for this purpose. The Macro-Cut package insert should be followed from the beginning through Step 15 of the SPECIMEN PREPARATION section. The removal of fecal lipids or ethyl acetate may be particularly beneficial if the fecal specimen is treated. This dilution will facilitate the adherence of fecal material to the treated microscope slide.

B. Specimen Processing

1. Remove a drop of the fecal or environmental material with a 10 µl pipet, a 10 µl calibrated urine exp., or equivalent, and place it on the prepared microscope slide as the slide well labeled "Patient".
2. Spread the specimen over the well using the side of an applicator stick to produce a very thin film. Do not scratch the treated surface of the microscope slide well.
3. Repeat steps 1-2 with 10 µl of the positive and negative control specimens as indicated on the microscope slide.
4. Allow the slide to air dry completely at RT (usually requires 15-30 minutes).
5. Place the contents of one PBS Buffer Packet in a one liter flask of beaker. Add 500 ml of purified water to the container and mix until the contents are dissolved. The container should be appropriately labeled, covered, and stored at 2°-8° C when not in use. Discard the solution if it becomes cloudy or contaminated.
6. To reduce waste of reagents, dilute the Primary Antibody Reagent in the prepared PBS solution using the following formula:

S = # of slides desired
Q = quantity of Primary Antibody Reagent to be diluted by prepared PBS
V = final volume of reagent PBS mixture
S x Q = ml - Q ml
Q ml x 10 = V ml

EXAMPLE: For a batch of six slides the amount of Primary Antibody Reagent would be 6 x 0.02 ml = 0.12 ml. To this is added enough PBS (10 ml) to bring the final volume to 1.2 ml (0.12 ml x 10). Mix the solution gently (see PRECAUTIONS #13). Carefully place 50 µl of diluted Primary Antibody Reagent in each of the 3 wells on a slide (see PRECAUTIONS #12), covering the entire surface of the well. Allow the slide to incubate in a humidified chamber for 30 minutes at RT.

7. Rinse the slide gently with the prepared PBS solution.



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HYDROFLUOR™-Combo
 Indirect Immunofluorescent Detection
 Procedure
 For Giardia Cysts and Cryptosporidium
 Oocysts in
 Environmental Samples
 Catalog #240025
 For Evaluation of Environmental Samples
 Only

INTENDED USE
 HYDROFLUOR™-Combo is an in vitro procedure for the simultaneous detection of Giardia cysts and Cryptosporidium oocysts from environmental sources which include secondary treated sewage as well as raw and finished water supplies. Monoclonal antibodies to Giardia cysts and Cryptosporidium oocysts bind to their respective cyst wall antigens. The reactions can be visualized by the addition of a fluorescent isothiocyanate (FITC)-conjugated anti-immunoglobulin and scanning with a fluorescence microscope.

EXPLANATION

Giardiasis has become one of the most common intestinal parasitic infections in the world (1). This is due, in part, to food contamination, defecation to human habitation, untreated surface water contamination with cysts containing human and other animal feces as well as inadequate filtration in water treatment facilities (1,2,3,4,5,6,7,8).

Organisms of the genus Giardia were first described in 1675 by Anthony van Leeuwenhoek upon examination of his own feces (1,9). Giardia spp. are protozoan flagellates which exist in two forms: 1) a motile and actively feeding trophozoite (9-21µm long and 5-15µm wide) found on the surface of the small intestine; 2) an inactive oval shaped cyst form which is smaller (8-12µm long and 7-10µm wide) and contains two to four nuclei, median bodies and heads. Cysts can survive outside a host for several days, whereas the trophozoite dies within hours after leaving the body (1,4,9,9,10). Giardia lamblia has not been shown to be a pathogen in humans. Thus far it has not been shown that other Giardia species are implicated in human disease.

Identification of Giardia cysts in water samples using color-staining methods is time consuming and tedious. The use of an immunofluorescent staining procedure permits a rapid and accurate method for the detection of Giardia cysts in water samples due to enhanced specific contrast between cysts and other particulates (11,12).

Cryptosporidium:

Organisms of the genus Cryptosporidium are protozoan parasites that produce gastroenteritis in humans (13,14,15,16,17).

The organism is shed 2 days (median) (18). It has been found in a variety of domestic animals including poultry, calves, lambs, and goats (19,20). In 1978 the first isolation of Cryptosporidium was reported (21).

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Test Procedures:

1. Presumptive positive test result: Any sample exhibiting one or more Giardia cysts and/or Cryptosporidium oocysts with apple-green fluorescence and showing typical size and shape should be considered presumptively positive for the presence of Giardia spp. and/or Cryptosporidium spp.

2. Confirmed positive test result: Must meet the criteria for the presumptive positive test results plus the following criteria: Giardia spp. - visually see two of three of the following internal structures: nucleus, median bodies and blebs; Cryptosporidium spp. - may visually see the sporozoites appearing as four crescent or sausage shaped structures within the oocysts; if internal structures are observed in one Giardia cyst or one Cryptosporidium oocyst the sample is considered confirmed positive for Giardia spp. and/or Cryptosporidium spp. NOTE: A microscope with phase contrast must be used to visualize internal structures.

3. Negative test result: A sample with neither apple-green fluorescence nor characteristic morphology should be considered negative for the presence of Giardia spp. and/or Cryptosporidium spp.

LIMITATIONS OF THE PROCEDURE

HYDROFLUOR™-Combo is an in vitro procedure to aid in the identification of Giardia cysts and Cryptosporidium oocysts in environmental samples. There is no consensus technique for the recovery of parasites from water. Any concentration technique for environmental water samples currently being used for the detection of Giardia cysts or Cryptosporidium oocysts is at best qualitative. It is advisable to report results as the presence or absence of Giardia cysts and Cryptosporidium oocysts (11,23).

The antibody to Giardia is pan specific and should be considered to react with all species of Giardia. The antibody to Cryptosporidium is directed against C. parvum but will cross-react with C. muris and C. nectopodii but not C. baileyi.

PERFORMANCE CHARACTERISTICS

A water treatment facility in western Pennsylvania compared two concentration techniques for the recovery of Giardia cysts from environmental water sources. The two techniques compared were a Percutaneous HYDROFLUOR™-Combo immunofluorescence assay and a zinc sulfate flotation Lugol's iodine stain method. Nine water samples from four rivers during a 23 day period were collected and tested. Both concentration techniques recovered Giardia cysts. Current techniques used as a recovery of Giardia cysts is not considered quantitative. Detection of even one cyst is significant (28).

In another study, water samples were spiked with a known number of Cryptosporidium oocysts. The Percutaneous method averaged 41% recovery in each of the samples. Cryptosporidium oocysts were not detectable using the zinc flotation Lugol's iodine method. The difference is probably due to the concentration technique and not to the detection method (27).

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13. Blunt-end membrane filter forceps (avoid forceps with serrated or toothed jaws).
14. Slide warming table.

REAGENTS NOT PROVIDED

- 1 Phosphate Buffered Saline (PBS) - 10X solution
Na₂HPO₄ · H₂O 3.87g
Na₂HPO₄ (anhydrous) 10.20g
NaCl 85.00g
NaN₃ (optional) 10.00g
Final pH 7.20 ± 0.05
- OS to 1L with purified water (deionized or distilled water). Adjust pH to 7.2 with HCl or NaOH. NOTE: This is a 10X preparation. It must be diluted 1:10 with purified water prior to use.
- 2 0.05% Tween-20 solution in pH 7.2 PBS
- 3 Glycerol
- 4 0.1% Propyl galate glycerol (optional use in place of glycerol; the propyl galate acts as an inhibitor that retards photobleaching of FITC)
- 5 0.1% Evans blue stock solution: Dissolve 0.1% Evans blue powder (Sigma #E-2129) in 50ml purified water. After the Evans blue is completely dissolved, adjust the volume to 100ml with additional purified water. Filter the solution through a 0.2µm membrane filter into a sterile container. Store at 2-8°C.
- 6 1.5M potassium chloride. Completely dissolve 1.16g KCl in 80ml purified water. After the KCl is completely dissolved, adjust the volume to 100ml with additional distilled water. Filter the solution through a 0.2µm membrane filter into a sterile container.
- 7 0.002% Evans blue working solution: 2ml 1.5M KCl 10ml purified water 80ml
- 8 0.1% Evans blue stock solution: 2ml 1.5M KCl 10ml purified water 80ml

Filter the solution through a 0.2µm membrane filter into a sterile container. Store at 2-8°C.

Alcohol/glycerol solutions for clarifying membrane filters. Prepare the following series of alcohol solutions containing 5% glycerol.

Final Ethanol 10%	Final Ethanol 20%	Final Ethanol 40%	Final Ethanol 60%	Final Ethanol 80%	Final Ethanol 95%	Purified Water	Glycerol	Final Volume
10ml	20ml	40ml	60ml	80ml	95ml	5ml	5ml	95ml
20ml	40ml	60ml	80ml	95ml	5ml	5ml	5ml	95ml
40ml	60ml	80ml	95ml	5ml	5ml	5ml	5ml	95ml
60ml	80ml	95ml	5ml	5ml	5ml	5ml	5ml	95ml
80ml	95ml	5ml	5ml	5ml	5ml	5ml	5ml	95ml
95ml	5ml	5ml	5ml	5ml	5ml	5ml	5ml	95ml

9 Clear detergent soap.

The following filter systems or their equivalent have been found to be satisfactory for routine use with transmitted or incident light dasked assemblies.

Excitation Filter	INCIDENT LIGHT		Red Suppression Filter
	Light Source - Mercury Vapor 200W, 100W or 50W	Mercury Vapor 200W, 100W or 50W	
MP 500	Dichroic Filter	Barrier Filter	
FITC	TK 510	K510 or K530	BQ 34
MP 500	TK 510	K510 or K530	BQ 38
FITC	TK 510	K510 or K530	BQ 38

10. Schneider and Schmal Calotte Alcolite membrane filter 0.2µm pore size #4-23600 (600-245-4024) (or equivalent).

11. GDS-00, Millipore Corporation, Bedford, MA or Ten Place Filter Assembly, 25mm with individual Teflon valves, catalog #FH225V, Hoyle Scientific Instruments, San Francisco, CA (or equivalent).

12. Microscope equipment with phase contrast, epifluorescence optics, and eyepiece with micrometer disc.

specimens from humans was reported (21). The first community outbreak from a water source was reported in 1984 (22). Several species have been described although little host specificity has been indicated (13). *Cryptosporidium* has worldwide distribution (23). Due to the three to layered wall, the cysts are resistant to most disinfectants and is unharmed after treatment by routine chlorination of drinking water (24). Because of the relative amount of drinking water consumption with other organisms in environmental samples, proper identification is very difficult using non-specific staining methods. The use of a specific immunofluorescent staining procedure permits rapid and accurate identification of the cysts (25).

BIOLOGICAL PRINCIPLES

The HYDROFLUOR-Combo immunofluorescent detection procedure utilizes the principle of indirect immunofluorescence. A prepared environmental sample is inoculated with murine monoclonal antibodies directed against surface antigen components of Giardia cysts and *Cryptosporidium* oocysts. The sample is rinsed and incubated a second time with FITC-conjugated antibody to murine immunoglobulin G. This conjugate will bind to murine immunoglobulin which has remained as a result of a specific reaction with Giardia cysts or *Cryptosporidium* oocysts. Unbound conjugate is washed away and the slides are examined, using an epifluorescence microscope, for organisms which display the typical apple-green color and characteristic morphologies of the Giardia cysts and *Cryptosporidium* oocysts.

MATERIALS PROVIDED

- 1. Primary Antibody Reagent. Concentrated murine anti-*Cryptosporidium* and anti-giardia monoclonal antibody preparation containing BSA and a preservative.
- 2. Labeling Reagent. Concentrated FITC-conjugated anti-murine antibody preparation containing BSA and a preservative.
- 3. Positive Antigen Control Reagent. Formalinized stool containing Giardia spp. cysts and *Cryptosporidium* spp. oocysts prepared with 10% formalin.

Component Quantities:

- 1. Primary Antibody Reagent #240023 2.75ml
 - 2. Labeling Reagent 2.75ml
 - 3. Positive Antigen Control Reagent 2.5ml
- Catalog #240023 provides sufficient material for 25 test replicates and 25 controls.

MATERIALS NOT PROVIDED

- 1. Microtainer paper (10-100µl)
- 2. Pipets (1, 5 and 10ml)
- 3. Pasteur pipets
- 4. Test tubes and racks
- 5. Purified water - distilled or deionized
- 6. One liter beaker or beakers
- 7. Lint free beaker or dilution beaker
- 8. Microscope slides
- 9. Microscope slide coverslips (25-50mm)
- 10. Schneider and Schmal Calotte Alcolite membrane filter 0.2µm pore size #4-23600 (600-245-4024) (or equivalent).
- 11. GDS-00, Millipore Corporation, Bedford, MA or Ten Place Filter Assembly, 25mm with individual Teflon valves, catalog #FH225V, Hoyle Scientific Instruments, San Francisco, CA (or equivalent).
- 12. Microscope equipment with phase contrast, epifluorescence optics, and eyepiece with micrometer disc.

Antibody Reagent. The final diluted working volume would be 3.3ml. Mix the solution carefully.
Add 0.5ml of diluted Primary Antibody Reagent to each membrane filter. Incubate for 30 minutes at room temperature. NOTE: It is important that the vacuum is OFF and the reagent stays on top of the membrane filter and does not pass through the membrane filter.

8. Rinse several times with PBS without Tween-20 (20-30ml total volume)

9. Dilute the 10X concentrated Labeling Reagent 1:10 in PBS without Tween-20 as described in PROCEDURE step #6. Add 0.5ml of diluted labeling reagent to each membrane filter. Incubate for 30 minutes at room temperature. NOTE: It is important that the membrane filter be shielded from light. NOTE: It is important that the vacuum is OFF and the reagent stays on top of the membrane filter and does not pass through the membrane filter.

10. Rinse as in PROCEDURE step #8

11. Apply 1µl of 0.002% Evans blue dye to the membrane filter for 10-15 minutes at room temperature. Remove the Evans blue solution with purified water. If phase contrast microscopy is desired, stop here and go to PROCEDURE step #12. If not, remove the membrane filter from the housing and place on a glass microscope slide using a membrane filter forceps. Apply a drop of glycerol or 0.1% propyl galate glycerol on the membrane filter and add a coverslip. Examine the membrane filter as in PROCEDURE step #13

12. If phase contrast microscopy is desired the membrane filter(s) need to be processed in the following manner while still in the filter housing unit.

- a. Apply the alcohol/glycerol solution to the membrane filter(s) starting with the 10% ethanol-5% glycerol solution. Pull each solution through the membrane filter before the next higher alcohol concentration is applied. (See REAGENTS NOT PROVIDED #12) and end with the 95% ethanol-5% glycerol solution.
- b. After the alcohol series has been run, remove the membrane filter(s) from the housing unit and place on a prewarmed slide (35-37°C) on top of a drop of glycerol or 0.1% propyl galate glycerol.
- c. Keep the membrane filter and slide on the warming table approximately 20 minutes. The membrane filter should become transparent. If the membrane filter begins to turn white apply a small amount of glycerol under the membrane filter.
- d. Apply a drop of glycerol or propyl galate glycerol to the center of each membrane filter and cover with a coverslip. Tap out air bubbles when they form. If the spread, the edges of the coverslip can be sealed with clear nail polish.

13. Examine the membrane filter at 200X magnification for Giardia and *Cryptosporidium* using epifluorescence microscopy. Slides can be stored at 2-8°C in a cool dark labeling deck for up to 5 days after preparation.

QUALITY CONTROL

- 1. At the time of each use, the Positive Antigen Control Reagent provided should be used to verify the proper performance of the reagents. Apply 100µl of Positive Antigen Control Reagent to a membrane filter and process in the same manner as the water samples.
- 2. At the time of each use, the kit components should be visually examined for obvious signs of contamination.

INTERPRETATION OF RESULTS

Control Reactions:

- 1. Giardia cysts are oval shaped organisms, 8-12µm long. The cyst wall will fluoresce bright apple green.
- 2. *Cryptosporidium* oocysts are 2-6µm in diameter. The oocyst wall will fluoresce bright apple green. They may be

PRECAUTIONS

- 1. All reagents are for in vitro use only.
- 2. Do not mix reagents from different kit lot numbers.
- 3. All reagents should be gently agitated before use.
- 4. The Positive Antigen Control Reagent contains killed Giardia cysts and *Cryptosporidium* oocysts, however, it should be handled as a potentially infectious sample after use.
- 5. Replace the reagent vial caps on their respective vials after use.
- 6. Wash hands thoroughly after completion of the test procedure.
- 7. Sodium azide is a skin irritant. Avoid skin contact with the kit components. Do not mix with acid as this may result in the formation of hydrazoic acid, an extremely toxic gas.
- 8. Never pipet by mouth.
- 9. Do not use kit components beyond the indicated expiration date on the kit label.
- 10. Protect the Labeling Reagent from exposure to light.
- 11. Mix the diluted Primary Antibody and diluted Labeling Reagents gently to avoid foaming. DO NOT VORTEX.
- 12. Do not allow the membrane filter to completely dry after the addition of the Primary Antibody Reagent.

WARNING: Some of the reagents in this kit contain sodium azide. Disposal of reagents containing sodium azide into lead or copper plumbing can result in the formation of explosive metal azides. This can be avoided by flushing with a large volume of water during such disposal.

SHIELD LIFE AND STORAGE

The expiration date of the HYDROFLUOR-Combo reagents is indicated on the kit label. To maintain optimal performance the kit should be stored at 2-8°C and returned promptly to the refrigerator after each use. The Labeling Reagent should be protected from light. CAUTION: DO NOT FREEZE.

PROCEDURE

- 1. Bring all reagents to room temperature.
- 2. Soak membrane filters in purified water post prior to use by floating the membrane filter on top of the water. When the membrane filter sinks to the bottom of the container the membrane filter is ready to use. NOTE: Always use a membrane filter forceps when handling membrane filters.
- 3. Place filter support and membrane filter on filter housing using the membrane filter forceps and assemble the unit.
- 4. Place 1ml of sample on the membrane filter (adjust the volume of the sample accurately if the particulates are too dense or widely spaced). Bring the level of PBS in the housing up to 10 ml and add 4-6µl of PBS in the housing.
- 5. Never allow the vacuum to pull air through the membrane filter or allow the membrane filter to dry.
- 6. Wash the membrane filter with 100ml of PBS containing 0.05% Tween-20.
- 7. To reduce waste of reagents, dilute the 10X concentrated Primary Antibody Reagent in PBS without Tween-20 solution as follows:
F = # of membrane filters desired
Q = volume of Primary Antibody Reagent or Labeling Reagent required
V = volume of PBS required to dilute Primary Antibody Reagent
F x 0.055 = Qml
F x 0.485 = Vml

Example: For a batch of six membrane filters the amount of Primary Antibody Reagent or Labeling Reagent would be 0.33ml (Qml = 6 x 0.055ml). Add 2.97ml (Vml = 6 x 0.485ml) of PBS to dilute the 0.33ml volume of Primary



GIARDIA-CEL IF TEST

INTENDED USE
The GIARDIA-CEL IF Test is a rapid in vitro direct immunofluorescence test for the detection of the protozoan parasite *Giardia* in faecal specimens.

INTRODUCTION
Giardia is a unicellular flagellate protozoan which exists in two forms, a non-infectious pear-shaped trichocystic 9-13µm which ingests the small intestine, and the highly infectious pear-shaped cyst which is elliptical in shape and ranges in size from 8-14µm (1). Survival outside its host varies greatly between the two forms. The trophozoite which is extremely fragile, lasting only a matter of hours outside the body while the cyst form may survive for several days in an external environment (1).
Giardia is a common cause of gastrointestinal disease worldwide (2,3) and is the most common cause of waterborne diarrhoea in the United States (1,3). The parasite has been responsible for infections due to water contamination (4) and travellers have been found to contract giardiasis from endemic areas (5). Transmission also occurs by direct contact and food contamination by asymptomatic carriers (6,7).
High risk categories include young children, immunocompromised patients and those without previous exposure (8). Clinical

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3. Specimens should have signs of normal faecal material. If none is visible the test should be repeated with a more gentle washing procedure.
4. If the reagent is diluted or not stored under the recommended conditions, sensitivity and accuracy will be affected.
5. It is recommended that not less than 20ul of the reagent be used to cover the specimen on the slide. A smaller volume may lead to difficulty in covering the area.

SENSITIVITY AND CROSS-REACTIVITY

The GIARDIA-CEL IF Test was used to evaluate 400 clinical specimens which included both light and heavy *Giardia* infections. It was found to be 100% sensitive when compared to the bovine wet mount.
The GIARDIA-CEL IF Test was tested for cross-reactivity against the following organisms and results are shown below. None of the organisms showed any degree of fluorescence.

	Reactivity with GIARDIA CEL IF TEST
Cryptosporidium	---
Dientamoeba	---
Entamoeba coli	---
Entamoeba histolytica	---
Blasotrypa	---
Acanthamoeba	---
Endolimax	---

- 1. Washed buffer solution pH 7.4 with Evans Blue (0.005%) as counter stain and 0.1% (w/v) sodium azide. Store at 2°C in the dark. Bring to room temperature before use.
- 2. Mounting Fluid
 - a) One bottle containing 2.5ml mounting fluid, a photoresistive emulsion on glycerol. Store at 2°C and bring to room temperature before use.
- 3. Positive Control Slides
 - a) Prepared unmounted fixed slides of a cyst positive suspension.

Materials Required but not Provided:

- 1. Distilled water.
- 2. Precision pipette for delivering 25ul
- 3. Microscope slides.
- 4. Coverslips
- 5. Pasteur pipettes.
- 6. Test tubes, caps and rack.
- 7. Acetone for specimen fixation.
- 8. Fluorescence microscope with filter system for FITC (maximum excitation wavelength 490nm, mean emission wavelength 530nm) and a 400 magnification.
- 9. Fecal concentration kit.
- 10. Sodium azide (1%) for sample preparation.
- 11. Phosphate buffered saline (PBS) pH 7.4 for sample preparation.
- 12. Humidified chamber.

- 1. For *in vitro* diagnostic use only.
- 2. The kit should be stored at 2°C, the reagent is sensitive to light and has been packaged in brown glass for protection. Reagents should not be used after the expiry date shown on the label.
- 3. Do not mix reagents from different kits.
- 4. The test reagent contains 0.1% (w/v) sodium azide which is a poison. It may react with copper and lead plumbing systems to form explosive metal azides. Always dispose of azide containing materials with large quantities of water.
- 5. Evans blue dye is present in the reagent. This is a possible carcinogen and contact with skin should be avoided.
- 6. Care should be taken when using the mounting fluid as it may cause skin irritation.
- 7. Patient specimens and control slides should be treated as potentially infectious and must be handled and disposed of accordingly.

INSTRUCTIONS FOR USE

- 1. Specimen Processing
 - a) If processing of specimens is not required immediately they may be stored at 4°C for 24-48 hours before examination. This procedure requires the use of faecal specimens not preserved by chemical means so that the antigenic sites recognised by the antibody are not destroyed. If a permanent record is

required, a separate sample should be taken and placed in the chemical preservative of choice for storage. The Evigreen Faecal Parasite Concentration Kit (No. 240 3074 030, Evigreen Scientific, CA90058) is recommended for specimen concentration, with care of local clause to the manufacturer. This is the substitution of 10% formalin for PBS (Steps 2 and 16).

Assay Procedure

- a) Prepare a suspension of faeces in PBS (10mm in diameter in 5ml of PBS with 0.1% sodium azide) or a faecal concentrate according to the amendment outlined in specimen preparation. Using a Pasteur pipette place a drop of the suspension onto a glass slide corresponding to the patient identity code.
- b) Allow the slides to completely air dry.
- c) Fix the slides for one minute in acetone and allow to air dry.
- d) Place 25ul of the FITC monoclonal antibody onto the fixed specimen (and positive control slide). Place in a humidified chamber for 30 minutes at room temperature.
- e) Wash the slide gently by irrigation in water.
- f) Drain the slide and add a drop of the mounting fluid supplied.
- g) Apply a coverslip to the slide and remove air bubbles.
- h) Scan the smear using a fluorescence microscope at a 400 magnification.

INTERPRETATION OF RESULTS

- 1. Giardia cysts should exhibit bright apple green fluorescence characteristic of all stages and be found in pairs. A test may be considered positive if more than one cyst is found. In some infections the cyst numbers are numbers as high as 100 in the asymptomatic stage. Cyst numbers are generally low if the test is negative and declining counts are noted in specimens that are considered as cyst excretors may vary from day to day. The positive control slide should demonstrate cysts with apple green fluorescence and characteristic shape and morphology. If these are not seen the test should be considered void and repeat.
- 2. Negative specimens should not exhibit bright green fluorescence and characteristic shape and morphology. If a specific fluorescence may occur in some specimens, however this is easily distinguished on morphological grounds.
- 3. The GIARDIA-CEL IF Test is an *in vitro* diagnostic procedure for the detection of Giardia in faecal specimens. The test does not preclude the presence of other underlying causative organisms other than Giardia.
- 4. Specimens preserved in formaldehyde (FA, SAF, MFMIF) are not suitable for this assay. Follow kit procedure for sample preparation.

LIMITATIONS

- 1. The GIARDIA-CEL IF Test is an *in vitro* diagnostic procedure for the detection of Giardia in faecal specimens. The test does not preclude the presence of other underlying causative organisms other than Giardia.
- 2. Specimens preserved in formaldehyde (FA, SAF, MFMIF) are not suitable for this assay. Follow kit procedure for sample preparation.

manifestations of giardiasis include asymptomatic carriage with the passing of cysts to chronic debilitating diarrhoea, weight loss and malabsorption (8,9,10).

PRINCIPLE OF THE TEST

Current diagnostic methods for giardiasis detect cysts or trophozoites by microscopy. Stool specimens are examined directly or with staining with or without concentration (11). Diagnosis by these conventional methods of staining is dependent on the skill of the microscopist and the success rate of stool examination varies between 60-70% (12,13). Sequential specimens are usually necessary to detect the presence of the cyst stage of the parasite. The GIARDIA-CEL IF Test utilizes direct staining of acetone fixed prepared faecal specimens. The fluorescein-labelled mouse monoclonal antibody binds specifically to cell wall components of the cyst. A washing step removes unbound antibody. When viewed under a fluorescence microscope, Giardia cysts are seen as bright apple green, oval shaped bodies, 8-10um in size. This technique has been reported to be more sensitive when compared to a non-fluorescent immunofluorescent stain (14).

CONTENTS OF KIT

- Each kit is sufficient for 50 patient specimens. Materials are supplied ready for use.
 - 1. Reagent
 - a) One bottle containing 1.35ml of fluorescein-labelled purified mouse monoclonal antibody reagent diluted in a protein

APPENDIX C: PUBLICATION

New Zealand Journal of Marine and Freshwater Research, 1992, Vol. 26: 275–278
0028–8330/2602–0275 © The Royal Society of New Zealand 1992

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Short communication

Concentrating *Giardia* cysts in water by tangential flow filtration compared with centrifugation

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Abstract Centrifugation, tangential filtration, and sedimentation were compared as methods of concentrating cyst-containing wash water from filters. A range of cyst concentrations were used on duplicate samples. The cysts were concentrated by one of the above methods and the detectable number of cysts noted. The ability of the methods to process samples with sediment and the overall time taken to process a sample was assessed. The method of choice for the concentration of *Giardia* cysts in water is centrifugation.

Keywords *Giardia* cysts; water; concentration; tangential filtration; centrifugation

INTRODUCTION

The presence of the pathogenic intestinal parasite *Giardia* has recently been reported in New Zealand in humans (Okell & Wright 1990; Walker et al. 1991), animals (Tonks et al. 1991), and water (Ainge & Jones 1991). The method generally used for detection of *Giardia* in water involves using a 1 µm nominal pore cartridge filter in a unit, and pumping more than 380 litres through it as recommended by the U.S. Environmental Protection Agency (Jakubowski pers. comm.) and American Public Health Association (1990). A breakdown and washing

of the filter, followed by centrifugation and staining, completes the process. Unless a large centrifuge is available, the concentration step is cumbersome and can lead to damage and loss of cysts.

Tangential filtration, where liquid is circulated across a membrane at a constant rate with a filtrate drawn off at a much lower rate, enables the membrane to remain unblocked while the liquid solution is concentrated. Tangential filtration has been used for concentrating *Giardia* trophozoites in liquid medium (Wieder et al. 1989) but is not routinely used in detection of *Giardia* cysts in water. Issac-Renton et al. (1986) have compared tangential flow filtration with a wound-orelon through filter system for *Giardia* detection. It has been used for other concentration purposes, e.g., the concentration of vaccine antibodies. Tangential filtration was tested as an alternative to centrifugation, because it was suggested it may cause less cyst damage yet provide the same level of detection as centrifugation (Wieder et al. 1989).

MATERIALS AND METHODS

In the evaluation of tangential filtration for use in the concentration step of the general method for testing for *Giardia* cysts, four different tangential filter units and a centrifuge unit were compared:

- i) Millipore Pellicon system: polyviaylidene difluoride membrane 0.1 m², pore size 0.45 µm. Pump: Master Flex high capacity 13 l min⁻¹.
- ii) Filtron Minisette Ultrafiltration system: polyethersulfane membrane 0.07 m², pore size 0.8 µm. Pump: Master Flex high capacity 13 l min⁻¹.
- iii) Sartoclon Mini Cross Flow System: cellulose acetate membrane 0.1 m², pore size 0.2 µm. Pump: Air Diaphragm PTFE 4-7, 500 l min⁻¹.
- iv) Sartoclon II: polyolefin membrane 0.6 m², pore size 0.2 µm. Pump: Air Diaphragm PTFE 4-7, 500 l min⁻¹.
- v) Sorval RC-3 centrifuge with a HG-41 rotor.

In vitro produced cysts (Schupp et al. 1988) were fixed in formalin, purified on a sucrose gradient, counted (using a beamcycotometer), and diluted to

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Received 20 September 1991; accepted 10 April 1992

Table 1 Number of *Giardia* cysts counted after concentration of 4 l retentate.

Cyst conc. in 4 l	Tangential filter units				Centrifugation	Sedimentation
	Millipore Pellicon unit (0.6 m ²)	Filtron Minisetite unit (0.07 m ²)	Sartocon Minicross unit (0.1 m ²)	Sartocon II (0.6 m ²)		
10 ⁶	>250	>250	>250	>250	>250	>250
5 × 10 ⁵	>250	>250	>250	>250	>250	>250
10 ⁵	>250	>250	>250	265†	>250	>250
5 × 10 ⁴	215†	190†	275†	75	>250	>250
10 ⁴	70	155	180	50	>250	>250
5 × 10 ³	5	95	60	35	>250	>250
10 ³	0	50	20	0	>250	>250
5 × 10 ²	0	5	0	0	170†	170†
10 ²	0	0	0	0	10	35
5 × 10 ¹	0	0	0	0	5	0
10 ¹	0	0	0	0	0	0
5	0	0	0	0	0	0
1	0	0	0	0	0	0
0	0	0	0	0	0	0

† the entire membrane was counted here

† only one value obtained

Table 2 Ability to function with sediment samples. -, Unit unable to function with soil in 4 l water; *, pump to small to function at high pressure; +, unit able to function with soil in 4 l water.

	Tangential filter units				Centrifugation	Sedimentation
	Millipore Pellicon unit (0.6 m ²)	Filtron Minisetite unit (0.07 m ²)	Sartocon Minicross unit (0.1 m ²)	Sartocon II (0.6 m ²)		
5 g soil	-	-	*	+	+	+
10 g soil	-	-	*	+	+	+

Table 3 Comparison of time taken to use each concentration method. N/A - not applicable.

	Tangential filter units				Sedimentation	Centrifuge
	Millipore Pellicon unit	Filtron Minisetite unit	Sartocon Minicross unit	Sartocon II		
Actual run (min)	2-5	5-10	5-10	2-5	Left overnight	20 (10 min at 1000g)
Backflush (min)	5-10	5-10	5-10	5-10	N/A	5-10
Cleaning method	Chemical	Chemical	Chemical	Autoclave or Chemical	Autoclaving	Autoclave RO rinse
Time taken (min)	15-20	15-20	15-20	15-20 (Autoclave 15)	15	15
Flushing (litres)	>50	>50	>50	>50	N/A	N/A
time (min)	20-30	30-40	30-40	20-30	N/A	N/A
Maximum time (min)	65	80	80	65	>8 h	55

appropriate concentrations. The experiment was performed in duplicate. Volumes of 4 l of water containing a known concentration of cysts were subjected to tangential filtration, centrifugation, or sedimentation. Sedimentation is an alternative method (American Public Health Association 1989) to centrifugation. The samples were left overnight allowing the cysts to settle and the supernatant to be removed. In the centrifugation method, the samples were loaded into 1-litre centrifuge buckets and centrifuged at 1000 g for 10 min. For tangential filtration the sample was placed in a bucket and circulated until 100–200 ml remained, this was then flushed out.

From all of these concentration techniques, the sample volume remaining (c. 100–300 ml) was further concentrated in a Heraeus Christ Centrifuge by centrifugation at 1000 g for 10 min. 10% of all samples were stained on the Hoefer Model FH 225V manifold using Meridian Merifluor *Giardia* monoclonal antibody stain. The membranes were viewed using an epifluorescent microscope with excitation at 450–490 μm . 250 cysts was the point at which no more cysts were counted.

Different amounts of sediment are obtained when natural water samples are collected. Neither sedimentation nor centrifugation methods are impaired by sediment. The effect of sediment on tangential filtration was investigated by passing 5 g soil per 4 litres and 10 g soil per 4 litres through the tangential filtration units. The total times for processing the samples and cleaning the units were also compared.

RESULTS

Averaged results of each method are shown in Table 1. As a control, the filtrate of the 10^6 concentration (tangential filtration) was spun and stained to demonstrate that no cysts penetrated the membrane. 4 l of water containing no cysts was put through each method to demonstrate its purity (no cysts present). The lowest number of cysts which could be detected using centrifugation was 50 cysts per 4 l, whereas with sedimentation this number was 10^2 per 4 l. Using the Filtron Minisette, the lowest number was 5×10^2 cysts per 4 l. The three other units (Millipore Pellicon, Sartocoon Minicross, and Sartocoon II) all had a lowest detection of 10^3 cysts per 4 l (Fig. 1).

The units containing the smaller surface area membranes (Sartocoon Minicross and the Filtron Minisette) were unable to process samples containing 5 g soil per 4 l (Table 2). The Millipore Pellicon unit

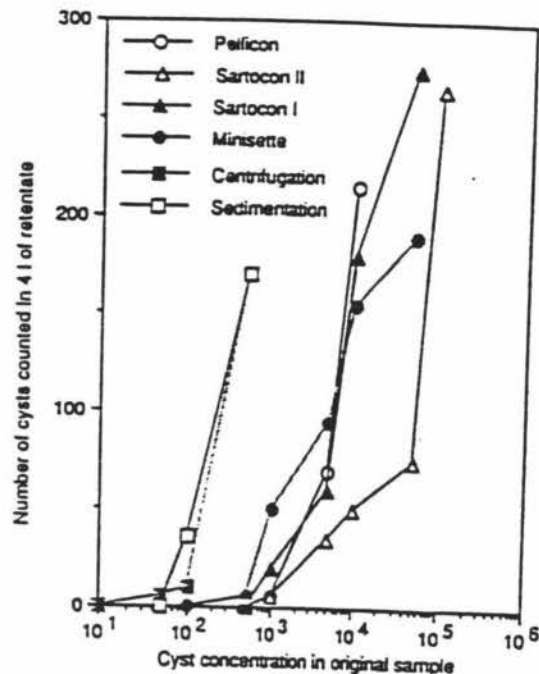


Fig. 1 Average number of *Giardia* cysts counted after concentration.

with a membrane surface area of 0.7 m² could, however, process samples containing 10 g soil per 4 litres. The pump of the Sartocoon II unit did not function at the high pressure developed. The times taken to run samples (not containing soil) are shown in Table 3. The most time-efficient method having the shortest overall time was found to be centrifugation.

DISCUSSION

Centrifugation allows detection of *Giardia* cysts at a concentration of 50 cysts per 4 l, even when high levels of sediment are present (Table 2). In addition to this, the shorter time taken to process a sample makes it preferable to tangential filtration. The use of additional centrifuge buckets can also decrease the overall time taken to process a sample. The tangential filter has to be thoroughly cleaned between samples. One set of centrifuge buckets can be used, while another set is decontaminated. Centrifugation is therefore the method of choice when concentrating the effluent of the filter washing in the process of detecting *Giardia* cysts in water.

ACKNOWLEDGMENTS

We acknowledge the financial support of the New Zealand Health Department for the *Giardia* project of which this study was part. We acknowledge the co-operation of Biolab Scientific for the use of the Millipore Unit, Life Technologies for the use of the Filtron Unit, and Medic Corporation for the use of the Sartorius Units. We acknowledge the Department of Chemistry/Biochemistry, Massey University, for the loan of their Sorvall® RC-3 centrifuge.

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