Viability of *Giardia intestinalis* cysts:
assessing viability under environmental conditions

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Abstract

Much work has been put into the detection and monitoring of *Giardia*, but once found, it is not easy to tell whether the cysts are viable and thus infective. There are fluorescently labelled monoclonal antibody kits which can be used to identify *Giardia*, but are the *Giardia* cysts viable?

Excystation has been the main method used to determine the viability of cysts. This is quite unreliable as varying excystation conditions seem to be required for different strains of cysts. Using samples of fresh cysts, certain batches consistently measured 80-95% viable, while others resulted in viability measurements of 0-10%. The cysts themselves displayed the normal morphology of viable cysts. The assumption that partially excysted trophozoites as well as completely excysted trophozoites are viable may also lead to over-estimation of viable cyst numbers.

Another commonly used method for estimating the viability of *Giardia* is staining with vital dyes, in particular the combination of fluorescein diacetate (FDA) and propidium iodide (PI). These also gave unexpected results where none of the cysts in a fresh sample stained with FDA, which usually stains viable cysts. An alternative dye, 4',6-diamidino-2-phenylindole (DAPI) was used in the place of FDA. The combination of DAPI and PI showed viabilities of 85.7% for cyst samples. This correlated well with 88% viability using excystation.

Using the DAPI/PI combination, the viability of *G. intestinalis* cysts over time was monitored under different temperature conditions, and in sea water. Temperature was quite significant in the viability of the cysts – cysts stored at 4°C remained viable for 62 days, while those stored at 25°C were non-viable after 5 days. Sea-water had an immediately lethal effect on the *G. intestinalis* cysts, with all cysts non-viable after 45 minutes.

Giardia intestinalis trophozoites can be cultured in the laboratory. By the addition of bile to the growth media, it is possible to transform these into cysts. Over the course of four days in encystation media, a large proportion of the trophozoites in the culture were

converted into cysts, 3.5 X 10^5 cysts/ml from an initial trophozoite concentration of 7.2 X 10^5 organisms/ml. However, the cysts generated from the strains of *G. intestinalis* used were completely non-viable, compared with viability rates for fresh *in vivo* cysts of 80-95%.

A population of hamsters was found to be carrying a *Giardia* which seemed different to recognised species. An analysis was carried out by PCR and sequencing of sections of the ribosomal DNA of this *Giardia*. Through this it was found to be closely related to *Giardia muris*, but perhaps not as closely related as to be a species of *G. muris*, possibly a sub-species. The rDNA analysis used may be very useful in typing other strains and species of *Giardia*.

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Chapter 1. Introduction

1.1 The *Giardia* organism

Giardia is the name given to the organism which causes the gastrointestinal disease giardiasis. Giardiasis is endemic in many areas of the world, with prevalence rates of 2-5% in industrialised countries and perhaps 20-30% in developing countries (Farthing, 1994). It has been reported as the most commonly occurring waterborne disease in the U.S.A. and is also widespread in New Zealand waterways. Since 1996, giardiasis has also become a notifiable disease in New Zealand.

Giardia causes intestinal infection in mammals, birds, reptiles and amphibians (Adam, 1991). The organism was first described by Antony van Leeuwenhoek in 1681 when he examined his own diarrheal stool:

> "My excrement being so thin, I was . . . persuaded to examine it . . . wherein I have sometimes also seen animalcules a-moving very prettily; some of 'em a bit bigger, others a bit less, than a blood-globule, but all of the one and the same make; their bodies were somewhat longer than broad and their belly, which was flatlike, furbished with sundry paws, wherewith they made such a stir in the clear medium, and among the globules, that you might e'en fancy you saw a pissabed running up against a wall; and albeit they made a quick motion with their paws, yet for all that they made but small progress." (Dobell, 1920)

Lambl made a more detailed description of the *Giardia* trophozoite in 1859, and the *Giardia* species which infects humans was named after him (Adam, 1991). *Giardia lamblia* is also known as *Giardia intestinalis* and *Giardia duodenalis*. In this thesis, the species of *Giardia* which affects humans will be referred to as *Giardia intestinalis*.

The *Giardia* organism is found in two forms. The trophozoite form is found in the gut of an infected body. It is a binucleate organism, unilaterally symmetrical, pear-shaped, with a pair of median bodies and four pairs of flagellae (anterior, caudal, posterior and ventral). A distinctive feature of *Giardia* is the large concave ventral disk for attachment to the host's internal mucosa. This disc covers approximately two-thirds of the anterior surface of the trophozoite (Adam, 1991). It is surrounded by a flexible rim which meets the surfaces of intestinal microvilli, disrupting and deforming them (Erlandsen and Chase, 1974). This extension is known as the ventrolateral flange. Internal plates support this flange, perhaps enabling it to contract and thus give it flexibility (Thompson *et al.*, 1993). *Giardia* trophozoites have two nuclei which are placed symmetrically on either side of the middle. Both are transcriptionally active and seem to replicate at almost the same time (Adam, 1991).

The cyst organism is the infective form of *Giardia*, found outside a host body. It is highly resistant to environmental factors, and does not replicate. It is elliptical in shape, with dimensions approximately 5μm by 8μm and a cyst wall about 0.3μm thick (Adam, 1991). By scanning electron microscope, the surface of the cyst wall appears smooth. Although the cyst wall is tightly applied to the organism, little of the internal structures of viable cysts are visible under phase contrast microscopy (Feely *et al.*, 1984).

1.2 Species differentiation

Giardia is classed in the Phylum Sarcomastigophora, Class Zoomastigophora and in the Order Diplomonadida, Family Hexamitidae (Adam, 1991; Feely *et al.*, 1984). The shape of the median bodies, body size and form of the trophozoites, were used in a system devised by Filice in 1952 as a basis for distinction between different species of *Giardia*. *Giardia* had been differentiated as species by host specificity, but in many cases this specificity was questionable. Using Filice's system of classification, the number of *Giardia* species dropped from more than forty, identified on the basis of host occurrence, to three based on morphology (Filice, 1952). These were *Giardia intestinalis*, found in many mammals including humans, as well as birds and reptiles, *Giardia muris*, found in rodents, and *Giardia agilis*, which infects amphibians (Adam,

1991). Trophozoites of the species *Giardia intestinalis* measure between 10 μm and 16 μm in length, and 5 μm and 9 μm in width. The median bodies take the appearance of claw-hammers lying across the trophozoite. Those of *Giardia muris* are small and round and are seen in the centre of the body. The *G. muris* trophozoite measures 10 μm in length by 7 μm in width. *Giardia agilis* is longer and narrower, 20 μm in length and 4.5 μm in width with tear-drop shaped median bodies lying lengthways along the body (Meyer, 1994). All three species though have a similar shape and have a ventral adhesive disc, so the median body morphology is the important criterion for determining the species (Erlandsen and Bemrick, 1987).

More recently, two other species have been identified using morphological characteristics observed under scanning electron microscopy (SEM). *Giardia psittaci* from budgerigars has claw-hammer shaped median bodies, but unlike *Giardia intestinalis*, it lacks a ventro-lateral flange, and so does not have a marginal groove bordering the anterior and lateral border of the adhesive disc (Erlandsen and Bemrick, 1987). *Giardia ardeae*, which is found in great blue herons, gray herons and some other wading birds such as egrets (van Keulen *et al.*, 1992), has the typical trophozoite morphology in its pyriform shape and ventral adhesive disc, but it has a single caudal flagellum, rather than the pair found in other species, and a deep notch in the ventral adhesive disc. In addition, it has a variable median body morphology. Median bodies seen varied from the round-oval *Giardia muris* type to the claw-hammer *Giardia intestinalis* type. Chromosome migration patterns were also distinctly different for *Giardia intestinalis*, *Giardia muris* and *Giardia ardeae*, despite the fact that the morphology of *G. ardeae* is similar to that of *G. muris* with its deeply notched ventral disc (Erlandsen *et al.*, 1990). Examination of morphology alone is not sufficient to determine species within the *Giardia* genus. These findings have been borne out by electrophoretic karyotyping. DNA patterns for *G. intestinalis*, *G. muris* and *G. ardeae* are all distinctly different (Campbell *et al.*, 1990).

The rRNA of *Giardia intestinalis* are smaller than those of other eukaryotes, and are also smaller than those of the eubacteria (Adam, 1991). The rDNA gene itself is 5,566 bp and is tandemly repeated in the genome (Healy *et al.*, 1990). The tandem repeat unit includes the large subunit (LSU) (23S), small subunit (SSU) (16S) and the 5.8S subunit.

The size of the rDNA repeat in *Giardia muris* is larger than for *Giardia intestinalis* at 7,668 bp. This size difference is mainly attributable to the different length of the internal transcribed spacer region (van Keulen *et al.*, 1991). The rDNA operon of *Giardia muris* is particularly different from that of *Giardia intestinalis* and *Giardia ardeae* in that the distance between the SSU rDNA and the LSU rDNA is shorter in *G. muris*; the spacer is longer than in *G. intestinalis* and is heterogeneous; and the SSU is the shortest of the three *Giardia* and has the lowest G + C content. Examination of the rDNA operon show that *G. intestinalis* and *G. ardeae* are more closely related to each other than to *G. muris* (van Keulen *et al.*, 1993).

Weiss *et al.* (1992) examined the *Giardia* rDNA sequence, using the polymerase chain reaction. They found that strains of *G. intestinalis* from a variety of sources and locations could be divided into three groups based on defined nucleotide changes within a 183 bp fragment of the 16S rDNA unit. These groupings correlated with groupings previously made based on surface antigen patterns and restriction enzyme analysis (Weiss *et al.*, 1992). Mahbubani *et al.* (1992) devised a system using PCR directed at the giardin gene and gene probes to distinguish *G. intestinalis* from *G. muris* and *G. ardeae* (Mahbubani *et al.*, 1992). The advantages of using the PCR system are that it is very sensitive (Weiss *et al.* (1992) found that the equivalent of 1 organism's DNA was a sufficient quantity to analyse), and that it is a highly specific system so that it is not as crucial to have a pure DNA sample.

In 1995, it was discovered that a colony of hamsters kept at the ESR-CDC (Environmental Science and Research – Communicable Diseases Centre) in Keneperu were infected with *Giardia*. Initial investigations of this 'strain' using standard staining techniques (McLenachan *et al.*, in preparation) indicated possible differences when compared to *Giardia muris*. It was decided to characterise this strain further by analysing the rDNA and comparing it with the rDNA of known species.

1.3 Life cycle

Giardia has a simple, asexual life-cycle. When a host ingests a cyst, stomach acid and other gastric conditions cause the cyst to undergo a process of excystation in the host's upper small intestine (Gillin *et al.*, 1987). In this process, the cyst wall is ruptured and a mass of protoplasm is released from one pole of the cyst, which differentiates into two binucleate trophozoites (Boucher and Gillin, 1990; Meyer, 1994). The exposure of the cysts to gastric acid in the host's stomach is thought to be an important trigger for excystation although the trophozoites do not emerge in the stomach as the low pH would kill them (Boucher and Gillin, 1990). The trophozoites emerge in the duodenum and colonise the epithelium of the small intestine. In rats trophozoites have been observed to adhere to the apical and lateral surfaces of intestinal villi (Erlandsen *et al.*, 1974). *Giardia* is typically non-invasive (Meyer, 1994). The trophozoites multiply by binary fission. Their rate of multiplication varies between strains, and is also dependent on host factors such as nutritional and immune status, but tends to be rapid (Thompson *et al.*, 1993).

Encystation, the mechanism where the trophozoites are transformed into cysts, occurs as trophozoites pass through the jejunum and to the posterior areas of the small intestine. The transformation takes 44 to 72 hours (Gillin *et al.*, 1987; Thompson *et al.*, 1993). Due to this lengthy period, trophozoites can be found in diarrhoeic samples where the transit time through the intestine is short, and cysts tend to be found in formed stools (Meyer, 1994). The mechanisms of encystment are not completely understood but the exposure of trophozoites to bile salts in the lower small intestine seems to be an important stimulus for encystation (Gillin *et al.*, 1987). These stimuli cause the appearance of encystation specific vesicles (ESVs) in the trophozoites. These then transport cyst wall components to the plasma membrane of the encysting trophozoite. Cyst wall components seem to be completely absent in non-encysting trophozoites (Mowatt *et al.*, 1995). Either before or after the cyst wall is formed, asexual reproduction occurs within the cyst with nuclear division resulting in a quadrinucleate cyst. Encystation begins 4-15 days after the colonisation of a host's small intestine.

Some cysts may require a maturation time of seven or so days before they become infective (Bingham and Meyer, 1979; Schaefer *et al.*, 1984).

1.4 The Disease

Studies of travellers to Leningrad, where it was common for travellers to contract waterborne giardiasis, have indicated that the incubation period averages 1-2 weeks, but can range from 1-45 days. Where volunteers were infected with *Giardia intestinalis* trophozoites by intubation, the incubation time was one week. The disease caused by *Giardia* can range from a complete lack of symptoms, to acute short-term diarrhoea to long-term illness lasting several weeks and more. Symptoms rarely last less than one week (Adam, 1991). For most untreated patients, giardiasis is self-limiting within a 2-4 week period, but in up to 25% of documented cases, illness persists for seven weeks and longer (Farthing, 1994). Giardiasis causes diarrhoea, nausea, abdominal discomfort and bloating, and often weight loss. Sometimes early in infection, fever can occur (Adam, 1991). For those with chronic diarrhoea, weight losses of 10-20% of body weight can be experienced (Farthing, 1994). In children, giardiasis can cause protein calorie malnutrition leading to retarded growth and development (Thompson *et al.*, 1993).

Infection with *Giardia* appears to cause disease through epithelial damage. This leads to increased epithelial turnover, villous shortening and disaccharidase deficiency. In rodent models infected with *Giardia muris* and *Giardia intestinalis*, villous atrophy and damage to microvilli correlated with brush border enzyme deficiencies. These returned to normal levels once the infection was cleared. Brush border injury was indicated by lower disaccharidase activity and a decreased microvillous surface area, leading to malabsorption which in turn meant reduced growth. The degree of brush border injury and the decrease in microvillous surface area, both factors affecting disaccharidase activity, seem to correlate to the parasite load. This may in part explain the symptoms of both diarrhoea and the failure to thrive (Thompson *et al.*, 1993).

Although there are many drugs available with which to treat giardiasis, most of these were originally developed to treat other infections. The first to be used was quinacrine, the antimalarial drug, in 1937. It is still often used in the United States to treat giardiasis (Jarroll, 1994). The class of drugs mostly used today are the nitroimidazoles, including metronidazole (5-nitroimidazole), which was developed to treat trichomoniasis in 1959, and tinidazole. Another drug used is furazolidone, a synthetic nitrofuran. However, studies have shown that treatment failures can be expected using these drugs. The efficacy of the four widely used drugs metronidazole, tinidazole, furazolidone and quinacrine are: furazolidone 58-95%, tinidazole 88-100%, metronidazole 46-95% and quinacrine 60-100%.

Unfortunately, these drugs cause some unpleasant side-effects. Mecaprine (quinacrine) can cause dizziness, headaches and mild gastrointestinal illness. Long-term administration of quinacrine can give rise to chronic dermatoses which can be lichenoid, eczmatoid or exfoliative. It may also cause anaemia. The nitroimidazoles, such as metronidazole and tinidazole can bring on gastrointestinal upset, headache, rashes and sometimes furred tongue, vertigo, urethral discomfort and darkening of the urine. As metronidazole can cross the placental barrier, it is not recommended during the first trimester of pregnancy. Paromomycin is recommended for pregnant women. Gastro-intestinal upsets are the only known side-effects, but this is rare. Side effects are also rare for furazolidone, but it may cause headaches, nausea and vomiting (Boreham, 1994).

1.5 Faecal-oral transmission

Giardiasis can be contracted by faecal-oral transmission and by waterborne transmission. The rate of infection with *Giardia* seems to be dependent on socio-economic status. In addition, the incidence of giardiasis is higher in urban areas rather than in rural ones. One study of giardiasis in New Zealand showed an urban incidence 2.3 times the rate of positive samples in rural areas (Walker *et al.*, 1991). This may however indicate lower rates of patients seeing doctors, and a lower likelihood of doctors requesting faecal samples in rural areas. It may also point to a more significant

role for faecal-oral transmission in urban environments where interpersonal contact is more frequent. An infective dose may be as low as 10 cysts, so that very little contaminating matter is required for transmission of *Giardia* to occur. Where the patient experiences severe diarrhoea, it is possible that disease can be transmitted through trophozoites as well as by cysts (Thompson *et al.*, 1993). A survey of New Zealand medical laboratories in 1990 indicated that there were at least 3,356 cases of *Giardia* a year (Walker *et al.*, 1991). These figures are probably gross underestimates, as a high number of cases are asymptomatic.

Factors which increase the chance of contracting giardiasis include overcrowding, high population density, poor hygiene standards, a lack of potable water and a lower level of education. *Giardia* is thus particularly prevalent in developing countries. All children in a sample group in rural Guatemala were found to have been infected by the age of three. By the age of six months, 40% of a sample group of children in Peru were found to have been infected with *Giardia.* Prevalence rates among children in Zimbabwe and Bangladesh were found to be approximately 20% (Adam, 1991). Disadvantaged groups in developed countries are also at risk, with a prevalence rate of 20-60% found among the Australian Aborigine population, with the higher rates particularly common in children (Thompson *et al.,* 1993). Children in institutionalised care, such as day-care centres, also seem to have a higher incidence of giardiasis than the general population. The risk factors in such situations are lack of toilet-training and personal hygiene (Keystone *et al.,* 1978). Prevalence of *Giardia* may be as high as 35% in some institutions (Thompson *et al.*, 1993). The children usually have asymptomatic infections but transmission to family members may result in symptomatic giardiasis (Adam, 1991). Food-borne transmission through an infected food handler is a well-recognised source of infection (Thompson *et al.*, 1993). Direct faecal-oral transmission probably also accounts for increasing rates of giardiasis in homosexual men (Adam, 1991).

1.6 Waterborne transmission

Giardia is so widespread in the environment that all surface water is subject to contamination by human or animal sources. A test carried out in fourteen U.S. states and one Canadian province found *Giardia* cysts in 69 out of 85 samples (81.2%) collected from 66 water treatment plants (LeChevallier *et al.*, 1991b). *Giardia* was first found in New Zealand among returning servicemen in the 1940's (Ampofo *et al.*, 1991). In research recently carried out in New Zealand, the country was divided into fifty-six grids and samples of fresh water taken from each grid in accordance with the USEPA method. Thirty-one of the fifty-six areas (55.4%) produced samples positive for *Giardia*. Some areas were sampled more than once; overall, 22.6% of the samples were positive for *Giardia* (Brown *et al.*, 1997). In New Zealand, there is a general lack of toilet facilities on tramping routes and in roadside rest areas resulting in people using anywhere convenient, such as behind bushes. This seems to be one way that *Giardia* is spreading into environments in which there is otherwise little human activity (Ampofo *et al.* 1991). Such sources can enable *Giardia* to spread into waterways and also expose animals to *Giardia*-infected faeces.

Although waterborne *Giardia* cysts can be inactivated by disinfectants, they are one of the most resistant waterborne pathogens. In the 90 waterborne outbreaks that occurred in the U.S. between 1965 and 1984, many of the treatment systems for the contaminated water supplies involved little more than disinfection with chlorine (Jakubowski, 1988). The effectiveness of chlorine, which is one of the most common disinfectants used for water supplies in inactivating cysts depends on the pH, temperature, water turbidity and chlorine contact time (Walker *et al.*, 1991). A variation in any one of these factors can result in treatment failure; considering the number of factors, the chance of failure is quite high. A number of communities in New Zealand, whose water sources are uncontaminated by wastewater discharge and appear pristine rely on chlorination alone or catchment closure or control for water treatment (Ampofo *et al.*, 1991). *Giardia* cysts however are well able to survive in clear, cold water. Although chlorine concentrations of 3 ppm are considered cysticidal, the standard chlorine levels in New

Zealand drinking water are 0.5 ppm (Walker *et al.*, 1991). Alone, routine levels of chlorine used in disinfection of drinking water are not adequate to inactivate *Giardia* cysts.

1.7 Zoonoses

A variety of animals, both domestic and wild, can serve as hosts for *Giardia*, and *Giardia* and *Cryptosporidium* are commonly found in farm animals in Canada (Olson *et al.*, 1997). The run-off from farms has been implicated in the contamination of waterways in the U.S.A. (LeChevallier *et al.*, 1991a). In the U.S.A. and in Canada, muskrats and beavers are thought to play a role as reservoir hosts of *Giardia*. Studies have found the prevalence of *Giardia* infection to be about 15% in beavers and up to 95% in muskrats (Jakubowski, 1988). Testing in New Zealand has shown that birds, domestic and feral animals here also carry *Giardia intestinalis*, which could be infectious to humans (Marino, 1993). A study of cats and dogs in Palmerston North and Hamilton found that 3% to 25% were infected with *Giardia* (Tonks *et al.*, 1991), while in a similar study in Perth 21% of dogs and 14% of cats were infected (Swan and Thompson, 1986).

However, although there are many reports of animals infected with *Giardia intestinalis*, the evidence on whether zoonotic transmission can occur is conflicting. Common domestic pets such as cats and dogs would appear to be prime candidates for zoonotic transmission of disease, and *Giardia* has been found in both. Yet in one study of an Aboriginal community where there was close physical association between humans and dogs, the *Giardia intestinalis* from the two populations were genetically dissimilar (Hopkins *et al.*, 1997). A single dog had a mixed infection including *Giardia intestinalis* of the type-sequence found in the human group, so it was possible that cross-transmission took place, although on a low level. On the other hand, the study by Isaac-Renton *et al.* (1993) found that human isolates of *Giardia* from an outbreak of giardiasis were genetically identical to isolates collected from diseased beavers caught at the water intake location for the affected town (Isaac-Renton *et al.*, 1993). From the

variety and number of animals carrying *Giardia*, it seems likely that other animals are also likely to be capable of transmitting *Giardia* to humans.

1.8 Culturing *Giardia*

Karapetyan carried out the first successful culturing of *Giardia in vitro* in 1960, growing *Giardia intestinalis* symbiotically with chick fibroblasts and *Candidia guilliermondii*. In 1970, Meyer achieved the isolation and axenic cultivation of *Giardia intestinalis* from rabbit, chinchilla and cat (Meyer, 1976). Early cultures were obtained from trophozoites isolated from the gut of animals. In 1979, Bingham and Meyer reported isolating trophozoites through the excystation of cysts from faeces. These were subsequently maintained for 7 months, with most cultivated axenically (Bingham and Meyer, 1979). This method made it possible to cultivate different strains of *Giardia* from a stool specimen from an infected human or animal, but due to the fastidious nature of *Giardia*, there are still relatively few successes in cultivating trophozoites. The difficulties of establishing axenic cultures is highlighted by Brown *et al.* (1992). Eight New Zealand strains of *Giardia intestinalis* were established from 129 attempts. The cultures themselves take several weeks to become well established (Brown *et al.*, 1992).

Establishing *Giardia* as a viable and axenic laboratory culture is a very time-consuming exercise, with no guarantee of success. Some strains, such as *Giardia intestinalis* from dogs, are more difficult than others. In the study by Mayrhofer *et al.*, (1992) it was found that different populations predominate in mice than in culture. In some cases, *in vitro* culturing resulted in a single genotype of *Giardia*, which on inoculation into suckling mice was discovered to be a mixture of genotypes (Mayrhofer *et al.*, 1992). Selective pressures during culturing may mean the loss of a predominant strain during the processing, giving a false idea of the make-up of the original population. Established *in vitro* cultures may lead to conclusions which are irrelevant for the majority of uncultured *Giardia*, such as susceptibility to drugs (Upcroft *et al.*, 1994).

Almost all *Giardia* cultures established *in vitro* successfully have been of the *Giardia intestinalis* species. *Giardia ardeae* was established as an axenic culture by Erlandsen *et al.* (1990), and there has been a report of axenic cultivation of *Giardia muris*, a species which has not been successfully cultivated in the past. Some strains of *Giardia intestinalis*, such as *Giardia* from dogs, are also notoriously difficult to culture. The problem of what ingredients to include in the complex medium took many years of experimentation, but Gillin and Diamond's modified TYI-S-33 medium, containing bovine serum, cysteine and bile is now the standard medium used in the cultivation of *Giardia* (Meyer *et al.*, 1987). L-cysteine, a thiol reducing compound, is highly important for attachment of the trophozoites to the surface of cell culturing containers and for survival. The presence of serum also stimulates attachment and growth of the trophozoites (Gillin and Reiner, 1982).

Different isolates of *Giardia* have different growth requirements. To start with, the initial cell concentration (ICC) needed to establish an isolate *in vitro* were found to differ for two different isolates studies by Binz *et al.*, (1992). This may mean that one isolate would require fewer cysts to establish an infection within a host *in vivo*. The two isolates also had different pH requirements and grew at different rates. The gastrointestinal pH varies widely in different parts of the intestine. While the gastric pH is quite low at 1.0-2.5, the mean pH in the proximal small intestine – the area which trophozoites tend to colonise – is 6.6 ± 0.5, the terminal ileum is pH 7.5 ± 0.4 and past the ileocecal valve is 6.4 ± 0.4. Different strains colonise different areas of the small intestine in the same host, which may be reflected in the differing growth requirements (Binz *et al.*, 1992). These are factors which may need to be considered in cultivating strains of *Giardia* which are difficult to grow and maintain in culture.

1.9 Viability

Giardia, once found, is not easily cultivated. With bacteria it is possible to identify the organism present, and also to ascertain the viable numbers by culturing on agar. The procedure is not as simple for *Giardia*. The positive identification of *Giardia* was

formerly quite a problem. There are now fluorescently labelled monoclonal antibody kits available which can do this reliably. The problem still remains of determining whether *Giardia* cysts are viable or non-viable.

For the study of viability of cysts from waterborne outbreaks, animal models have been used extensively. While mice are excellent models for the study of *Giardia muris* (Hoff *et al.*, 1985) mice cannot be infected with *Giardia intestinalis* cysts. Neo-natal mice can be infected with *Giardia intestinalis* trophozoites but this requires that cysts first be excysted *in vitro*, for which large numbers of cysts are needed. Gerbils can be infected with *Giardia intestinalis* cysts, and are very convenient for work with *Giardia*, as they can be infected with low infectious doses of cysts (ID_{50} 5-15 cysts have been reported) and the prepatent time and pathogenesis are similar to those of the original hosts (Wallis, 1994). There are some disadvantages with using animal infectivity. Not all *Giardia intestinalis* isolates have produced infection in gerbils, and some isolates while infecting the animals, have not produced cysts (Schaefer, 1988). In any case, animal infectivity is not very useful in quantitative measurements of cyst viability. Unfortunately, gerbils are not permitted in New Zealand, so alternative methods of determining viability and thus infectivity are needed.

Although excystation of *Giardia* had been observed earlier, there was no reproducible method of *in vitro* excystation prior to Bingham and Meyer's report of 1979. Many other methods of excystation have since been reported but they all follow the general pattern of attempting to duplicate *in vivo* conditions that cysts would encounter within a host organism. The conditions in a host's stomach are approximated *in vitro* by a half hour acid-induction step, followed by a reducing step, as the cysts would encounter as they passed into the small intestine.

One of the problems with excystation is that large numbers of cysts are required to ensure a statistically significant sample size, and to overcome losses in numbers during the process. Water samples seldom yield high numbers of cysts, so alternative methods are needed with which it will be possible to ascertain the viability of small numbers of cysts. One of the other methods used to determine the viability of cysts included the use of dyes such as eosin and trypan blue. A comparison of excystation with eosin

exclusion found that the numbers of cysts excluding eosin (viable cysts) was consistently higher than the number of viable cysts as counted by excystation (Bingham *et al.*, 1979).

Schupp and Erlandsen in 1987 reported on the use of the fluorogenic dyes fluorescein diacetate (FDA) and propidium iodide (PI), based on the method of Jones and Sneft (1985), to determine the viability of *Giardia muris* cysts. Comparison of the staining behaviour of the cysts with mouse infectivity and excystation showed that cysts which stained with PI (non-viable cysts) were incapable of infecting mice, and were not observed to excyst. Cysts which stained with FDA (viable cysts) were seen to excyst, releasing FDA-staining trophozoites (Schupp and Erlandsen, 1987a). Schupp and Erlandsen (1987b) also found a direct correlation between FDA/PI staining behaviour and the morphology of the cysts as viewed under differential interference contrast (DIC) and brightfield optics.

Sauch *et al.* (1991) demonstrated good correlation between cysts which stained with PI and a lack of excystation in cysts which had been exposed to heat or to a quaternary ammonium compound. However they found that there was no correlation between PI staining and lack of excystation for cysts which had been exposed to chlorine and monochloramines (Sauch *et al.*, 1991). This was also the finding of Donaghy (1993), who in addition found no correlation between excystation and fluorogenic dye staining in cysts exposed to sea water. After 1-2 days the readings using FDA indicated 100% viability, while the PI readings indicated 0% viability (Donaghy, 1993). Smith and Smith (1989) found that FDA over-estimated cyst viability, while PI under-estimated non-viable cysts compared with *in vitro* excystation. One isolate in their study did not stain at all with either PI or FDA, but underwent excystation (Smith and Smith, 1989).

The difficulties in measuring viability mean there have been few studies done on the length of time and the conditions under which *Giardia* cysts can remain viable. There has been a report of *Giardia muris* cysts stored for a year in a faecal slurry being capable of causing *Giardia* infection in rats, but other studies have not come near reproducing this. Bingham and Meyer (1979) used excystation to determine viability. Cysts remained viable for 77 days in water at 8°C (Bingham and Meyer, 1979). In DeRegnier

et al.'s (1989) study, *Giardia* cysts suspended in either lake or river water in the winter at temperatures below 10°C remained viable for 56 to 84 days and that cysts exposed to tap water were non-viable by the 14th day. Measurement of water quality parameters showed that the only factor of significance in the viability of cysts in environmental water such as in lakes and rivers was the water temperature. Low water temperatures prolonged the survival of the cysts. The viability readings using the fluorogenic dyes fluorescein diacetate and propidium iodide correlated reasonably well with animal infectivity tests (DeRegnier *et al.*, 1989). This thesis seeks to find a reproducible method of measuring cyst viability using fluorogenic dyes.