Giardia in New Zealand Animals

Prevalence, Viability in the Environment and Preservation

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ABSTRACT

Little is known about the epidemiology of giardiasis in New Zealand. Most interest has been focused on the occurrence of *Giardia* cysts in the water ways of this country and in particular in municipal water supplies. Little is known about the occurrence of asymptomatic or even symptomatic giardiasis in people as this is not a notifiable disease, even less is known about giardiasis in domestic or wild animals.

Giardia can now be cultured in the laboratory. *G. intestinalis* may be cultured *in vitro* while *G. muris* is often cultured in the mouse. *G. intestinalis* cysts when harvested are often only a portion of the total cells present with trophozoites and incompletely formed cysts being a larger portion of the cell population. For ease of counting cysts it was desirable to destroy the trophozoites and incompletely formed cysts. On average 64% of the trophozoites can be destroyed when harvested cultures are incubated in double distilled water overnight. Trophozoites were found to remain present in the water for weeks when stored at 4°C. Sonication destroyed trophozoites within two minutes while incompletely formed cysts persisted in suspension with completely formed cysts. The only way to quickly and easily destroy trophozoites and incompletely formed cysts was to incubate the cell suspension in 0.1% SDS for approximately two minutes and then wash the cysts remaining by slow centrifugation. The result is a clean suspension of non-viable completely formed cysts.

New Zealand animals shown to harbour the parasite *Giardia* include farm animals; cattle, sheep, dogs and chickens, of importance to anyone using animal manure on their gardens especially on vegetables that do not require cooking before consumption. Domestic animal wastes can enter the water supplies on farms and get into rivers that supply town water supplies. Wild animals infected with *Giardia* studied in more detail were the possum, house mouse and ship rat. These animals may be a reservoir for contaminating water ways in less populated areas of New Zealand though they were more likely to just maintain the infection within their own population due to little contact with running water. Other wild animals defecating near water ways could serve as sources of infection.

Little is known about the zoonotic potential of *Giardia* found in animals. In past trials with *Giardia* cysts from beavers were shown to infect 2 out of 3 people. Dogs and cats have been implicated as possible sources of household infections and it is recommended when treating a family for giardiasis to also treat household pets.

Giardia intestinalis cysts cultured *in vitro* are commonly used for experimental work due to the ease of harvesting large numbers. It was not known if the *in vitro* cultures truly reflected the characteristics of *Giardia* isolates from people and animals. Morphologically, few cysts harvested from the flask are elliptical in shape, most being round. It is thought

in vitro culturing is highly selective and resulting cultures would only represent a small portion of the wild population.

G. intestinalis cysts cultured *in vitro* were compared to *G. intestinalis* isolated from human faeces and *G. muris* cultured in the mouse. Cysts could not survive in the absence of water. Laboratory trials found that *Giardia* cysts were able to survive and remain viable for months in cold water (4°C) and for shorter periods of time at higher storage temperatures. Cysts suspended in water free of faecal matter were viable and detectable for a longer period of time than those cysts exposed to faecal matter. Cysts incubated at 4°C had the best survival rate with respect to viability and the length of time cysts were present.

Preservation of cysts in 10% formalin did not necessarily prolong the length of time *Giardia* cysts could be stored for. Cysts stored in water at 4°C survived for as many months as cysts fixed in 10% formalin. *Giardia* cysts studied in the laboratory are often fixed in 10% formalin or Schaudin's fixative (PVA) to enable long term storage of specimens in suspension or on slides. Using Schaudin's fixative, *Giardia* cysts were destroyed and the internal morphology of the cysts was greatly distorted. Fixing with osmium tetroxide was not found to distort internal morphology as no difference in morphology was viewed under Nomarski optics between viable organisms and those fixed with osmium tetroxide.

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CHAPTER 1. INTRODUCTION

1.1. The Organism

1.1.1. Morphology

Giardia is a flagellate protozoan that can exist in two forms, as an actively growing and dividing trophozoite usually found in the intestine and as an infective, resistant cyst that is excreted within faeces into the environment.

The trophozoites measure approximately 12-15 μm in length and 5-9 μm wide and look like a pear with a flattened side ie the ventral disc (Plates 1, 3A and B). The anterior half of the organisms contain a pair of nuclei. The axonemes of the four pairs of laterally placed flagella originate from basal bodies at the anterior pole of the nuclei and the axonemes of the three posterior directed flagella run between the nuclei. The posterior half of the organism contains a prominent structure, the median body. This structure is described as a 'claw-hammer' shape in *Giardia intestinalis*, and as 'short and round' for *G. muris*, which is of use in taxonomy. This structure consists of a bundle of microtubules. Four pairs of flagella originate from basal granules at the anterior pole of the nuclei.

The cysts are elliptical in shape and are approximately 6-10 μm long (Plates 2, 3c-3e and 4a). A few internal structures may be recognised. There may be two or four nuclei in the cyst depending on whether cell division has taken place. Axonemes of the flagella and crescent-shaped fragments of the ventral disc are often seen. The cyst wall is easily detected by light microscopy but little of its structure can be seen if the cyst is viable and refractive under phase-contrast microscopy.

With the use of scanning electron microscopy (SEM), greater detail of the *Giardia* trophozoite surface structures are visible (Plate 4). The dorsal surface of the trophozoite has few named structures. It may be slightly rough in appearance and bear slight depressions or pits related to underlying cytoplasmic vacuoles. Three of the four pairs of flagella emerge at anterior, posterior, and caudal positions. The fourth pair, the ventral flagella, emerge from the crevice between the posterior portion of the ventral disc and the body of the trophozoite. The dorsal surface is separated from the ventral surface by the ventrolateral flange which is often marked by the presence of short microvilli. The ventral disc is found in the anterior half of the trophozoite. The prominent lateral crest marks the periphery of the disc and produces a characteristic overlapping spiral on the surface of the disc. The surface of the disc is smooth but depressions may be present in the centre of the disc.

Figure 1 shows the morphological features of the species *G. intestinalis, G. muris* and *G. agilis*, (Kulda and Nohynkova, 1978).

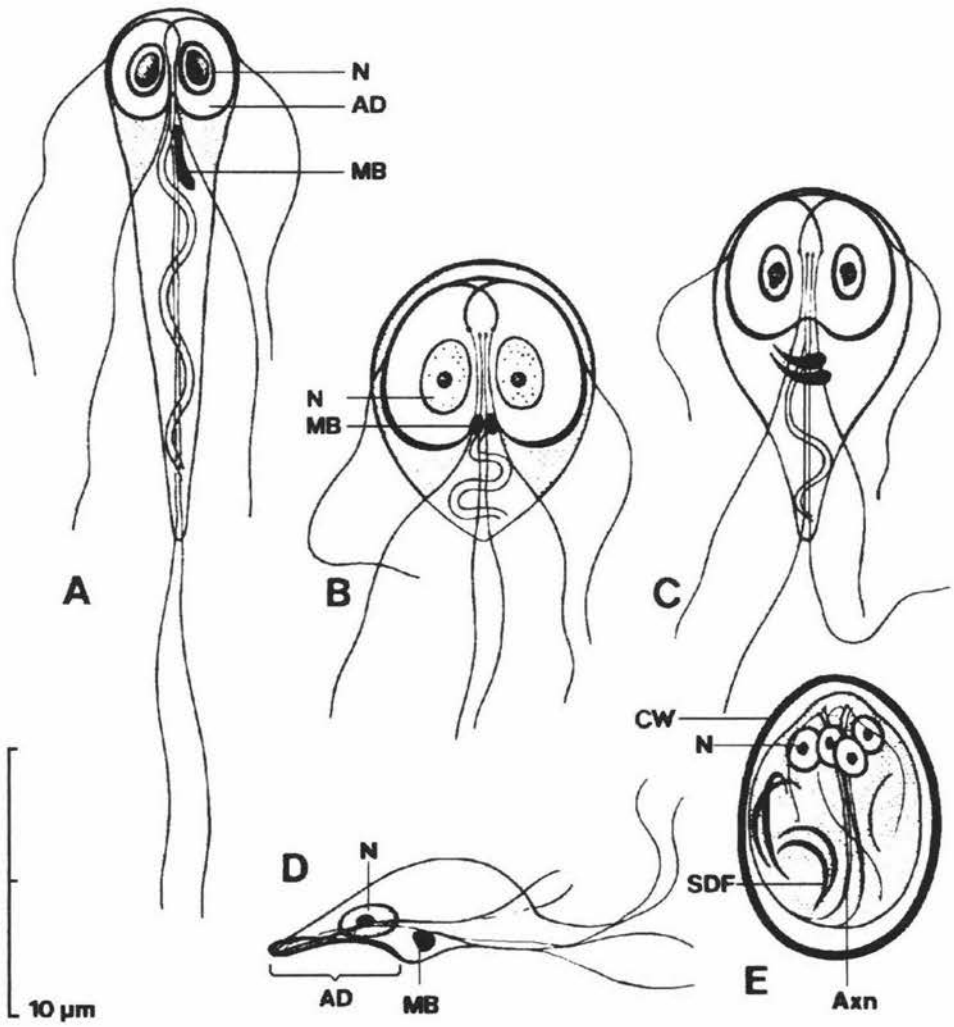


Figure 1. Semidiagrammatic representation of three morphological types of *Giardia* viewed in the light microscope. Ventral view of trophozoites of *G. agilis* from amphibians (A), *G. muris* from mice (B), and *G. intestinalis* from man (C). *G. intestinalis*, lateral view of a trophozoite (D) and cyst (E). Trophozoites of the *G. agilis* group (A) have a long and narrow body, a relatively short adhesive disc (AD) and long club-shaped median bodies (MB) situated parallel to the longitudinal axis of the cell. Trophozoites of the *G. muris* group (B) have a broad, short body with a large adhesive disk overlapping more than one-half of the body length. Median bodies are small and round, situated parallel to the longitudinal axis of the cell. Trophozoites of the *G. intestinalis* group (C) have a pyriform body with an adhesive disk shorter than one-half of the body length. Nucleus (N), Cyst Wall (CW), Suction Disc Fragments (SDF), Axonemes (Axn).

1.1.2. Life cycle

Giardia is transmitted from person to person and requires no intermediate host, being transmitted via the faecal-oral route with only 10-100 cysts ingested found to cause infection (Rendtorff, 1954).

Cysts are ingested, pass through the acidic stomach into the duodenum where they excyst to form two trophozoites. These then attach to the lining of the intestine. The trophozoite colonises the upper small intestine by attaching to the mucosal epithelial cells of the lumen, with its ventral adhesive disc (Morecki, 1964; Gillin, 1981), or by swimming in the intestinal fluid (Gillin, 1986). Attachment is firm and may result in an impression on the ventral surface of the trophozoite attached to the microvillous border. Attached trophozoites may detach from the epithelium which is replaced every 72 hours and sloughed off the villi. The trophozoites may undergo mitotic division within the intestinal lumen or they may encyst and pass out of the host in their faeces.

The trophozoite encysts, ie, the morphology of the organism changes from a flagellate trophozoite to a cyst, in the distal part of the small intestine and is excreted in the faeces. The cysts are transmitted to a new host through contaminated water or ingestion of food contaminated with faeces.

The efficiency of *Giardia* surviving passage from one intestinal tract to the next depends on the resistance of the cysts wall to dehydration, soap, physical forces, the antimicrobial actions of the mouth (amylase in saliva) and the stomachs hydrochloric acid (pH 2.0). *Giardia* is well adapted for transmission via the faecal-oral route with gastric acid being found necessary to facilitate excystation and duodenal colonisation (Bingham and Meyer, 1979). They can survive for months if kept wet and cold but cannot withstand desiccation even for a few seconds as observed during this study.

Giardia can be transmitted in several ways via the faecal-oral route eg through close contact as well as through ingestion of food or drinking water contaminated with human or animal faeces (Erlandsen *et al*, 1988). There has probably been an overemphasis on water-borne giardiasis with little attention paid to other forms of transmission. Direct faecal-oral transmission, whether food-borne, person-to-person or animal-to-person, has been as frequently implicated as the source of *Giardia* as water-borne transmission (Wallis and Hammond, 1988a). Cysts involved in water-borne outbreaks may also be from a range of animal origins (Erlandsen and Bembrick, 1988).

1.1.3. Taxonomy

There have been many different ideas of how to define species of *Giardia*. Hegner (1930) believed that *Giardia* were strictly host specific but this could not be verified on a

morphological basis (Ackers, 1980). Later workers, on the basis of Hegners work, recognised over 20 species based on the host animal and minor morphological and dimensional differences. There have now been at least 49 "species" named.

This is compared with Filice (1952) who earlier recognised only three species: *G. agilis*, a morphologically distinct parasite, being slimmer and longer, found in amphibians; *G. muris* with rounded median bodies found in mice, rats, other rodents, and some birds and reptiles; and *G. intestinalis* with median bodies shaped like a claw hammer, found in humans and other mammals including rodents and some birds and reptiles. *G. intestinalis* is also known as *G. lamblia, G. duodenalis,* or *Lamblia intestinalis.*

Grant and Woo (1978a, 1978b), after careful cross-species transmission studies, concluded that some species, eg *G. simoni* in rats, *G. muris* in mice and *G. peromysci* in deermice, are highly host specific while others are not, eg *G. microti* in voles and *G. mesocricetus.* They also found that *G. muris* from laboratory mice were more host specific than *G. muris* found in wild rats, suggesting *in vivo* culturing was selective.

Erlandsen *et al* (1990) isolated and axenically cultured *Giardia ardeae* from the great blue heron (*Ardea herodias*). Based on morphological characteristics, namely the presence of a single caudal flagellum, the other one being rudimentary in structure, and chromosomal migration patterns, they considered *G. ardeae* to be a separate species and considered species classification based on median body structure alone to be inadequate.

Grant and Woo (1978) identified five species and suggested that species be defined on a combination of morphology, morphometrics and host specificity based on cross transmission studies.

The use of trophozoite morphometrics was found to show significant differences in the measurements of five isolates (Bertram *et al*, 1984) but significant differences were also found within the isolates so the use of morphometrics should not be used as the sole basis of classification. There was a necessity to establish standard classification procedures. The usefulness of small differences in defining species is at best limited, especially as the hosts diet has been shown to significantly affect the trophozoite number and cyst size. Also when studying *Giardia* isolates for morphometric comparisons it is important to realise there may be more than one strain present. With this in mind, the use of clones is necessary to ensure that only one strain at a time is being studied in detail eg for cross-species infection, detailed morphometric traits and establishing required optimal growth environments.

Because of the different methods of classification, an organism may be given several names or conversely, one name for what in reality are several species.

There have been several new methods of species classification proposed. These include gel electrophoresis of the genome or iso-enzymes (Korman *et al*, 1986), immunofluorescence, DNA-DNA hybridisation and many more (Wallis and Hammond, 1988b).

Differentiation of *G. intestinalis* and *G. muris* was achieved by immobilisation in various sera (Lehmann and Wallis, 1988). Tests have shown antigenic differences between cysts of various animal origins and human origin and it may be feasible to use this to identify the animal of origin (Wallis and Hammond, 1988b) though not necessarily the *Giardia* species. This is because the antigenic differences may be physiological manifestations induced by the different intestinal environments of animals. An experiment utilising DNA-DNA hybridisation (Uji *et al*, 1988) has suggested that *G. intestinalis* strains isolated from a specific geographical area, regardless of their original host, are closely related genetically. It was concluded that some *G. intestinalis* strains have a broad host specificity and can cause outbreaks of giardiasis by transmission among different animal species.

In all cases, the current research is only at the developmental stage and the above methods have been proposed as a basis for the future studies in establishing a standard method of species classification. It has been recommended that strains be referred to as isolates of the animal of origin until such time as a standard classification and a characterisation method is agreed upon.

The classification of *Giardia* from Phylum to Family is as follows:

Phylum: **Protozoa**.

Subphylum: **Sarcomastigophora** (Honigberg and Balamuth, 1963), with a single type of nucleus, vesicular; sexuality, if present, syngamy; with flagella, pseudopodia, or both types of locomotory organelles; typically no spore formation.

Superclass: **Mastigophora**, one or more flagella typically present in trophozoites; asexual reproduction, basically by longitudinal binary fission; sexual reproduction unknown in many groups.

Class: **Zoomastigophorea**, chromatophores absent; one to many flagella; amoeboid forms, with or without flagella in some groups; sexuality absent in most groups; predominantly parasitic.

Order: **Diplomonadida**, with two karyomastigonts, each with four flagella; at least one of these flagella is recurrent; with two nuclei; without mitochondria or Golgi apparatus; with cysts; free-living or parasitic.

Family: **Hexamitidae**, six or eight flagella, two nuclei and sometimes axostyles and median or parabasal bodies; bilaterally symmetrical.

This study uses the genetic classifications of Filice, 1952, ie *Giardia intestinalis*, *G. muris* and *G. agilis* when referring to the different species of *Giardia*.

1.2. The Disease

1.2.1. Pathogenesis

Clinical effects of the human infection with *Giardia* range from the asymptomatic carrier state to a severe malabsorption syndrome. Histopathological changes in the affected mucosa may be minimal, or there may be significant enteropathy with enterocyte damage, villus atrophy and crypt hyperplasia. Reasons for this extreme variation is host susceptibility are poorly understood. It is thought that host factors such as nutritional status, systemic immune responses and mucosal immunity contribute significantly to the tissue damage in giardiasis, almost independently of the local effects produced directly by the parasites and their secreted products.

Trophozoites are readily detected in the duodenum and proximal jejunum of infected patients. Wright *et al* (1977) found evidence of vitamin B_{12} malabsorption in some patients with giardiasis. The degree of B_{12} malabsorption seemed to correlate with the severity of histological change in the jejunal mucosa, and absorption returned to normal in the majority of treated patients

Not studied in humans is whether the distribution of the parasites along the intestine alters in abnormal circumstance, ie in disorders of intestinal motility. When conditions are favourable, the parasite may be found in various extra intestinal sites such as the gall bladder (Godstein *et al*, 1978) and urinary tract (Meyers *et al*, 1977).

Evidence suggests that trophozoites do not normally penetrate the intestinal epithelium in humans. Brandborg *et al* (1967) found evidence of mucosal invasion in patients who had diarrhoea and large numbers of trophozoites in the lumen, but not in asymptomatic subjects. However, Owen *et al* (1979), in a careful ultrastructure study using the murine model of the infection, found mucosal invasion only in areas where necrosis or mechanical trauma was present.

The intensity and time course of *Giardia* infection was monitored by performing serial counts, and total small intestinal trophozoite load was measured by a vibration technique (Gillin *et al*, 1982a). Results for mice of the CBA strain were recorded. Both cyst excretion and trophozoite count peak at 2 weeks, cysts being undetectable in the majority of animals after 4 weeks. However, a small number of trophozoites could be found in 50% of the animals studied at 3 or more months post infection. The existence of a latent infection, undetectable by faecal cyst counts, was confirmed by using systemic corticosteroids. In a majority of previously infected mice, residual trophozoites were found to proliferate rapidly when the host mice were treated with cortisone acetate, a glucocorticoid (Nair *et al*, 1981).

1.2.2. Diagnosis

Giardia cysts can be isolated from faecal samples, while the trophozoite form can be isolated from the intestinal fluid or from diarrhoeal stools. Viable trophozoites may be isolated from fresh stools. They are not adapted for exposure to the external environment and therefore do not remain viable for many hours, but they may still be detected several days later (see Results). For the best results when isolating cysts from stools, use fresh samples or preserve the sample for future isolation.

Feely and Erlandsen (1981) developed a simpler method for the isolation of trophozoites for rat intestine based on the trophozoites ability to attach to warm surfaces and detach at low temperatures leaving intestinal debris still attached. The washed trophozoites were then reattached at 37°C with viability remaining greater than 90%. Trophozoites have also been cultivated from the duodenal fluid of humans using a medium developed by Gordts *et al*, (1985).

The detection of *Giardia* cysts in faeces requires multiple examinations as some hosts seem to excrete cysts intermittently. Two to three faecal samples need to be collected one to three days apart (Logsdon, 1988). Because of the possibility of intermittent cyst excretion, a negative test does not mean the patient is *Giardia* free, only a positive result has any meaning.

There are a variety of techniques available for the recovery and detection of *Giardia* cysts from faecal samples. The simplest methods used involve a smear of faecal material stained with a non-specific stain eg a Trichrome, Grams iodine or Lugols iodine stain.

Giardia cyst recovery methods include flotation methods using gradients of sucrose, percoll, percoll-sucrose, sheather-sucrose or zinc sulphate and many more. Zinc sulphate should not be used if cysts are required for culturing as this solution renders cysts non-viable within minutes.

There are several commercial stains available for the detection of *Giardia* cysts. These include, from Cellab; a Direct Fluorescent Antibody assay (DFA), and an Enzyme Linked Immuno Sorbant Assay (ELISA), and from Meridian; an Indirect Fluorescent Antibody assay (IFA) IFA-*Giardia* (Plate 2), IFA-Combo, and a DFA developed specifically for testing faecal samples. Also available is the ProSpectT/*Giardia*® test kit (Alexon Biomedical Inc), an enzyme immunoassay (EIA) specific for a 65 kilodalton (kDal) antigen called GSA 65 (Rosoff *et al*, 1989).

The fluorescent antibody technique is an immunological procedure in which a fluorescent dye is attached to an antibody molecule which in turn reacts specifically with its antigen. The reaction is observable with a fluorescent microscope. However, a monoclonal antibody developed by Riggs *et al*, (1984) only reacted with four out of 647 animals tested

(information supplied by Cellab Laboratories for the Cellab DFA kit). It was suggested that the monoclonal antibody was too specific, ie it was directed against an uncommon antigen, and the use of polyclonal antibodies would give better results, being directed against many more antigens on the cyst wall and hence should hopefully detect all *Giardia* cysts present.

A combination of flotations and stains were compared in this study. See Results.

Sauch (1985) found the indirect immunofluorescent assay (IFA) for detection of cysts in faeces to be much faster and more sensitive than the use of phase contrast microscopy to detect cysts stained non-specifically with iodine or trichrome or not stained at all. This is especially important when there are few cysts to be found.

1.2.3. Treatment

There are several drugs available for the treatment of a *Giardia* infection. They include tinidazole (fasigyn), metronidazole (flagyl and trichozole), nimorazole (naxogin) and ornidazole (tiberal).

Treatment protocol for human adults:

Metronidazole - 2 g orally as a single does daily for 3 days.
Nimorazole - 2g as a single dose or 1g as 3 doses 12 hourly.
Ornidazole -1500g as a single dose for 1 or 2 days.
Tinidazole - 2g as a single dose (1 day).

Side effects include:

Metronidazole - stomach upset, angioedema, drowsiness, skin reactions, dark urine, anaphylaxis. Prolonged or high doses may cause neuropathy, encephalopathy.
Nimorazole - pyrosis, nausea, skin reactions, drowsiness.
Ornidazole - mild dizziness, headache, gastrointestinal intolerance
Tinidazole - stomach upset, allergic reactions, furry tongue, dark urine, transient leucopenia.

Drug treatments are not always successful. The reasons for this are not known but other factors may play a part. There may be rapid reinfection by the host resulting in drug ineffectiveness not failure. This is more a problem involving social contact between animals and humans rather than drug failure. Drug resistance has not been demonstrated to date. Other factors contributing to treatment failure besides reinfection include failure of the patient to comply with treatment instructions, variations in the pharmacokinetics of drugs, inactivation of the drug by superinfection with other organisms and sequestration of the organism from the drug. Drug resistance has only been "indicated" by a natural variation in drug sensitivity in which case a combination of chemical treatments is recommended. In all cases of treatment, follow-up is necessary to ensure eradication of the infection.

1.2.4. Epidemiology

There is a wide variation in the prevalence of *Giardia* infection between countries and within regions of the same country. Children are more commonly infected than adults. Social, environmental, and climatic factors may influence the prevalence of infections.

In population studies, a high prevalence in children has been demonstrated in developing countries. In a study by Gilman *et al*, 1985, where it appeared that *Giardia* infection rapidly declined with increasing age, it was highest in 5-10 year old children and rare after the age of 40.

Prevalence of *Giardia* between urban and rural populations has not been studied in New Zealand, but in studies in African countries, urban children had higher incidences of infection. It was suggested that a high population density coupled with inadequate water supply and sanitation in the towns may contribute to this (Mason *et al*, 1986).

In developed countries where pre-school children are enrolled into day care centres the most significant characteristic associated with a high incidence of diarrhoea was the presence of young, non toilet trained children (Sullivan *et al*, 1984). If these children were allowed to maintain close contact with other children then there is a good chance that *Giardia* infection occurred via direct contact, or via toys and food also handled by infected children. Staff and parent hygiene practices are also an important consideration. Hands not washed adequately between nappy changes and food preparation can aid the spread of infection.

1.3. Cultivation

1.3.1. Isolation of *Giardia* from animals

To establish *in vitro* or *in vivo* cultures, isolates of the organism must first be isolated from an animal host. There are several procedures used and may depend on the availability of the animal host. Isolates can be obtained by collecting faecal samples and isolating cysts or the animal may be sacrificed and trophozoites harvested directly from the intestine. The methods available are discussed in more detail below.

1.3.2. Axenic cultures

A convenient method of obtaining *Giardia* is by *in vitro* axenic cultures. Axenic culturing is required to grow up the large number of isolates which are required for various studies eg gel electrophoresis, DNA analysis, and morphological characteristics of both cysts and trophozoites. Being able to axenically culture isolates was a major step forward for

researching this organism. Axenic cultures have been successfully established for human *Giardia* strains. There have been no report of the establishment of axenic cultures or even *in vivo* cultures for *G. agilis* found in tadpoles and frogs. Nor have long term *in vitro* cultures been established for *G. muris*, let alone through its whole life cycle.

To establish an axenic culture from isolated cysts, it is necessary for the cysts to undergo excystation. Methods available include those used by Feely (1986), Feely *et al* (1991), Sauch (1988), Boucher and Gillin (1990), and Schupp *et al* (1988). The excystation method may use an acid medium to induce excystation and final incubation in a growth medium though acid induction is not always used eg Feely *et al* (1991). The excystation medium used in this study was modified TY1-S-33 which had extra L-cysteine (0.2%) (Kasprzak and Majewska, 1983a, 1983b) and 50/50 foetal calf and bovine serum (Tonks, 1988).

It had been found that trophozoite attachment was necessary for replication, and L-cysteine was an important vector for trophozoite attachment and therefore trophozoite viability. However cysts have been seen to attach to each other *in vitro* and replicate while suspended in medium. L-cysteine was also found to have a protective effect upon trophozoites exposed to oxygen (Brown *et al,* 1992).

Because of significant physiological differences between *G. muris* and *G. intestinalis* strains, a different method was required for the excystation of *G. muris*. A method was developed by Schaefer *et al* (1980) in which greater than 90% excystation rates were reported. Feely (1986) developed a simpler method reporting 96% excystation. Excystation of cysts occurred within 30 minutes.

It should be noted that rats may concurrently harbour *G. muris* and *G. intestinalis* so it would be necessary to clone organisms for identification and future experiments when isolating *Giardia* from these animals to ensure only one species at a time is being studied or it may only be possible to draw generalised conclusions from experiments performed using mixed cultures.

Modified TY1-S-33 is the medium of preference for axenic culturing of human *Giardia* (Keister, 1983). There is also a commercially available medium in Switzerland developed by Gordts *et al* (1985).

TY1-S-33 is a complex medium used to culture *G. intestinalis*. It contains serum, even though serum is not to be found in the intestinal lumen. Gillin *et al* (1986) has shown that a defined mixture of pure biliary lipids can support the growth of *G. intestinalis* in serum-free medium. Unfortunately the trophozoites were only maintained for 24 serial subcultures, indicating that biliary lipids do not fulfil all the functions of serum. They also found that iron, occurring naturally in bile and duodenal mucous, increased the growth of *G. intestinalis*.

Wieder *et al* (1983) managed to mass cultivate *G. intestinalis* in serum-free medium getting 30% less growth than would occur in medium with serum. It was concluded that surface

area also played a major role in mass cultivation with yields better in tubes than in flasks. Whether unlimited subculturing was possible was not indicated.

Incubation temperature was also important with the maximum number of trophozoites becoming attached at 37°C compared with none at 12°C. The optimum pH was found to be between pH 6.85 and 7.0 (Gillin and Reiner, 1982).

1.3.2.1. *In vitro* cultures

Giardia intestinalis trophozoites were grown *in vitro* in the growth media TY1-S-33 (Keister, 1983). Cultures were incubated at 37°C in a sealed 50 ml or 250 ml nunc culture flask. The flasks were turned once a day to allow a monolayer of trophozoites to form on all sides of the flask. Twice weekly, or before the trophozoites reached the end of their log growth phase, the cultures were subcultured. This involved placing the flask on ice for 10 minutes, allowing most trophozoites to detach. The flask was inverted twice and half the media was discarded into medol (diluted 1/50). The flask was then immediately refilled with pre-warmed growth media and placed back in a 37°C incubator. However, if the cultures were growing rapidly and required subculturing daily, it was advised to sit the culture flasks on ice until only a few trophozoites remain attached to the flask, wash out detached trophozoites and refill the flask with pre-warmed growth media.

1.3.2.2. Encystment

When cultures were encysted, the culture flask was not placed on ice so most trophozoites remain attached. Instead the flask was stood on end so all detached trophozoites congregated at the bottom of the flask. Most of the growth media was discarded with a small amount left containing free trophozoites. The flask is then immediately refilled with pre-warmed encystment media, a modified form of growth media containing 5 mg/ml bile.

Cultures were induced to encyst on the third day after subculturing while they were still in the log growth phase. Cysts along with trophozoites were harvested on day 3.

According to Gillin *et al (*1988), and Boucher and Gillin (1990), it is possible to obtain a suspension of intact *in vitro* cultured cysts free of trophozoites and incompletely formed cysts. This would be of use as cysts are usually only 30% of the total organisms harvested as counted by the author. For the experiments performed by this author, counting intact *in vitro* cysts in such a mixed suspension was found to be unnecessarily tedious and difficult. Also when doing viability experiments, the presence of trophozoites may mislead an observer into thinking their suspension of cysts is still viable and producing trophozoites when it was not. This suspension may then be used to inoculate animals when it is in reality composed of non-viable cysts, leaving the scientist puzzled as to why *in vivo* culturing failed. Various methods to separate viable trophozoites from cysts and incompletely formed cysts cultured *in vitro* were tried in this study. (Section 3.1.)

1.3.2.3. Excystment

This is the process by which the cyst form of *Giardia* undergoes transformation resulting in the production of two viable trophozoites. The cyst may be induced *in vitro* to excyst by a number of different methods as mentioned above. These methods are for specific species ie *G. muris* or *G. intestinalis* and all report to have a high level of excystation ie greater than 90%.

Cysts may also be induced to excyst naturally by passing through the stomach and into the intestine of an animal host. To establish axenic cultures from *Giardia* cysts isolated from environmental samples it is necessary for them to be induced to excyst.

1.3.3. *In vivo* culturing

For *in vivo* culturing, animal models have been established, with human *Giardia* strains cultured in the Mongolian gerbil (*Meriones unguiculatus*) and mice, and *G. muris* cultured in mice.

The animal chosen as a model for diseases in humans should reflect the human body in its sensitivity to infection as it occurs in nature and the pathology that will develop later. When selecting a suitable animal the following points should be considered:

1. Sensitivity of the animal to *G. intestinalis* from various animals;
2. Successful infection including colonisation and multiplication of trophozoites in the small intestine;
3. The formation and release of cysts in the faeces;
4. Infection must be transferable from humans or other hosts by oral inoculation;
5. The course of the infection and associated pathology in the animal host should mimic the changes observed in human giardiasis;
6. The animal should be inexpensive, breed under laboratory conditions and be easy to maintain in the laboratory;
7. Background knowledge of the biological properties of the animal;
8. Ethical implications.

The Mongolian gerbil is considered by Faubert and Belosevic, (1990), to be an ideal model for human giardiasis for the following reasons: (1) the animal is highly susceptible to infection with *G. intestinalis* organisms; (2) adult animals can be infected with either cysts or trophozoites cultured *in vitro*; (3) trophozoites recovered from gerbils can be used to initiate new *in vitro* cultures; and, (4) it is an ideal model to study the pathophysiologic changes caused by the infection.

Mice are useful models for infection with *G. muris* but not so for *G. intestinalis.* This is because mice have been demonstrated to show age-dependent susceptibility to *G.*

intestinalis, reducing their usefulness for studies on immunology and pathology of human giardiasis. They are however, useful for *in vivo* culturing of *G. intestinalis* and *G. muris*.

The use of *Giardia*-free animal colonies for *in vivo* culturing is very important because of acquired resistance from previous infections. It is best that a *Giardia*-free colony be established over several generations and not by treatment eg with flagyl. This is because it has been found that infected animals treated with flagyl, though being rendered *Giardia*-free, appear to acquire resistance to re-infection by this parasite (Roberts-Thomson *et al*, 1976a). This would have an adverse affect on the results of any experiment on the infectivity rate of different strains and make *in vivo* culturing even more difficult.

1.4. Cryopreservation

Two methods are currently used in this laboratory. The method of Phillip *et al* (1982) with cultures stored at -80°C and a modified method of Phillips *et al* (1982) and Wallis and Wallis (1986) was used to retrieve them.

To preserve the organism, either in the cyst or trophozoite form, a suspension of organisms of 1.2-1.5 x 10^6 was used. Trophozoites were harvested on day 3 of incubation when they were in late log phase. Culture tubes or flasks were placed in ice water for 10 minutes to detach trophozoites from the surfaces of the culture flasks. The trophozoites, or cysts, harvested were concentrated by centrifugation at 700 x g for 5 minutes. The concentration of the organisms was determined using a haemocytometer with the trophozoite suspension adjusted to the correct concentration with warm medium and the cyst suspension was adjusted with sterile milli-Q water. 0.25 ml of the suspension was pipetted into 1 ml Nunc cryopreservation tubes and 0.25 ml of a 15% dimethyl sulphur oxide (DMSO) solution was added drop by drop. The tubes were mixed by inversion and then well wrapped in paper tissues, placed in a polystyrene container which was in turn placed in a -80°C freezer. The cryopreservation tubes were well wrapped with paper tissues to reduce the rate of freezing, protecting the fragile trophozoites from violent forces of freezing which would normally kill them. When frozen, some tubes could be stored in liquid nitrogen and the rest in the -80°C freezer.

To retrieve frozen trophozoites, a modified method of Phillips *et al* (1982) and Wallis and Wallis (1986) was used. The frozen tubes were quickly thawed in a 37°C water bath, transferred to a sterile and clean Kimax tube which was immediately filled with warm growth medium and incubated horizontally at 37°C. After 15-25 minutes, during which time health trophozoites are expected to attach to the surface of the kimax tube, the medium was discarded and the tube refilled and incubation was continued at 37°C. After 4 hours, the medium was again discarded and the tube refilled with warm growth medium. This series of washed in which the growth medium is replaced was required to remove all traces of DMSO and cell membranes and other degrading enzymes released by dead

trophozoites. The cultures are then incubated for a further three days before they are refilled with fresh medium. It may be two weeks before a monolayer of trophozoites is established. It is recommended that sample tubes of the frozen cultures are retrieved a week after freezing to ensure that the stored trophozoites remain viable ie that the process has worked.

For the retrieval of cysts, it is necessary to thaw as for the trophozoites but the cysts can then be washed free of DMSO and other contaminants by centrifugation at 1000 x g, discarding the medium and refilling the kimax tube with sterile milli-Q water. The washing process is done at least three times to ensure a clean suspension. The cysts may then be induced to excyst to ensure they have survived the process.

1.5. Animal Hosts

Many domestic and wild animals have been identified as possible reservoirs of *Giardia*, including strains that infect humans. Buret *et al* (1990), postulated that domestic ruminants (sheep and cattle) may be a reservoir for human infection and vice versa. Humans may become infected from their pets through zoonotic transmission (Hewlett and Andrews, 1982), eg dogs licking people after anal grooming, or a child getting into the cats dirt box, though such transmissions have not been proven. Davies and Hibler (1979) found that cats may have been responsible for the introduction of *Giardia* into two households but there was a question of who infected who and how.

Dogs were indicated as a possible indicator of imminent giardiasis outbreaks within a community. This is because the prevalence of *Giardia* in dogs was found to increase several-fold when there were outbreaks of human giardiasis. Dogs may be important reservoirs because of their close association with people, and also they may carry cysts on their fur which may be picked up by rolling in dung (Lewis, 1988). Davies and Hibler (1979), found cysts in 2/34 coyotes and 10/78 dogs. They did not state whether these strains could infect humans. Also 2/34 cattle were found to have *G. bovis* which may be transmitted to humans directly or transmitted via dogs.

The incidence of *Giardia* in calves in India was studied with indications of a high rate of infection (Deshpande and Shastri, 1981). This is important in India where dung is commonly used in fires for cooking, and to build huts and where the water supply is not treated in the country or city. Cattle should be considered an important potential reservoir in this environment.

Giardia is not strictly host specific, though the ability to cross infect different animals seems to differ between strains, and has been shown in many laboratory experiments (Hegner, 1930; Grant and Woo, 1978a, 1978b; Centers for Disease Control, 1979; Meyer and Radulescu, 1979; Dykes, *et al*, 1980; Erlandsen, *et al*, 1988; Lewis, 1988; Tonks, 1988).

Cross transmission between primates and handlers has been documented in a zoo (Centers for Disease Control, 1979). It was found when better procedures were followed by staff, the prevalence of *Giardia* in the primates was reduced.

There have been positive cross transmissions of *Giardia* from humans to domestic animals eg puppies (Davies and Hibler, 1979; Hewlett and Andrews Jr, 1982), mongrel dogs (Hewlett and Andrews Jr, 1982), and cats (Brightman and Slonka, 1976). Budgerigars have also been found to have human *Giardia* indicating that domestic birds may be a source of infection though such cross transmissions have not been demonstrated (Hirai *et al*, 1980; Box, 1981).

Cross transmissions animal-to-animal have been documented with some strains eg from a golden hamster to mice in some experiments (Roberts-Thomson, *et al*, 1978), but not in other experiments (Stevens, 1978). This indicates hamsters could have more that one strain of *Giardia* which at least one was infective to mice. Strain definition would be useful to determine if hamsters could be a source of human *Giardia*.

Many water-borne outbreaks of giardiasis have occurred in which faecal contamination was suggested as a source of infectivity, and in many cases where there was little human activity in the water source, beavers and muskrats were indicated as a possible source of infection.

Davies and Hibler (1979) concluded that beavers are an important reservoir of *Giardia* as cysts were found in 44 of 244 faecal samples tested. It was found that beavers retained the infection in summer, lost it in winter and were reinfected the following summer. Beavers were considered an important reservoir as they often defaecated into the cold water where cysts survive well (Wickramanayake *et al*, 1985). Davies and Hibler (1979) were successful in infected 2 out of 3 human volunteers with *Giardia* isolated from beaver. Dykes *et al*, (1980) has also implicated the beaver as a source of infection and used cysts isolated from beavers to infect beagle puppies, but whether the strains were able to infect humans could not be determined.

Hibler (1984) indicated that up to 51% of muskrats shed cysts and therefore were also a possible reservoir.

Cross transmission has been found to occur between humans, dogs, cats, and beaver, suggesting these cysts are not host specific and are either very closely related or are even the same strain.

A tendency has developed to blame beavers and possibly muskrats for water-borne outbreaks of giardiasis in parts of USA where the human population is sparse. This could lead to other sources like birds or unhygienic practices by people being overlooked. Pacha *et al* (1987) found that microtines and other small rodents of mountain meadows have the potential to be a reservoir of *Giardia* and should be included in any surveys. All possible

animal reservoirs should be considered in any country before any one particular animal is labelled as the source of *Giardia*.

Erlandsen *et al* (1988) showed that a large number of human cysts could infect beavers and muskrats, the muskrats could be infected with beaver isolates, but beavers were not infected with cysts characteristic of *G. ondatrae*, named after their host. Beavers carry a strain that is different from those described for humans so it is possible that beavers carry more than one strain.

Though organisms of the *G. intestinalis* type are encountered in rodents, they occur in these animals less frequently than the organisms of *G. muris* type. This could be due to exclusion by *G. muris* which has already colonised an animal and/or to the fact that *G. intestinalis* strains are not as well adapted for survival in the intestines of rodents.

Cross transmission studies in the past were often contradictory, non-reproducible, or inconclusive because cloned *Giardia* isolates were not used or maintained, and often the test strain is only identified as a human or animal isolate. It should be noted that an animal may be the source of more than one strain eg mice can be infected with both *G. intestinalis* and *G. muris* at the same time. This is why strain differentiation and the use of cloned isolates for experimental work is important - so it is known which strain is being studied.

It is likely that the presence of *Giardia* in animals is more common than currently believed. As clinicians become more aware of the possibility of giardiasis and make a concerted effort to detect these parasites by appropriate methods, the number of reported cases will probably increase, thereby aiding research into the epidemiology and the significance of animals as reservoirs for human *Giardia*.

1.6. Introduction to This Study and Its Justification.

Giardia cysts can not be used to differentiate the species as the medium bodies are not visible. However, there were some morphological differences observed in this laboratory between *in vivo* derived cysts of *G. muris* and *G. intestinalis*. When *G. muris* cysts were freshly isolated from mice faeces they tended to be evenly shaped, like a rectangle with rounded ends. On the other hand *G. intestinalis* isolated from human faeces tended to be shaped similar to a hens egg, ie it is more pointed at one end and tends to be shorter than *G. muris* freshly isolated from mice faeces. Whether these differences are due to the species of *Giardia*, the host species, or simply to the diet of the host was unknown.

In general, there have been no in depth studies performed to determine the role that animals, wild and domestic, may play in the spread of the parasite *Giardia* between animal and human hosts, if this does indeed occur.

The following experiments examined the isolation of pure suspensions of *in vitro* cultured *Giardia intestinalis* cysts for experimental work to make counting easier for the author and

for viability assays. Various combinations of methods were also assessed to see which combination of methods to isolate and detect *Giardia* cysts from animal host faeces were the most sensitive. Culturing of *G. muris* in mice was done to determine the best method for harvesting cysts and a possible mode of transmission between mice.

Giardia intestinalis cysts were harvested from *in vitro* cultures and from human faecal samples, and *G. muris* cysts were harvested from mice. The cysts were then subjected to a range of environmental conditions ranging in temperature, faecal contamination with their associated faecal coliforms and other contamination organisms, and to different levels of humidity ie cysts were suspended in a water environment or the faeces were allowed to dry out. This was done to give an indication of the importance of animals as a continuous source of *Giardia* contamination in New Zealand's mountain streams and rivers.

The common methods of storage used in this laboratory involved suspending *Giardia* cysts in water or 10% formalin and storage at 4°C over a period of months. It was initially assumed by this laboratory that if cyst were stored in 10% formalin there should not be any significant decrease in the number of cysts present over long periods of storage. It was also recommended (Garcia and Ash, 1979) that faecal samples collected from the environment be fixed in PVA (Schaudins fixative) so that they can be tested in the laboratory at a later date. Different methods for the storage of *Giardia* cysts were studied to determine which distorted the internal morphology of the cysts the least and which allowed permanent or long term storage.

CHAPTER 2. MATERIALS AND METHODS

2.1. *In Vitro* Cultivation of *Giardia intestinalis*

Giardia intestinalis cultures isolated from humans were grown in the growth medium of Keister, modified TY1-S-33. The *Giardia* cultures were induced to encyst by adjusting the concentration of bile in the growth medium to 5 mg/ml and adjusting the pH to 6.8.

2.1.1. Growth media (TY1-S-33)

Trypticase Soy Broth (BBL. No. 11768 or Oxoid No. CM129)	20.0 g
Yeast Extract Powder (Difco No. 0127-01)	10.0 g
Glucose	10.0 g
NaCl	2.0 g
K_2HPO_4	1.0 g
KH_2PO_4	0.6 g
L-Cysteine Monohydrochloride (Sigma No. C-7880)	1.5 g
Ferric Ammonium Citrate (Brown Pearls)	0.023 g
L-Ascorbic Acid	0.2 g
NCTC 135 (Gibco No 440-1100)	100 ml solution
or NCTC 109 (Difco No 5927-23)	or 9.6 g
Bile Bacteriological (Sigma No B-8381)	0.8 g
Benzyl Penicillin	0.06 g
Gentamycin sulphate	0.05 g
Vancomycin	0.02 g
Bovine serum	100 ml
Distilled water added to make volume up to	1000 ml

The above ingredients were added to 500 ml of distilled water and mixed with a magnetic stirrer. Once all the ingredients were added, the volume was made up to 1000 ml (1.0 L) with RO water. After mixing, the medium was adjusted to pH 7.0-7.2 with 1M NaOH.

The medium was filtered under positive pressure through a series of non-sterile filters ie a layer of 4 Whatmans #1 filters, then 0.45 μm and 0.2 μm pore membranes. This was necessary to prevent clogging of the final sterilising membrane. Sterilising was achieved by filtering through a 0.2 μm pore membrane that had been sterilised by being autoclaved within the filter unit at 121°C for 15 minutes. Sterile medium was stored at 4°C for a maximum of 10 days.

Bovine serum was obtained through Waitake freezing works and processed in the laboratory in which this study was done.

When the normal set of antibiotics could not control the bacteria the following were used:

Tetracycline	10 µg/ml
Streptomycin Sulphate	50 µg/ml
Chloramphenicol	50 µg/ml

To control fungal and yeast contamination, Amphotericin B was used at a final concentration of 10 µg/ml.

When culturing trophozoites from animals, pepricillin was used in the growth medium at a final concentration of 10mg/ml.

2.2. Recovery of *In Vitro* Cultured *Giardia intestinalis* Cysts from Trophozoites.

Giardia cultures in 250 ml flasks were induced to encyst by adjusting the concentration of bile in the growth medium TY1-S-33 to 5 mg/ml and adjusting the pH to 6.8. Cysts were harvested on day 3. The culture flask was not placed on ice as a few trophozoites as possible were required ie the trophozoites attached to the sides of the flasks were encouraged to remain attached, reducing the amount of trophozoites harvested. The flask was inverted twice and the contents decanted into sterile 50 ml conical flasks. The tubes were centrifuged at 1000 x g for 3 minutes and the supernatant was discarded. The tube was refilled with sterile milli-Q water and spun at 1000 x g for 3 minutes. This washing step was done three times. The *Giardia* cysts, completely and incompletely formed cysts, and trophozoites were suspended in 10 ml sterile milli-Q water.

Five different methods for disrupting trophozoites and incompletely formed cysts with the aim of leaving completely formed cysts intact were examined. The aim was to isolate a pure suspension of cysts free from incompletely formed cysts and trophozoites.

2.2.1. Sonication to remove trophozoites and incompletely formed cysts

A Soniprep 150 Ultrasonic disintegrator was used. This means a sonicating probe was placed in the cyst and trophozoite suspension and sonicated. The distance the probe vibrates across is determined multiplying 5.5 by the intensity of amplitude ie at 5µ the probe moves 27.5µ.

The concentration of the cysts in suspension (ignoring the number of trophozoites present) was determined using a haemocytometer (see 2.1.1.1.). Triplicate 3 ml aliquots of cysts at different concentrations were placed in bijous. The concentrations were:

(i) 10^5 cysts/ml (3X)
(ii) 10^6 cysts/ml (3X)

The bijous were placed in iced water for sonicating. Each cyst concentration was sonicated at a) 5μ, b) 6μ, and c) 7μ, for 1, 1.5 and 2 minutes.

The concentration of the cysts remaining was determined and it was noted if any trophozoites or incomplete cyst walls remained.

2.2.1.1. Suspension density determination using the improved Neubauer (bright-line) Haemocytometer.

The haemocytometer consists of two chambers separated by a transverse trench and bordered bilaterally by longitudinal trenches. Each chamber was ruled and consisted of nine squares, each 1 mm x 1 mm x 0.1 mm with a volume of 0.1 mm^3. Each square mm was bordered by a triple line. The centre line of the three is the boundary line of the square.

According to the U.S. Bureau of Standards' requirements, the cover glass must be free of visible defects and must be optically plane on both sides within ± 0.002 mm. **Only haemocytometer cover glasses may be used. Ordinary cover glasses and scratched haemocytometers are unacceptable**, as they introduced errors into the volume relationships.

The suspension to be counted must be evenly distributed and free of large debris, so that the chamber floods properly. The suspension to be counted should contain 0.01% Tween 20 solution (TDW) to prevent *Giardia* cysts from sticking and causing improper haemocytometer chamber flooding. Cyst suspensions should be adjusted so that there were a total of 60 to 100 cysts in the four corner counting squares. Counts were statistically accurate within this range. If the suspension was too dilute to give a statistically reliable count, the number should be recorded and labelled questionable.

To use the haemocytometer:

1. The suspension was diluted or concentrated as required.
2. A clean cover glass was applied to the haemocytometer and the haemocytometer chamber was loaded with 8-10 μl of vortexed suspension per chamber. If this operation was properly executed, the liquid would fill the entire chamber without bubbles or overflowing into the surrounding moats. This step was repeated with a clean, dry haemocytometer and cover glass, if loading had been incorrectly done. See step 12 below for the haemocytometer cleaning procedure.
3. It was important not to attempt to adjust the cover glass, apply clips, or in any way disturb the chamber after it was filled. The *Giardia* cysts were allowed to settle for 30 to 60 seconds before the count was started.
4. The *Giardia* cysts were counted using a magnification of 200-600X.

5. The chamber was moved so that any ruled area was centred underneath the objective lens.

6. The objective was adjusted so it was close to the cover glass while watching from the side of rather than through the microscope.

7. The view was focused up from the coverslip until the haemocytometer ruling appeared.

8. At each of the four corners of the chamber there are 1 mm^2 ruled areas divided into 16 squares in which *Giardia* cysts were to be counted. The count was started at the top left hand corner square using a hand tally counter. Counts were done from left to right for the top row, right to left for the second row, left to right for the third row and so on. To avoid counting *Giardia* cysts twice, only those touching the top and right boundary lines were counted, not those touching the left and bottom boundary lines. Each of the four corners (1 mm^2 areas) were counted in this fashion.

9. The formula used for determining the number of *Giardia* cysts per ml suspension was:

$$\frac{\text{number of cysts counted}}{\text{number of sq. mm counted}} \times \frac{10}{1\text{ mm}} \times \frac{\text{dilution factor}}{1} \times \frac{10^3\text{ mm}^3}{1\text{ ml}} = \text{Number of cysts/ml}$$

10. The data was recorded.

11. A total of six different haemocytometer chambers were loaded, counted, and then averaged for each *Giardia* cyst suspension to achieve counting precision. Counting precision was measured by calculating the coefficient of variation (CV), ie standard deviation/mean. For values greater than 20%, the analytical results was examined for counting problems, and the procedure was corrected as required. Coefficient of variation values less than 20% should be in the upper 95% confidence limit of the mean.

12. After use, the haemocytometer and coverslip was cleaned immediately to prevent the cysts and debris from drying on it. Since this apparatus was precisely machined, abrasives could not be used to clean it as they would disturb the flooding and volume relationships.

    a. The haemocytometer and cover glass were rinsed first with tap water, then 70% ethanol, and finally with acetone.

    b. The haemocytometer chamber and cover glass were dried and polished with lens paper. They were stored in a secure place.

13. A number of factors were known to introduce errors into haemocytometer counts. These include:

a. Inadequate suspension mixing before flooding the chamber.

b. Irregular filling of the chamber, trapped air bubbles, dust, or oil on the chamber or coverslip.

c. Chamber coverslip not flat.

d. Inaccurately ruled chamber.

e. The enumeration procedure. Too many or too few *Giardia* cysts per square, skipping or recounting some *Giardia* cysts.

f. Total number of *Giardia* cysts counted were too low to give statistical confidence in results.

G. Error in recording tally.

h. Calculation error; failure to consider dilution factor, or area counted.

2.2.2. Flotation on a series of aqueous sucrose solutions

Harvested cysts were suspended in 30 ml sterile milli-q water in a 50 ml conical centrifuge tube. The suspension was underlaid with 10 ml of 0.5 M sucrose. This layer of sucrose was then underlaid with 5 ml of 1.0 M sucrose. This series of layers was underlaid with 5 ml of 1.5 M sucrose. Care is taken not to disturb the interface of each layer. The tube was centrifuged at 500 x g (1800 rpm.) for 10 minutes without brakes applied in a Heraeus Christ centrifuge. 5 ml fractions were collected, washed three times with 0.01% Tween 20 and viewed under phase contrast to determine if intact cysts could be recovered separate from trophozoites and incompletely formed cysts.

All 5 ml fractions collected contained cysts, trophozoites and incompletely formed cysts except the bottom fraction which contained neither. Trophozoites appear to have the same specific gravity as complete cysts and incomplete cysts.

2.2.3. Percoll flotation

Stock Percoll diluted with 0.15 M NaCl was used.

Harvested cysts were suspended in 3 ml of TDW in kimax tubes (130 mm x 10 mm). The cyst suspension was underlaid with 3 ml of 20% percoll. These two layers were then underlaid with 3 ml of 40% percoll. Care was taken not to disturb the interface between each layer. The tubes were centrifuged at 500 x g for 10 minutes without a brake in a Heraeus Christ centrifuge. 1 ml fractions were collected and viewed under phase contrast microscopy at 400 X magnification to determine if complete cysts could be recovered free of trophozoites and incomplete cysts.

Completely formed cysts could not be separated on a series of percoll gradients from incompletely formed cysts or trophozoites.

2.2.4. Trophozoite destruction in water

Cysts (including trophozoites and incompletely formed cysts) were harvested, suspended in water and incubated under the following conditions.

2.2.4.1. *Giardia* incubated at room temperature (20°C)

This study used the method of Boucher *et al* 1990.

Encysted cultures were harvested on day 3. 250 ml flasks were inverted eight times and the non-adherent cells decanted into 50 ml conical centrifuge tubes containing 15 ml sterile milli-Q water. The tubes were incubated for 30-45 minutes at room temperature (20°C). After incubation the tubes were centrifuged at 135 x g (925 rpm) for 5 minutes at 4-10°C and the supernatant was decanted. The organisms were resuspended in TDW and viewed under phase contrast microscopy at 400 X magnification for the presence of trophozoites and incomplete cyst walls.

2.2.4.2. *Giardia* incubated at 4°C overnight

This study used the method of Gillin *et al* 1988.

Cultures were induced to encyst and were harvested on day 3. The 250 ml culture flasks were inverted and media decanted into 50 ml conical centrifuge tubes. The tubes were spun at 833 x g for 3 minutes. The isolates were resuspended and washed twice with sterile milli-Q water and then incubated overnight at 4°C in a total volume of 20 ml. The next day the isolates were vortexed and centrifuged at 833 x g for 3 minutes, and washed again in TDW. The supernatant was discarded into medol. The cysts were suspended in 1 ml TDW and viewed by phase contrast microscopy at 400 X magnification for the presence of trophozoites and incomplete cyst walls.

2.2.5. Trophozoites destruction in sodium dodecyl sulphate (SDS)

In vitro cultured *Giardia* cysts were incubated, with trophozoites and incompletely formed cysts, in 0.1% SDS for 2 minutes. The suspension was centrifuged at 135 x g for 5 minutes in a Heraeus Christ centrifuge, the supernatant was decanted and the cyst suspension was viewed to see if trophozoites and incomplete cysts walls still remained. The method used to detect viability of the remaining cysts was Feely's 1986 method.

2.2.5.1. Feely's 1986 method to induce excystation of *Giardia* cysts

Isolated cysts were incubated at 37°C for 30 minutes in HBSS adjusted to pH 2.0 with 2N HCl immediately prior to use. The HBSS was prepared daily from a 10 X stock solution. Sodium bicarbonate was added to bring the pH to 7.2. A portion of this was then prepared for the acid induction step by the addition of 2 N HCl to adjust it to pH 2.0. The cysts were concentrated by centrifugation, washed with HBSS (pH 7.2) and resuspended in 2-3 ml of modified TY1-S-33 medium with the presence of a 50/50 mixture of foetal bovine serum (Sigma No 200-6140) and bovine serum. The cysts were incubated in uncapped kimax tubes (130 x 10 mm) at 37°C.

2.3. Techniques to Recover and Detect *Giardia* Cysts from Environmental and Laboratory Samples

2.3.1. *In vitro* cultured *Giardia intestinalis* cysts

Cysts originally isolated from humans, were cultured using the method of Schupp *et al* (1988). A comparison of the flotation techniques using different flotation gradients was done with *Giardia* cysts isolated into 0.01% Tween 20 solution. The suspensions were floated on the gradients 1 M sucrose (Roberts-Thomson *et al*, 1976), Percoll-sucrose (Sauch, 1985), Sheather-sucrose (Melvin *et al*) and Percoll diluted to 40% and 70%. Each flotation was repeated 3-4 times and the age of the cysts used varied by weeks. The cysts had their volume adjusted, as required for each flotation technique, with 0.01% Tween 20 solution. The flotation gradient was added under the cyst suspension. Cysts were then recovered according to the individual flotation techniques, suspended in 1 ml of 0.01% Tween 20 solution and the number, and percent, recovered was determined with a haemocytometer under phase contrast microscopy .

2.3.1.1. Sucrose centrifugation

1. In each 50 ml centrifuge tube containing not more than 1.0 ml of pellet, the volume was adjusted to 10 ml with TDW.
2. The suspension was homogeneously dispersed in each 50 ml conical centrifuge tube by vortexing.
3. The volume in each 50 ml centrifuge tube was adjusted to 35 ml by the addition of TDW and the suspension was vortexed.
4. Using a 10.0ml serological pipette, the pellet suspension in each tube was carefully underlaid with 10.0 ml of 1.0 M sucrose.

5. The tubes were centrifuged for 10 minutes at 500 x g in a swinging bucket rotor. The centrifuge was started slowly and accelerated up to the speed where the tubes were horizontal to avoid disturbing the interface between the 1.0 M sucrose and the pellet suspension. Similarly, the centrifuge was decelerated slowly below the horizontal level, to avoid disturbing the interface. **The brake was not used.**

6. 30 ml of the supernatant above the interface was aspirated and discarded from each conical centrifuge tube.

7. With a Pasteur pipette, the interface contained in the next 10 ml was transferred from each tube into clean, individual 50 ml conical centrifuge tubes. The tubes containing the remaining layers were discarded.

8. The volume of each tube containing the interfaces was adjusted with TDW to 50 ml to dilute the sucrose. The volume of diluent used should be at least five times the volume of interface.

9. The interface particulates in each tube were concentrated by centrifugation at 500 x g for 3 minutes.

10. The pellet was resuspended and washed with TDW a minimum of three times by centrifugation at 500 x g for 3 minutes. The pellet in each tube was resuspended in 1 ml of TDW. This material was then stained and examined microscopically or viewed without staining under phase contrast microscopy at a magnification of 200-400 X magnification.

2.3.1.2 Percoll-sucrose centrifugation

1. In each 50 ml centrifuge tube containing not more than 1.0 ml of pellet, the volume was adjusted to 20 ml with eluting solution.

2. The pellet was homogeneously dispersed in each 50 ml conical centrifuge tube by vortexing.

3. Each pellet suspension was sonicated in a Bronson cleaning bath for 10 minutes.

4. Again each suspension was homogeneously dispersed in each 50 ml conical centrifuge tube by vortexing.

5. Using a 10.0ml serological pipette, the pellet suspension in each tube was carefully underlid or overlaid onto 30.0 ml of Percoll-sucrose (specific gravity 1.10).

6. Without disturbing the pellet suspension/Percoll-sucrose interface, the preparation was centrifuged for 10 minutes at 1050 x g in a swinging bucket rotor. The centrifuge was slowly accelerated up to the speed where the tubes were horizontal to avoid disrupting the interface. Similarly, the centrifuge was decelerated slowly, to

lower the tubes below the horizontal level to avoid disturbing the interface. **The brake was not used.**

7. Using a pasteur pipette rinsed with eluting solution, the top 25 ml was collected into clean, individual 50 ml conical centrifuge tubes. The tubes containing the remaining bottom layer were discarded.

8. The volume of each tube containing the top 25 ml layer, was adjusted with eluting solution up to a volume of 50 ml. The volume of diluent used should be at least two times the volume of the collected layer.

9. The interface particulates in each tube were concentrated by centrifugation at 1050 x g for 10 minutes with the brake on.

10. The supernatant in each tube was discarded except for the bottom 5 ml containing the pellet. The pellet was homogeneously dispersed by vortexing. This material was then stained and examined microscopically or viewed without staining under phase contrast microscopy at 200-400 X magnification.

2.3.1.3 Sheather-sucrose centrifugation

1. In each 50 ml centrifuge tube containing not more than 1.0 ml of pellet, the volume was adjusted to 20 ml with eluting solution.

2. The suspension in each 50 ml conical centrifuge tube was homogeneously dispersed by vortexing.

3. Using a 10.0ml serological pipette, the pellet suspension in each tube was carefully underlaid/overlaid onto 10.0 ml of Sheather-sucrose (sp. gr. 1.10).

4. Each 50 ml conical centrifuge tube was centrifuged for 10 minutes at 1900 rpm (450 x g) in a swinging bucket rotor. The centrifuge was started and slowly accelerated up to the speed where the tubes were horizontal to avoid disturbing the interface between the Percoll-Sucrose and the pellet suspension. Similarly, the centrifuge was decelerated slowly, to lower the tubes below the horizontal level and to avoid disturbing the interface. **The brakes were not used.**

5. Using a pasteur pipette rinsed with eluting solution, the interface was collected and then the supernatant with as little of the flotation gradient as possible. The interface and supernatant were collected into a clean, centrifuge tube. The tube containing the remaining bottom layer was discarded.

6. The volume of each tube containing the top 25 ml layer was adjusted with eluting solution to 50 ml.

7. The interface particulates in each tube were concentrated by centrifugation at 2500 rpm for 10 minutes with the brake on.

8. The pellet was resuspended and the particulates washed with eluting solution a minimum of three times by centrifugation at 2500 rpm for 10 minutes. The pellet was then resuspended in 1 to 2 ml of eluting solution or PBS. This material was then stained and examined microscopically or viewed without staining under phase contrast microscopy at 200-400 X magnification.

2.3.1.4. Percoll centrifugation

1. In kimax tubes (130 mm x 10 mm), containing not more than 1.0 ml of pellet, the suspension volume was no greater or less than 3.0 ml. If it was greater, then the tubes were centrifuged 3-5 minutes at 500 x g and the supernate was reduced to the 3.0 ml mark. If it was less than 5.0 ml, then the volume was adjusted up to 3.0 ml with TDW.

2. To each tube containing a 3.0 ml suspension, 3.0 ml of 40% Percoll, (sp. gr. 1.04) was carefully underlaid with a pasteur pipette resulting in two distinct layers.

3. In each tube, the bottom layer containing 40% Percoll was underlaid, using a pasteur pipette, with 3.0 ml of 70% Percoll, (sp. gr. 1.09). There were now three distinct layers in each tube.

4. The kimax tubes were centrifuged for 10 minutes at 500 x g. The centrifuge was started and slowly accelerated to avoid disturbing the band interfaces. Similarly, the centrifuge was decelerated slowly to avoid disturbing the band interfaces. **The brake was not used.**

6. For each tube, the top 1-2 ml of the supernatant was aspirated and discarded into medol. The TDW/1.04 and the 1.04/1.09 interfaces were collected into individual clean, kimax tubes.

7. The particulates from each interface was concentrated by centrifugation for 3 minutes at 500 x g. The particulates were washed a minimum of three times with TDW by centrifugation for 3 minutes at 500 x g. The particulates were then resuspended in 1 to 2 ml of TDW or PBS. This material was then stained or examined microscopically or viewed without staining under phase contrast microscopy at 200-400 X magnification.

2.3.2. *Giardia intestinalis* cysts isolated from human faeces

The cysts were isolated using the 1 M sucrose flotation gradient method. This was taken arbitrarily as a 100% recovery as the original number of cysts present could not be determined. The cysts were stored in 10% formalin at 4°C. The cysts were floated on the gradients Percoll-sucrose, Sheather-sucrose and Percoll diluted 40% and 70%. The flotations were repeated four times, in some cases with cysts that were several weeks older

ie had been incubating at 4°C for a longer period of time. The cysts had their volume adjusted, as required for each flotation technique, with 0.01% Tween 20 solution. The flotation gradient was added under the cyst suspension. Cysts were then recovered according to the individual flotation techniques, suspended in 1 ml of 0.01% Tween 20 solution and the number, and percent, recovered was determined with a haemocytometer under phase contrast microscopy.

2.3.3. *Giardia muris* cysts isolated from mice

As for 2.3.2. with isolated *Giardia muris* cysts cultured in mice in the laboratory.

2.3.4. *Giardia* cysts recovered from domestic animals

Faecal samples were collected from farm animals including dogs, hens, sheep and cattle. *Giardia* cysts were isolated on 1 M Sucrose, Percoll-sucrose and Sheather-sucrose flotation gradients and were stored in 10% formalin at 4°C. These cysts were then stained with the four stains described below. The cysts isolated on zinc sulphate (Giarcia and Ash, 1979) (only for the first 25 samples tested) were transferred directly to a known volume of Lugols iodine and stained. Only for the first 25 samples tested was zinc sulphate flotation compared with the other flotation techniques. The number of cysts per gram faeces were calculated. A total of 125 samples were used to determine which flotation technique consistently recovered the highest number of cysts per gram faeces.

2.3.4.1 Zinc sulphate flotation

1. ½ teaspoon of stool was transferred (more if the stool was very fibrous) to a test tube containing 1 to 2 ml of water and vortexed thoroughly. The tube was filled to within 2 to 3 mm of the top with water.
2. The test tube was centrifuged at 1500 rpm for 1 minute with the brakes on. The supernatant was discarded.
3. 1 to 2 ml of the zinc sulphate flotation medium was added to the pellet. The pellet was resuspended by flicking the tube at the bottom with one finger while holding the top of the tube tightly with the other hand.
4. The tube was filled to within 2 to 3 mm of the rim with the addition of more zinc sulphate solution.
5. The suspension was strained through gauze into a glass beaker. The gauze was discarded. The filtrate was placed into a clean test tube and zinc sulphate solution was added to fill the tube to within 2 to 3 mm of its rim.

6. The tube was centrifuged at 1500 rpm for 1 minute without the brake on.
7. While the centrifuge was slowing, 1/3 to ½ a drop of Lugols iodine stain was placed on a slide.
8. Without removing the tube from the centrifuge, with a flamed and cooled wire loop (diameter 5 mm), 1 or 2 drops from the centre of the suface film was removed and added to the drop of Lugol's iodine on the slide.
9. A coverslip was placed over the stained preparation and the preparation was viewed under phase microscopy at 100-400 X magnification for the presence of *Giardia* cysts.

2.3.5. The detection of *Giardia* cysts with different stains

The cysts recovered from above (Section 2.3.4.) were then stained with four different stains. The immunofluorescent detection kits used in the comparisons were Merifluor (Meridian Diagnostics Inc.), Hydrofluor (Meridian Diagnostics Inc.), and *Giardia*-Cel (Cellabs Diagnostics Pty Ltd). A polyclonal labelled antibody was supplied by J. Chan (Massey University). Of the immunofluorescent tests used, only *Giardia*-Cel is a direct method, the others being indirect. The Hydrofluor test kit is designed to detect the presence of *Giardia* cysts in environmental samples which include secondary treated sewage as well as raw and finished water supplies. The Merifluor kit is also designed to test faecal material as well as environmental samples. The *Giardia*-Cel kit is designed for testing faecal samples, and this like all other commercial kits does not identify particular species of *Giardia*, nor their host source. A comparison of these immunofluorescent stains was made using 96 animal faecal samples.

2.4. Comparison of Commercial Diagnostic Kits with Commonly Used Diagnostic Methods

11 faecal samples were collected for a brief comparison, 10 samples were known to be positive for the presence of *Giardia* cysts and one had tested negative for the presence of *Giardia* cysts. The faecal samples were composed of five human samples collected over a period of time, including the negative sample. Also collected were samples from two dogs, one rat and three mice. These samples were numbered with the date of collection and the presence of cysts, as determined when the samples were fresh, noted as shown:

1.	Human	25/9/91	positive
2.	Human	3/10/91	positive
3.	Human	29/11/91	positive
4.	Human	9/12/91	positive
5.	Dog	6/12/91	positive
6.	Dog	6/12/91	positive

7.	Mice	15/11/91	positive
8.	Mice	19/11/91	positive
9.	Mice	7/12/91	positive
10.	Rat	27/11/91	positive
11.	Human	13/6/91	negative control

Each faecal sample had sterile milli-q water added to it to form a thick slurry, from this suspension aliquots were taken for each diagnostic method used.

Seven different methods for diagnosis were compared:

1. 2 ml of each faecal suspension was suspended in TDW and floated on a 1.0 M sucrose gradient (Schaefer *et al*), cysts were collected in 0.01% Tween 20 solution with a final volume of 1 ml. 10 µl of the suspension was placed on a slide with an aliquot of Lugol's iodine stain (a non-specific stain), and mounted for viewing under phase contrast microscopy.

2. 2 ml of the faecal suspension was floated on a 1.0 M sucrose gradient, cysts collected were suspended in 1 ml of 0.01% Tween 20 solution. 10 µl were taken and stained with the Meridian Hydrofluor IFA-combo stain (a specific stain developed for water tests). This preparation was viewed under an epi-fluorescent microscope.

3. 2 ml of the faecal suspension was floated on a 1.0 M sucrose gradient, cysts collected were suspended in 1 ml of 0.01% Tween 20 solution. 10 µl were taken and stained with the *Meridian Merifluor DFA-combo stain (a specific stain developed for testing faecal samples).

4. The faecal suspensions were stained without first isolating cysts on a flotation gradient. The samples were tested with the *Meridian Merifluor DFA-combo test kit.

5. 2 ml aliquots of each faecal suspension was fixed in PVA and trichrome stained according to the Palmerston North Hospital Laboratory Manual (a non-specific stain).

6. 2 ml aliquots of each faecal suspension was tested for the presence of *Giardia* cysts using the *Cellabs - *Giardia* CELISA test kit.

7. 2 ml aliquots of each faecal suspension was tested for the presence of *Giardia* cysts using the Prospect 65 (an ELISA) test kit.

This series of samples and tests were done with the co-operation of Natalie Walker (Technician, NZCDC, Porirua, NZ) and Phillip Kelly (Technician, Massey University, NZ).

2.4.1 Trichrome staining

1. The faecal sample fixed in PVA for 30 minutes was placed on paper towels and allowed to stand for 3 minutes for the paper towels to absorb the PVA out of the faecal mixture. This was a very important step.
2. With an applicator stick, some of the faeces was spread onto a glass slide and allowed to dry for several hours at 37°C or overnight at room temperature.
3. The dry slides were then placed in 70% ethanol for 5 minutes.
4. The slides were then placed in 70% ethanol containing D'Antaoni's iodine (the solution was a port-wine colour) for 2 to 5 minutes.
5. The slides were then placed in two changes of 70% ethanol, one for 5 minutes and one for 2 to 5 minutes.
6. The slides were then placed in trichrome stain solution for 10 minutes.
7. After staining, the slides were placed in 90% ethanol containing 1% acetate for up to 3 seconds.
8. The slides were then dipped in absolute ethanol and placed in two changes of xylene or toluene for 2 to 4 minutes each.
9. The slides were mounted and viewed with a light microscope at 100-400X magnification to detect the presence of *Giardia* cysts.

2.5. Sucrose Flotation Reviewed

The 1.0 M sucrose flotation used previously requires that no brakes are used when centrifugation finished so spinning slows done without any interference. This is recommended so as not to disturb the interface containing the *Giardia* cysts required. 1.0 M sucrose flotation was carried out without a brake as standard and with brakes to determine if brakes could be used, saving time, without incurring greater cyst loss.

2.5.1. *In vivo* cultured *Giardia muris*

Giardia muris cysts were harvested from fresh mice faeces and suspended in 0.01% Tween 20 solution. Their concentration was determined with a haemocytometer under phase contrast microscopy. 5 ml aliquots of the cyst suspension was placed in 8 50 ml conical tubes, adjusted to a volume of 10 mls with 0.01% Tween 20 solution and floated on a 1.0 M sucrose gradient Four tubes were spun without the brake on and four tubes were spun with the brake off. Cysts were collected from the interface and washed three times with 0.01% Tween 20 solution and resuspended in a final volume of 1.0 ml. The number of

cysts recovered from each tube was determined with a haemocytometer under phase contrast microscopy.

2.6. New Zealand Animals Surveyed

Faecal samples were collected with the help from DSIR Land Resources Division from different parts of New Zealand and tested to see if they contained *Giardia*. DSIR collected samples from rats, mice, possums, and various birds from parts of the North Island. Samples from cows, sheep, chickens, dogs, cats, cockatiels, horses and other domestic animal samples were collected from areas in the Manawatu and from Dannevirke. Other samples were donated by hunters and other interested people.

The faecal samples were tested initially by Zinc Sulphate flotation and Lugols Iodine staining, with samples viewed under phase contrast microscopy at 400 X magnification. Later, faecal samples were floated on a 1.0 M Sucrose gradient and viewed without staining under phase contrast microscopy or stained using commercial indirect fluorescent test kits and viewed under epifluorescence for the presence of *Giardia* cysts.

2.7. Methods Used to Differentiate Species

Trophozoites of *G. muris* and *G. intestinalis* can be differentiated on the basis of their general body morphology and the shape of their median bodies. Using Nomarski optics (direct interference contrast microscopy) median bodies can be viewed.

It is not possible to differentiate the species when viewing cysts under the microscope because they have no median bodies present or any other differentiating feature. However, a general trend was observed, during this study, in the proportions of the cysts for each species as mentioned in the introduction ie *G. muris* seemed to be longer and more even in shape than *G. intestinalis* which varied from elongated to very short cysts, more pointed at one end than the other when they were freshly isolated.

2.7.1. Separation by specific gravity

Separate cyst suspensions were floated on two percoll gradients. *Giardia intestinalis* cysts isolated from human faeces and *G. muris* cysts isolated from mice faeces were suspended in 0.01% Tween 20 solution in separate kimax tubes (130 mm x 10 mm). The cyst suspension was underlaid with two different specific gravity percoll gradients. The bottom layer was 3 ml 70% percoll, 1.09 specific gravity, and the middle layer of percoll was 3 ml of 40% percoll, specific gravity 1.04. The tubes were centrifuged at 500 x g for 10 minutes without a brake in a Heraeus Christ centrifuge and each interface was collected and observed for the presence or absence of cysts. *G. muris* and *G. intestinalis* isolated from human faeces were floated on percoll gradients in separate kimax tubes (130 mm x 10 mm).

2.7.2. Differentiation on the basis of cyst size

This was based on cyst dimensions of each species to determine if there were significant differences in cyst size.

G. muris cysts isolated from mice faeces and *G. intestinalis* cysts isolated from human faeces had their length and width measured at 400 X magnification under phase contrast magnification to determine if there was any significant differences between the dimensions of these two species. The cysts also used in the Meridian Merifluor - *Giardia* test kit positive control were measured.

2.8. Culturing *Giardia muris* in the Mouse

Group 1 and 2 mice supplied were treated with Metronidazole or Tinidazole to ensure that no *Giardia* were present in their intestinal tract. Fresh faecal specimen were collected every day (when possible) and tested for the presence of *Giardia* by 1.0 M Sucrose flotation concentration and viewing under phase contrast or staining with a commercial indirect fluorescent test kit specific for *Giardia* cysts. Mice were place in cages with wire grill floors over a tray with water so faeces could drop down through the wire and be kept moist. Faeces were collected between 8-10 am. The mice, after testing negative for the presence of *Giardia*, were inoculated with at least 5 x 10^3 *G. muris* cysts. Faecal samples were collected everyday and tested for *Giardia* to determine when *Giardia* were established in the gut and encysting to be excreted in the faeces.

Group 3 - 7 are mice born to infected parents. They were surveyed to determine, if possible, at what point they are colonised with *Giardia* and were also excreting cysts.

The number of cysts excreted per mouse or per group of mouse was expressed as the number of cysts per gram of faecal material.

2.9. Establishing Viability

There are a variety of methods available to establish viability of *Giardia* cysts, including vital stains and different methods said to work better with different species ie *G. muris* or *G. intestinalis*. Two methods developed by Feely were tested.

2.9.1. The method of Feely, 1986

This study used the method of Feely 1986.

This was an excystment method used for *in vivo* derived *G. muris* cysts. Fresh faeces were floated on 1.0-M sucrose gradient. Isolated cysts were washed and suspended in 1.0 ml sterile milli-Q water in a 130 mm x 10 mm kimax screw top glass tube. 1.2×10^7 cysts were incubated at 37°C for 30 minutes in HBSS adjusted to pH 2.0. After the induction period, the cysts were washed three times with HBSS adjusted to pH 7.2 and were incubated in 2 - 3 ml of TY1-S-33 growth media at 37C in an uncapped kimax tube. HBSS was prepared fresh for use on the day and adjusted to pH 7.2 just prior to use in excystation experiments.

At set time intervals, the isolates were gently suspended and 10 µl was taken and placed in 10µl of 20% formalin so the isolates were fixed (inactivated) in 10% formalin. These aliquots were placed on a glass slide, with a coverslip and sealed with clear nail polish. Aliquots were taken and fixed in formalin at the set time intervals of 5, 15, and 30 minutes, 1 hour and 1 day. Samples were viewed under phase contrast microscopy for excystment and viability was determined using the following equation:

$$\% \text{ Excystation} = [ECW + PET]/[ECW + PET + IC] \times 100 \quad \textbf{(1)}$$

where IC = the number of intact cysts, PET = the number of partially excysted trophozoites, (the cyst had started the excystation process and progressed to the point where the trophozoite had either started to emerge or had completely emerged and was still attached to the cyst wall) and ECW = the number of empty cyst walls (Schaefer *et al* 1984).

2.9.2. The method of Feely, 1991

This study used the method of Feely *et al* 1991.

0.1M Potassium buffer, at pH 7.0 was made and just before used it had $NaHCO_3$ mixed in to get a final concentration of 0.3 M $NaHCO_3$, pH 7.5.

1.2×10^7 cysts were incubated in 15 ml of the pH 7.5 buffer for 5 minutes.

The cyst suspension was centrifuged at 600 x g for 5 minutes in a Heraeus Christ Centrifuge. The suspension was washed with 0.1 M KPO_4 buffer (pH 7.0) and centrifuged at 600 g for 5 minutes.

The cysts were suspended in 1 - 2 ml of PBS and incubated at 37°C.

Samples were fixed in 10% formalin as above and viewed under phase contrast microscopy to determine viability of the cysts at the incubation times of 5, 15, and 30 minutes, 1 hour and 1 day using equation 1.

2.10. Environmental Effects on *Giardia* Cyst Presence and Viability

Giardia cysts were subjected to a variety of conditions similar to what may occur in the environment. Cysts were obtained from *in vitro* cultures (*G. intestinalis*), from human source (*in vivo* cultured *G. intestinalis*) and from mice faeces *(in vivo* cultured *G. muris*).

The cysts were stored under varying conditions ie a range of temperatures and *in vivo* cysts were either isolated on a 1.0 M sucrose gradient and stored in sterile milli-Q water or their faecal suspension was diluted to 20-30 ml with sterile milli-Q water. Samples were taken over a period of time to establish how long cysts remained present and viable.

For cysts to be incubated in a faecal suspension, the weight of the fresh faeces and final volume of the faecal suspension was recorded so the amount of faeces per ml suspension could be calculated. The sample was vortexed to get an even suspension and 2 ml aliquots of faecal material was collected. Cysts were isolated on a 1.0-M sucrose gradient and suspended in 1 ml of TDW. The number of cysts in that 1 ml were determined with a haemocytometer. From this it was possible to determine the number of cysts found per gram faeces.

For cysts suspended in water, samples were vortexed to gain an even suspension and the number/ml was determined with a haemocytometer.

Viability was established using the Feely 1986 method and calculated using equation (1).

Counts of cysts present on the day of collection (day 0) were considered to be 100% present, counts after day 0 were adjusted to get the percent remaining. Each set of conditions were repeated and the results were averaged.

2.10.1. *Giardia* Cysts stored at -20°C

2.10.1.1. *Giardia intestinalis* cultured *in vitro*

Giardia intestinalis were cultured according to the method of Schupp *et al* (1988) and harvested on day 3 after encystation. The cysts were washed and suspended in sterile milli-Q water. 1 ml aliquots of the suspension were placed in sterile bijous and stored at -20°C. Individual bijous were retrieved and thawed at 20°C. The number of *Giardia* cysts present and their viability were determined over a period of time.

2.10.1.2. *Giardia muris* cultured in mice

2.10.1.2.1. *Giardia muris* cysts suspended in mouse faeces

Fresh faecal samples collected from mice containing *G. muris* were evenly suspended in water. 2 ml aliquots were placed in bijous and stored at -20°C. *G. muris* cysts were

isolated from the faecal suspensions using the 1.0 M sucrose flotation technique. The number of cysts present per gram faeces and their viability was determined over a period of time.

2.10.1.2.2. *Giardia muris* cysts suspended in water.

Giardia muris cysts isolated from fresh mice faeces were suspended in sterile milli-Q water. 1 ml aliquot's were placed in bijous and stored at -20°C. Individual bijous were taken out, thawed at 20°C and the number of *Giardia* cysts present and their viability was determined over a period of time.

2.10.2. *Giardia* cysts stored at 4°C

2.10.2.1. *Giardia intestinalis* cultured *in vitro*

As for 2.10.1.1. except samples were stored in sterile universals - not as separate aliquots in bijous. Samples were stored at 4°C. The percent of cysts remaining and their viability were determined over a period of time.

2.10.2.2. *Giardia intestinalis* isolated from humans

2.10.2.2.1. *Giardia intestinalis* cysts suspended in human faeces

Fresh faecal samples collected from people were suspended in sterile milli-Q water in universal bottles. The suspension was vortexed to get an even suspension and the bottles were stored at 4°C. The percent of cysts recovered and their viability were determined over a period of time.

2.10.2.2.2. *Giardia intestinalis* cysts suspended in water

As for 2.10.2.2.1. except cysts were isolated from faeces and stored suspended in sterile milli-Q water.

2.10.2.3. *Giardia muris* isolated from mice

2.10.2.3.1. *Giardia muris* cysts suspended in mouse faeces

As for 2.10.1.2.1., except the faecal suspensions were stored in universal bottles and not as separate 2 ml aliquots in bijou bottles. The cysts were stored at 4°C and their numbers and viability were determined over a period of time.

2.10.2.3.2. *Giardia muris* cysts suspended in water.

As for 2.10.1.2.2., except the cysts were stored in a universal bottle and not as separate aliquots in bijou bottles. The cysts were stored at 4°C and their numbers and viability were determined over a period of time.

2.10.3. *Giardia* cysts stored at 20°C (room temperature)

2.10.3.1. *Giardia muris* cultured in mice

2.10.3.1.1. *Giardia muris* cysts suspended in mouse faeces

As for 2.10.2.3.1. The cysts were stored at 20°C and their numbers and viability were determined over a period of time.

2.10.3.1.2. *Giardia muris* cysts suspended in water.

As for 2.10.2.3.2. The cysts were stored at 20°C and their numbers and viability were determined over a period of time.

2.10.4. *Giardia* cysts left in sawdust for 24 hours

Faeces from mice were collected fresh at the hours designated 0 and 24 (8 am each day). Between these two collection points, the mice were returned to their cages containing a layer of sawdust on the floor. The faeces deposited during this 24 hour period were collected into 50 ml centrifuge tubes and *G. muris* cysts were isolated on a 1 M Sucrose gradient and the number of cysts per gram faeces calculated as above.

The values of the hours 0 and 24 was averaged to determine the "Expected number of cysts per gram faeces" to be excreted throughout the day. The number of cysts per gram faeces was calculated for faeces collected from the sawdust was compared to the "Expected" value to determine if cysts numbers and/or their viability was reduced when exposed to dry conditions.

2.10.5. *Giardia* cysts stored at 37°C

As for 2.10.2. except some faeces containing cysts were either allowed to dry out or were suspended in water and contained in a tightly sealed sterile universal.

2.11. Preservation of *Giardia* Cysts

Giardia cysts were collected as in 2.10. and were suspended in a faecal suspension or isolated into sterile milli-Q water. The suspensions were then placed in different fixing solutions. 2 ml aliquots were taken from these faecal suspensions, *Giardia* cysts were isolated on a 1.0 M sucrose gradient and resuspended in 1 ml of 0.01% Tween 20 solution. The number of cysts per gram faeces was calculated. Cysts suspended in solution and stored were enumerated to determine the % cysts remaining over a period of time.

2.11.1. PVA fixation

2.11.1.1. *Giardia muris* cultured in mice

2.10.1.1.1. *Giardia muris* cysts suspended in mouse faeces

Fresh faecal samples collected from mice infected with *G. muris* were evenly suspended in milli-Q water. The *Giardia* cysts in the faeces were fixed with PVA by mixing faeces and PVA at a ratio of 3 parts PVA to 1 part faeces. 2 ml aliquots were collected at various time intervals and *Giardia* cysts were isolated using the 1.0 M sucrose flotation technique. The number of cysts present were determined over a period of time. The cysts were enumerated before and after PVA fixation to determine if there was any decrease in cyst numbers due to the fixing step. Day 0 was taken as the time after the cysts had been fixed in PVA. Cyst suspensions were stored at room temperature (20°C).

2.11.1.1.2. *Giardia muris* cysts suspended in solution.

G. muris cysts isolated from fresh mice faeces were suspended in 0.01% Tween 20 solution and were fixed with the same ratio of PVA. The number of cysts present were determined over a period of time. The cysts were enumerated before and after PVA fixation to determine if there was any decrease in cyst numbers due to the fixing step. Day 0 was taken as the time after the cysts had been fixed in PVA. Cyst suspensions were stored at room temperature (20°C).

2.11.2. 10% formalin

2.11.2.1. *Giardia intestinalis* cultured *in vitro*

Giardia intestinalis were cultured according to the method of Schupp *et al* (1988) and harvested on day 3 after encystation. The cysts were washed with sterile milli-Q water and resuspended in a final concentration of 10% formalin and stored at 4°C. Over a period of time the percent of cysts recovered was determined.

2.11.2.2. *Giardia intestinalis* isolated from humans

2.11.2.2.1. *Giardia intestinalis* cysts suspended in human faeces

Fresh faecal samples collected from people were suspended in a final concentration of 10% formalin in universal bottles. The suspension was vortexed to get an even suspension and the bottles were stored at 4°C. At various time intervals the percent of cysts recovered was determined as for 2.10.2.2.1.

2.10.2.2.2. *Giardia intestinalis* cysts suspended in solution

As for 2.11.2.2.1. except the cysts were first isolated from faeces, suspended in 10% formalin and stored at 4°C.

2.11.2.3. *Giardia muris* isolated from mice

2.11.2.3.1. *Giardia muris* cysts suspended in mouse faeces

Fresh faecal samples collected from mice infected with *G. muris* were suspended in a final concentration of 10% formalin in universal bottles. The suspension was vortexed to get an even suspension and the bottles were stored at 4°C. At various time intervals the percent of cysts recovered was determined as for 2.10.2.2.1.

2.11.2.3.2. *Giardia muris* cysts suspended in solution.

As for 2.11.2.3.1. except the cysts were first isolated from faeces and then suspended in 10% formalin and stored at 4°C.

2.11.3. 0.1% SDS

2.11.3.1. *Giardia intestinalis* cultured *in vitro*

Giardia intestinalis were cultured according to the method of Schupp *et al* (1988) and harvested on day 3 after encystation. Cysts were rendered non-viable by suspension in a final concentration of 0.1% SDS for 1-2 minutes (cysts viability was determined by the method of Feely, 1986). Cysts were washed 3 times with sterile water by spinning at 500 x g for 3 minutes with the brake on and resuspended in sterile milli-Q water. The percent of cysts recovered was determined over a period of time.

2.11.4. Cryopreservation

The method of Phillips *et al* 1982 was used for the cryopreservation of cultures at -80°C. *In vitro* cultured *Giardia intestinalis* trophozoites were harvested in their late log phase of growth by placing tubes or flasks in ice water for 10 minutes. The trophozoites that detached were decanted into conical centrifuge tubes and centrifuged at 700 x g for 5 minutes with a brake on to concentrate them. The pellet was resuspended in fresh TY1-S-33 and the concentration of trophozoites was determined with a haemocytometer. The suspension was adjusted to 1.2-1.5 x 10^6 trophozoites per ml with growth medium. 0.25 ml aliquots of the suspension was placed in 1 ml Nunc cryopreservation tubes and 0.25 ml of a 15% solution of Dimethyl Sulfate (DMSO) in TY1-S-33 was added drop-wise. The tubes were then mixed, wrapped in paper and tissue and placed in a polystyrene container which was then placed within the -80°C freezer. The tubes were wrapped up to reduce the rate of

freezing, protecting the trophozoites from the violent effects of rapid cooling. When frozen the tubes were stored in a -80°C freezer.

The cultures were retrieved with a modified method of Phillips *et al* 1982 and Wallis and Wallis 1986. The cultures were quickly thawed in a pre-warmed 37°C water bath and were transferred to a Kimax tube (130 mm x 10 mm) which were immediately filled with growth medium and incubated horizontally at 37°C for 15-25 minutes. During this time, healthy trophozoites could attached to the sides of the kimax tube. After this incubation time the medium was discarded and the tube refilled and incubated at 37°C for 4 hours. The medium was discarded again and replaced with pre-warmed growth medium. This process of washing was necessary to ensure all the DMSO was washed out of the medium and cell membranes. The tubes were left for three days before either subculturing or being refilled with fresh growth medium.

Retrieval was carried out a week after freezing to check that the process had worked.

It was up to a month before any *Giardia* trophozoites were detected. The rate of recovery of healthy trophozoites was very low and it required care with the method to ensure a successful recovery of any organisms. With such a low recovery of viable trophozoites, it was necessary to be very patient and allow the trophozoites time to multiply up to sufficient numbers so they are visible under a light microscope.

2.11.5. Osmium tetroxide fixation

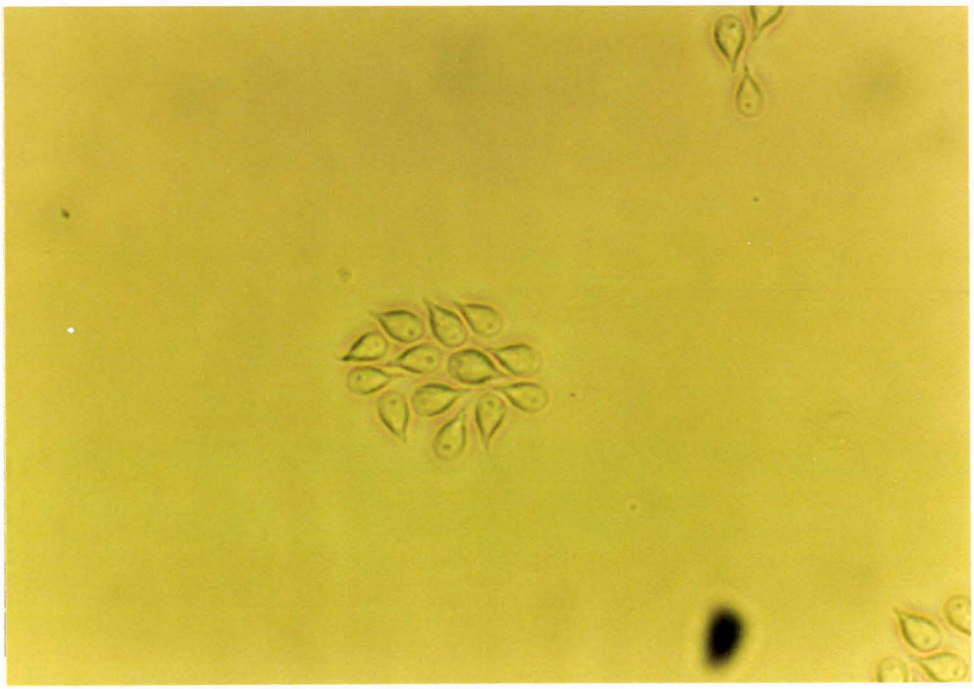
A 4% solution of Osmium tetroxide ($Os0_4$) was prepared and stored in a chemically and biologically sterile bottle. It was important that absolutely no organic or inorganic material was present that could interact with the osmium tetroxide reducing it to an inactive dioxide form. The following method was done in a fume hood to ensure no fumes from osmium tetroxide interacted with the person.

A drop of cysts or trophozoites suspended in sterile milli-Q water was placed on a clean glass slide. The slide was placed in a sterile petri-dish with a small square piece of parafilm™ next to it. Using a clean, sterile pasteur pipette, a drop of osmium tetroxide (equal volume) was placed on the piece of parafilm and the petri-dish lid was replaced onto the petri-dish. The osmium tetroxide reaction was allowed to continue for one minute after which the cyst or trophozoite forms should be fixed, and then the osmium tetroxide was discarded into a separate discard bottle. The full volume of the osmium tetroxide, now osmium dioxide, is easy to retrieve due to a high surface tension. The total volume placed on the parafilm™ can be sucked back up into the pasteur pipette without leaving any traces behind. The parafilm™ can then be discarded into medol and/or a decontamination bin. A glass cover slip is placed over the cyst or trophozoite suspension and sealed with clear nail polish.

When the cysts fixed with osmium tetroxide were viewed under Normaski optics, it was not possible to distinguish them from viable cysts. This process of fixing organisms did not distort the internal organisation of the cysts as much as PVA fixation did.

Plate 1: *Giardia* trophozoites viewed under direct light microscopy. 400 X magnification.

A. *Giardia intestinalis* trophozoites cultured *in vitro*.



B. *Giardia muris* trophozoites isolated from the intestines of mice. Some trophozoites are angled and it is possible to see their suction disc (→). These trophozoites are smaller and rounder than the *G. intestinalis* trophozoites seen in Plate 1A.

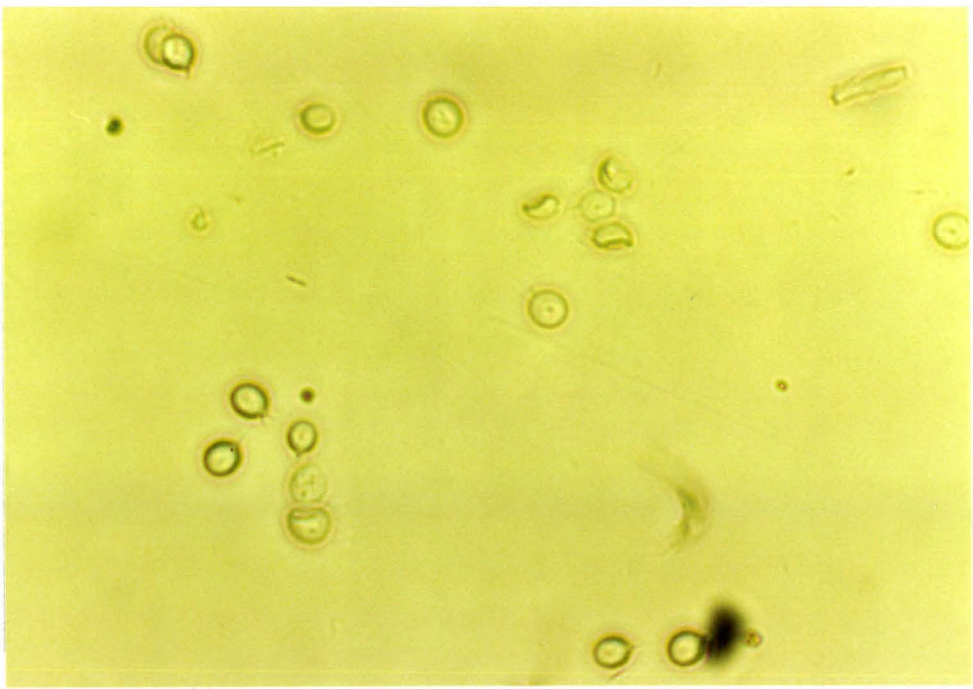
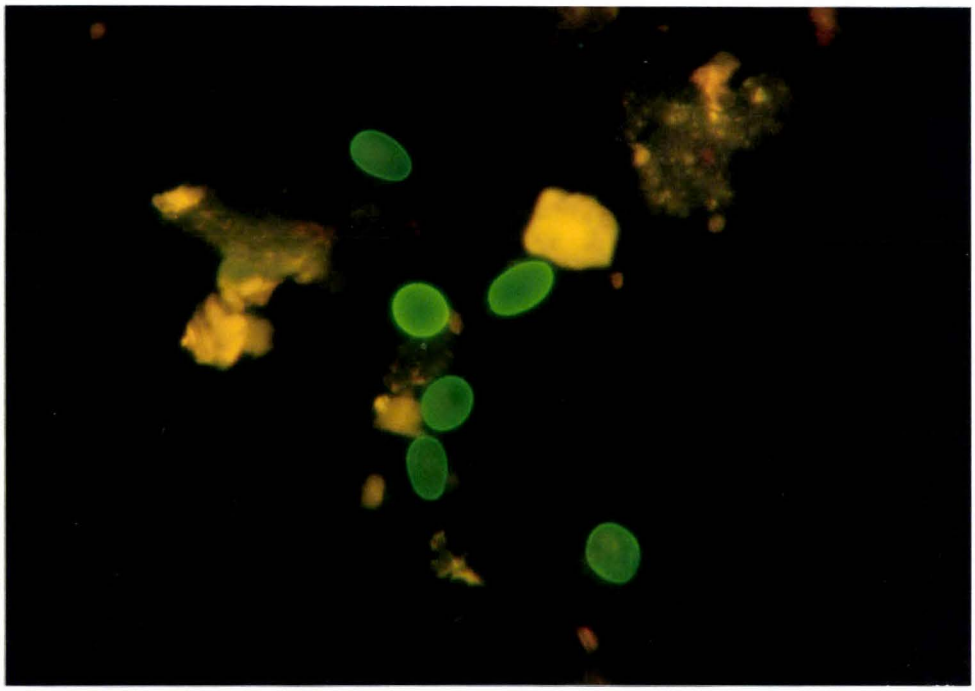


Plate 2: *Giardia* cysts are stained using the Meridian™ Merifluor-*Giardia* kit and viewed under epifluorescence. 400 X magnification.

A. *Giardia intestinalis* isolated from human faeces.



B. *Giardia muris* isolated from mouse faeces.

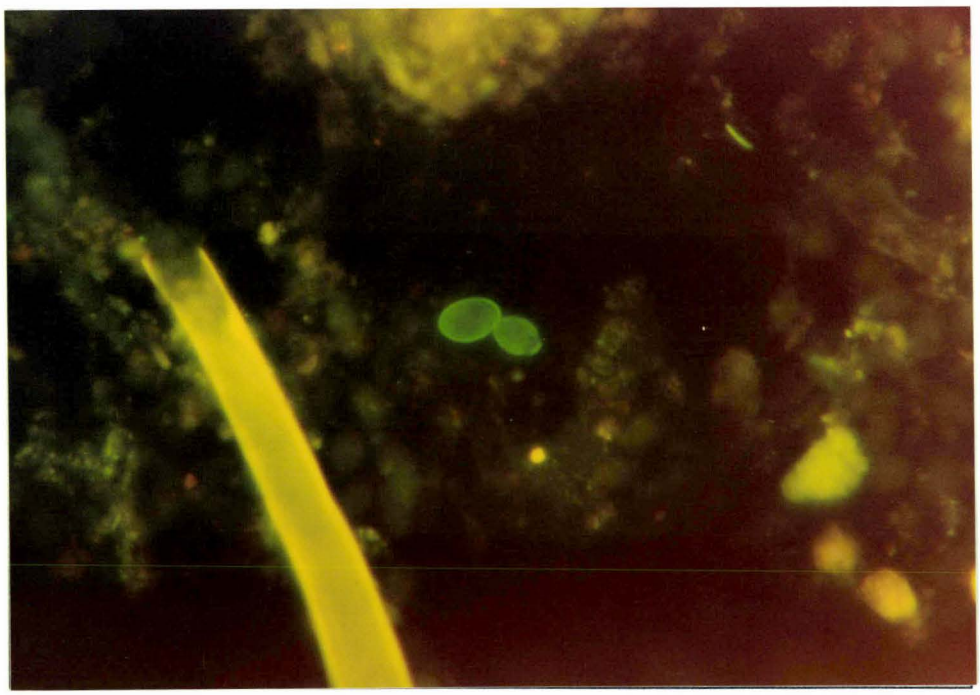
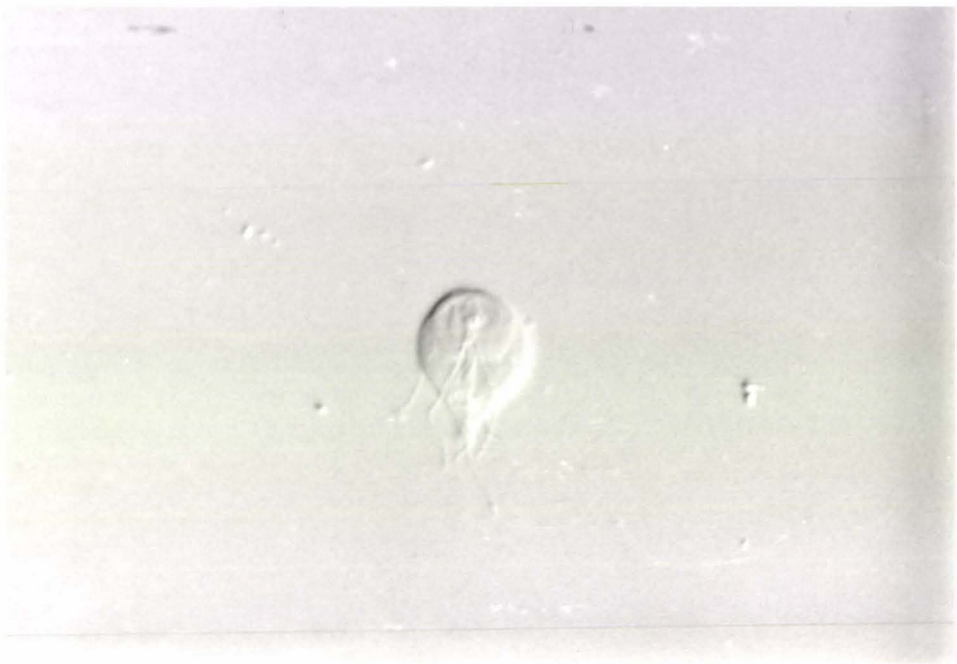


Plate 3: *Giardia* cysts and trophozoites viewed under Nomarski optics. Magnification 400 X except for 3B with 800 X magnification.

A. *Giardia intestinalis* trophozoite cultured *in vitro*. This trophozoite was initially viable but after several minutes exposure to oxygen on a glass slide its flagella had ceased movement. It is possible to see nuclei, the suction disc and axonemes as well as the flagella.



B. *Giardia muris* trophozoites isolated from mouse faeces. The trophozoite is non-viable.

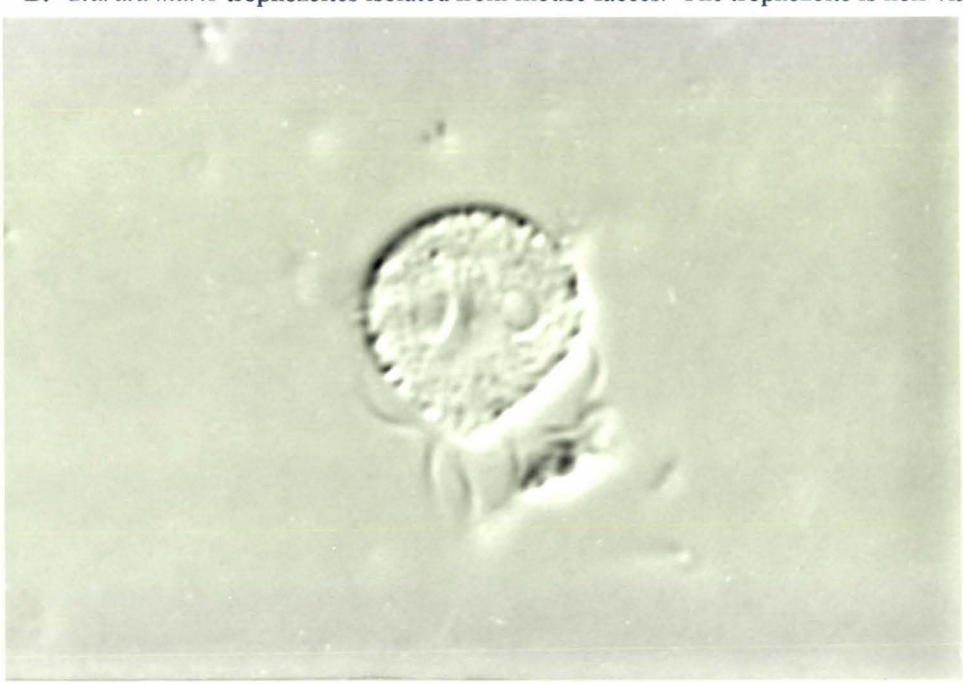
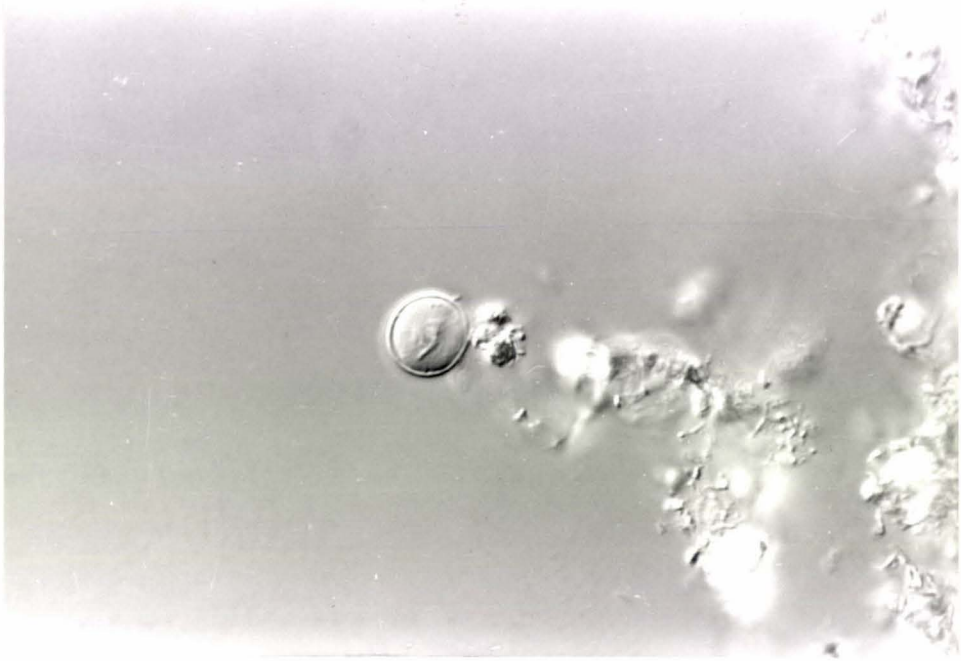


Plate 3 (continued)

C. *Giardia intestinalis* cysts cultured *in vitro*. This cyst is morphologically different from *G. intestinalis* cysts isolated from humans ie it is round not optical in shape. The cyst is viable showing little internal morphology.



D. A *Giardia* cyst isolated from a hamster (New Zealand Communicable Disease Centre, Porirua) showing some internal morphology ie axonemes and suction disc fragments.

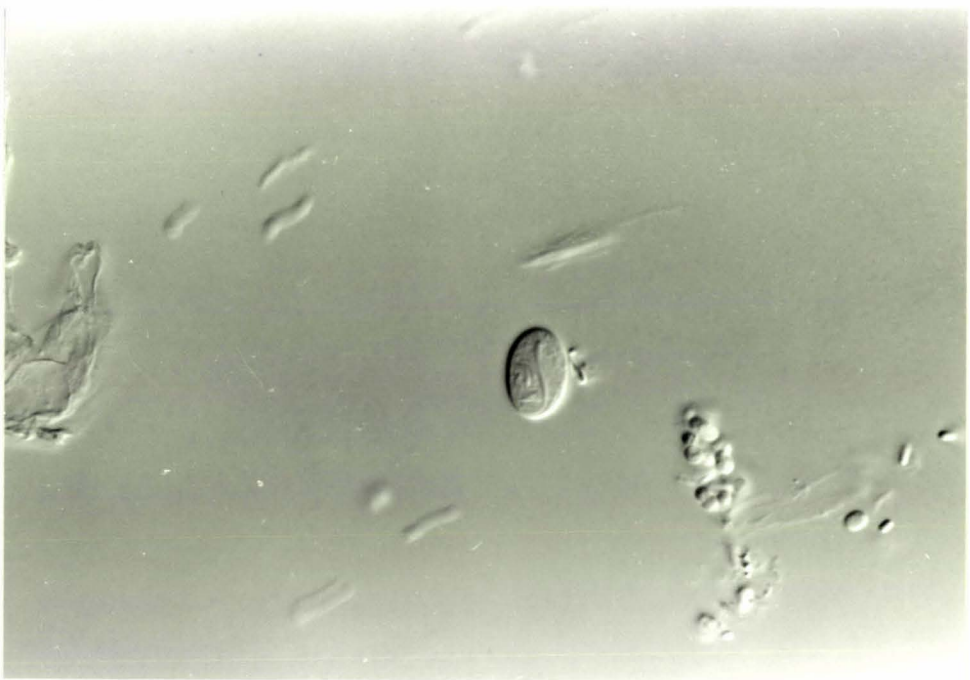


Plate 3 (continued)

E. A *Giardia muris* cyst fixed with PVA showing distortion of the cytoplasm. The cytoplasm has pulled away from the cyst wall. The axonemes and two nuclei are visible.

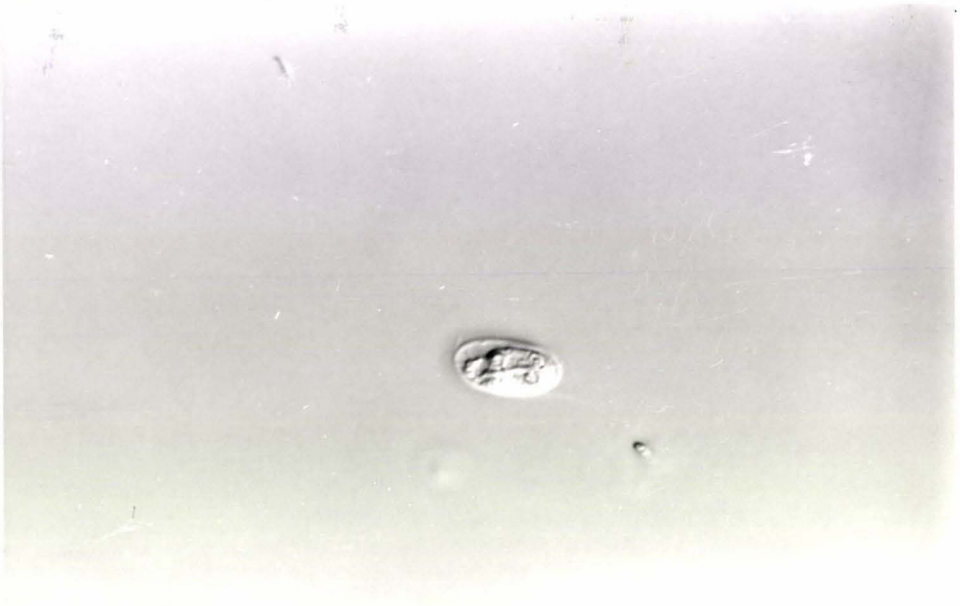
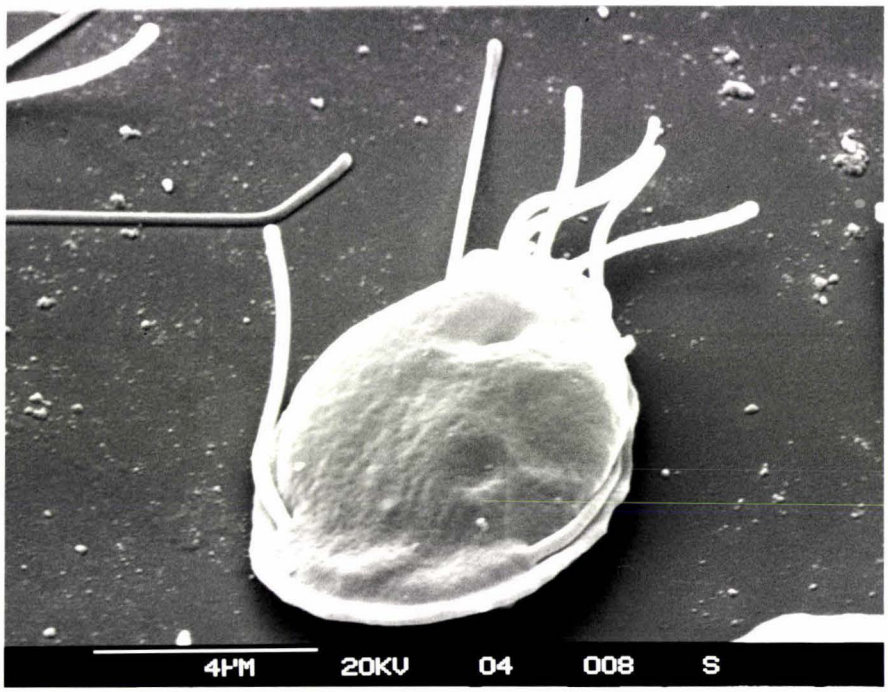
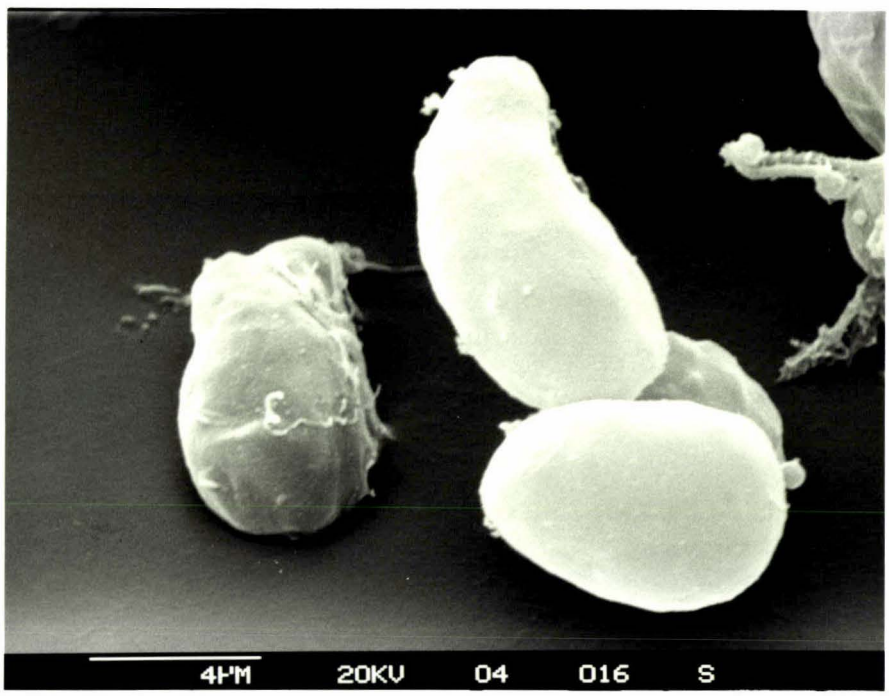


Plate 4: Scanning electron micrographs taken of *Giardia intestinalis* cysts and trophozoites cultured *in vitro*.

A. *Giardia intestinalis* trophozoite showing the edge of the ventral suction disc and flagella as well as dimples in the dorsal surface.



B. *Giardia intestinalis* cysts.



CHAPTER 3. RESULTS

3.1. Recovery of *In Vitro Giardia* Cysts from Trophozoites

3.1.1. Sonication to remove trophozoites and incompletely formed cysts

3.1.1.1. 10^5 cysts/ml

For all the different sonication intensities (5μ, 6μ and 7μ) trophozoites disappeared within the first minute, incompletely formed cysts remained present and completely formed cysts decreased over a period of time. The results for the percent recovery of completely formed cysts is presented in Figure 2 and tabulated in Appendix A.

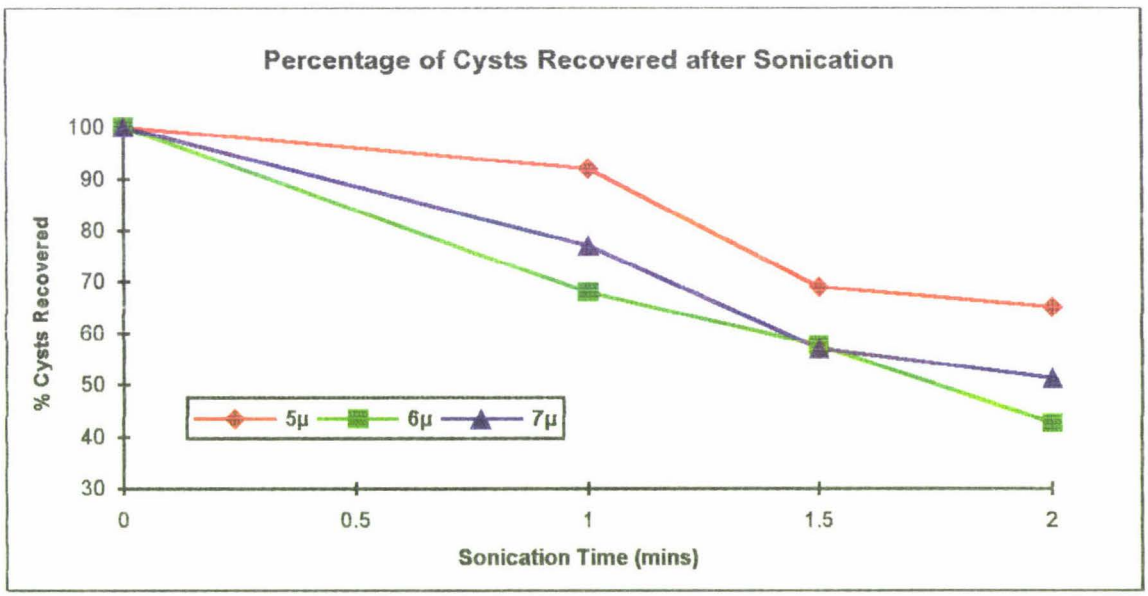


Figure 2: The % of 10^5 *Giardia* cysts cultured *in vitro* recovered after a period of time subjected to sonication at various intensities.

3.1.1.2. 10^6 cysts/ml

With a higher concentration of cysts present, and therefore a higher concentration of trophozoites and incompletely formed cysts present, sonication was not as effective in destroying trophozoites and incompletely formed cysts remained present. Trophozoites were destroyed after 1 minute of sonication at 6μ, and 7μ, and there was again a steady decrease in the percent of completely formed cysts remaining after sonication. The results for the recovery of completely formed cysts is presented in Figure 3 and the data is tabulated in Appendix A.

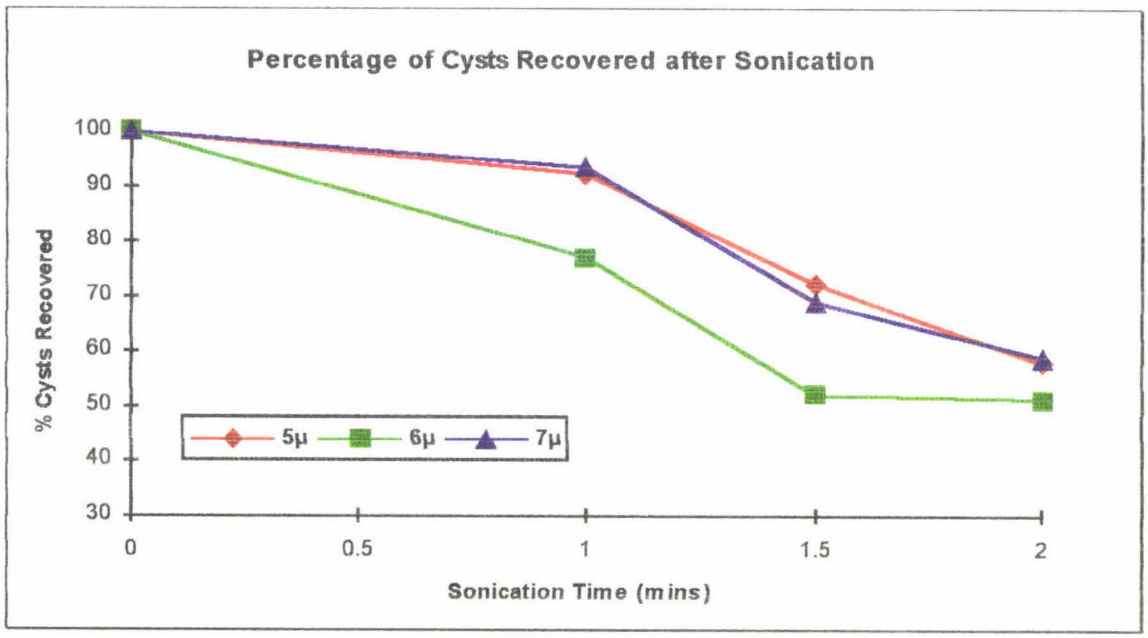


Figure 3: The % 10^6 *Giardia* cysts cultured *in vitro* recovered after a period of time subjected to sonication at various intensities.

3.1.2. Trophozoite destruction in water

3.1.2.1. Trophozoites destroyed by incubation in double distilled water at 20°C.

Intact trophozoites and incompletely formed cysts were still present.

3.1.2.2. Trophozoites destroyed by incubation in double distilled water at 4°C overnight.

When samples were viewed under phase contrast, intact trophozoites were still present. In fact intact trophozoites were still seen several weeks later after storage at 4°C.

Table 1: Data for recovery of lysed trophozoites in double distilled water at 4°C

Tube	Initial Count (#/ml)	Final Count (#/ml)	% Destroyed
1	1.38×10^6	6.08×10^5	55.98
2	1.58×10^6	5.85×10^5	62.98
3	1.51×10^6	6.07×10^5	59.80
4	1.58×10^6	3.52×10^5	77.72

Average number of trophozoites destroyed = 64.12 (standard deviation = 9.51).

3.1.3. Trophozoite lysis in sodium dodecyl sulphate (SDS)

Incomplete cysts and trophozoites were destroyed with no fragmented remains when incubated in 0.1% SDS for a very short period of time. The cysts remaining were determined to be non-viable using Feely's 1986 method of excystation.

3.2. Techniques to Recover and Detect *Giardia* cysts from Environmental and Laboratory Samples

3.2.1. *Giardia intestinalis* cysts cultured *in vitro*

Table 2: Data for recovery of *in vitro G. intestinalis* cysts from faeces

Flotation Gradient	% Cysts Recovered	Number of Repeats
1 M Sucrose	31.00 ± 33.08	3
Percoll-Sucrose	48.93 ± 42.90	3
Sheather-Sucrose	68.32 ± 41.67	3
Percoll	66.76 ± 11.45	3

3.2.2. *Giardia intestinalis* cysts Isolated from human faeces

Table 3: Data for recovery of *G. intestinalis* cysts isolated from human faeces

Flotation Gradient	% Cysts Recovered	Number of Repeats
1 M Sucrose	100	4
Percoll-Sucrose	74.98 ± 8.05	4
Sheather-Sucrose	84.21 ± 7.37	4
Percoll	0.00†	4

3.2.3. *Giardia muris* cysts isolated from mice

Table 4: Data for recovery of *G. intestinalis* cysts isolated from mice faeces

Flotation Gradient	% Cysts Recovered	Number of Repeats
1 M Sucrose	100	4
Percoll-Sucrose	70.31 ± 10.51	4
Sheather-Sucrose	75.50 ± 13.34	2†
Percoll	64.73 ± 0.59	3†

3.2.4. *Giardia* cysts recovered from domestic animals

For the first 25 samples compared, not once did the zinc sulphate flotation technique recover the largest number of cysts ie the number of times zinc sulphate yielded the highest recovery

† Cysts stored for 24 weeks in 10% formalin were used in the Percoll flotation. It appears that the cysts have had an increase in their specific gravity.

equals zero. The data is presented in tabulated format in Appendix A. After this, the comparisons excluded the zinc sulphate flotation technique.

For the total 125 samples compared (data in Appendix A) the results are grouped according to the origin of the faecal samples. Beside each flotation medium used to recover *Giardia* cysts is recorded the number of times each technique yielded the highest number of *Giardia* cysts per gram of faeces.

Dogs: n = 40			**Sheep: n = 44**		
Sucrose	14	(35.0%)	Sucrose	19	(43.18%)
Percoll-sucrose	17	(42.5%)	Percoll-sucrose	14	(31.82%)
Sheather-sucrose	11	(27.5%)	Sheather-sucrose	11	(25.00%)
Hens: n = 14			**Cows: n = 27**		
Sucrose	7	(50.0%)	Sucrose	19	(70.37%)
Percoll-sucrose	5	(35.71%)	Percoll-sucrose	2	(7.41%)
Sheather-sucrose	2	(14.29%)	Sheather-sucrose	6	(22.22%)

For all 125 samples used the overall results are:

Sucrose	59	(47.20%)
Percoll-sucrose	38	(30.40%)
Sheather-sucrose	30	(24.00%)

3.2.5. The detection of *Giardia* cysts with different stains

For a total of 97 samples compared the results are grouped according to the origin of the faecal samples. Beside each commercial staining kit or laboratory produced stain used to detect *Giardia* cysts are recorded the number of times each technique detected the highest number of *Giardia* cysts per gram of faeces.

Table 5: Comparison of different stains by faecal origin

Stain	Dogs: n = 30	Sheep: n = 33	Hen: n = 13	Cows: n = 20
Meridian™ Merifluor-*Giardia*	10 (33.00%)	10 (33.00%)	7 (53.8%)	8 (40.00%)
Meridian™ Merifluor-*Combo*	6 (33.33%)	9 (27.33%)	5 (38.46%)	2 (20.00%)
Cellab-*Giardia*	3 (10.00%)	8 (24.24%)	1 (7.69%)	2 (20.00%)
Polyclonal Antibody	13 (43.33%)	8 (24.24%)	3 (23.08%)	9 (45.00%)

For the total of the 97 faecal samples compared the overall results are:

Meridian™ Merifluor-*Giardia*	38	(39.58%)
Meridian™ Hydrofluor-*Combo*	22	(22.91%)
Cellab-*Giardia*	14	(14.58%)
Polyclonal Antibody	33	(34.38%)

3.3. Comparison of Commercial Diagnostic Kits with Common Diagnostic Methods

Table 6: Comparison of new diagnostic kits vs. common diagnostic methods

Sample	Test Method Used						
	1	2	3	4	5	6†	7†
1	–	+	+	+	–	++++	+++
2	+	+	+	+	+	++++	++++
3	+	+	+	+	+	+++	+++
4	+	+	+	+	+	++	++
5	–	–	–	+	+	+	++
6	–	–	–	–	–	+	–
7	–	+	–	–	–	–	–
8	–	–	–	+	–	+	–
9	+	+	–	+	+	++	++
10	–	+	+	+	+	+	+
11	–	–	–	–	–	–	–
False Negatives (%)	**60**	**30**	**50**	**20**	**40**	**10**	**30**

Key of Methods:

1 = 1.0 M sucrose flotation to isolate cysts, stain with Lugol's iodine.
2 = 1.0 M sucrose flotation to isolate cysts, stain with Meridian Hydrofluor IFA - combo.
3 = 1.0 M sucrose flotation to isolate cysts, stained with Meridian Merifluor DFA-combo.
4 = Faecal suspension stained with Meridian Merifluor DFA-combo.
5 = Faeces were fixed with PVA and trichrome stained.
6 = Faecal suspension was tested with the Cellab *Giardia* CELISA test kit.
7 = The faecal suspension was tested with the Prospect 65 kit.

3.4. Review of Sucrose Flotation

3.4.1. *Giardia muris* cysts

3.4.1.1. Without brakes

Table 7: Data for sucrose flotation of *G. muris* cysts, without brakes.

Sample	Original Number of Cysts	Final Number of Cysts	% Recovery
1	3.91×10^5	1.92×10^5	49.03
2	3.44×10^5	2.17×10^5	62.98
3	3.87×10^5	2.87×10^5	74.16
4	3.74×10^5	1.73×10^5	46.26

Average % Recovery = 58.11%, Standard Deviation = 12.96%.

† The number of + symbols used for Methods 6 and 7 indicates colour intensity observed visually.

3.4.1.2. With brakes

Table 8: Data for sucrose flotation of *G. muris* cysts, with brakes.

Sample	Original Number of Cysts	Final Number of Cysts	% Recovery
1	3.86×10^5	1.77×10^5	45.89
2	3.75×10^5	2.01×10^5	53.60
3	4.00×10^5	1.82×10^5	45.53
4	3.89×10^5	2.32×10^5	59.59

Average % Recovery = 51.15%, Standard Deviation = 6.75%.

3.5. New Zealand Animals Surveyed

The animals surveyed are listed in alphabetical order with the number tested (n) and the number positive for the presence of *Giardia* (% positive in brackets) recorded.

Blackbird	n = 8	3 positive	(37.50%)
Cattle	n = 32	7 positive	(21.88%)
Chaffinch	n = 3	1 positive	(33.33%)
Cockatiel	n = 1	0 positive	(00.00%)
Deer	n = 2	1 positive	(50.00%)
Dogs	n = 10	6 positive	(60.00%)
Ducks	n = 5	0 positive	(00.00%)
Ferret	n = 1	0 positive	(00.00%)
Goats	n = 3	1 positive	(33.33%)
Hedgehog	n = 3	1 positive	(33.33%)
Hedge Sparrow	n = 2	0 positive	(00.00%)
Hens	n = 16	3 positive	(28.75%)
Horse	n = 1	1 positive	(100.00%)
Magpie	n = 1	1 positive	(100.00%)
Mice	n = 263	60 positive	(22.81%)
Possums	n = 177	28 positive	(15.81%)
Pukeko	n = 1	0 positive	(00.00%)
Rabbits	n = 1	0 positive	(00.00%)
Rats	n = 94	59 positive	(62.77%)
Sheep	n = 80	24 positive	(30.00%)
Sparrow	n = 17	1 positive	(5.88%)
Swan	n = 4	0 positive	(00.00%)
Thrush	n = 3	3 positive	(100.00%)
Turkey	n = 6	0 positive	(00.00%)

3.6. Methods Used to Differentiate Species

3.6.1. Separation by specific gravity

Giardia intestinalis isolated from a fresh human faecal sample was harvested from the interface of 0.01% Tween 20 solution and the 40% percoll layer above the faecal pellet. *Giardia muris* cysts which had been stored for 8 weeks in 10% formalin were isolated from the interface of the cyst suspension of 40% and 70% percoll. This experiment was repeated 8

weeks later using the same cyst samples and both species, *G. intestinalis* and *G. muris* moved through the percoll gradients and pelleted on the bottom of the test tubes. This showed and increase in specific gravity from <1.0 to >1.35, when cysts were stored over long periods of time.

3.6.2. Differentiation on the basis of cyst size

Giardia intestinalis cysts isolated from a single human faecal and used in 2.6.1. experiment had an average length of 13.29 μm (standard deviation = 0.82 μm) and an average width of 9.68 μm (standard deviation = 0.53 μm, n = 117).

Giardia intestinalis isolated from a variety of human faecal samples had an average length of 12.68 μm (standard deviation = 0.96 μm), and average width of 9.21 μm (standard deviation = 0.75 μm, n = 105).

Cysts cultured from mice and used in the Meridian-*Giardia* (IFT) kit for the positive control, had an average length of 13.58 μm (standard deviation = 2.04 μm) and average width of 9.76 μm with a standard deviation of 1.25 μm (n = 79).

Giardia muris cysts isolated from mice faeces and used in previous experiments, ie had been stored in 10% formalin for at least 4 months, had an average length of 12.28 μm (standard deviation = 0.85 μm) and an average width of 7.99 μm (standard deviation = 0.51 μm, n = 170).

3.7. Culturing *Giardia muris* in the Mouse

Table 9: A general description of each mouse group studied.

Group 1	6 adult mice
Group 2	9 adult mice (not fully matured)
Group 3	Mice born to infected females
Group 4	Mice born to infected females
Group 5	Female mice born to infected females
Group 6	Male mice born to infected females
Group 7	Female mice born to infected females

The results are presented in the figures and tabulated in Appendix A.

3.7.1. Group 1

6 adult Swiss albino mice were inoculated with *Giardia muris* cysts. On day 3 after inoculation all tested positive for *Giardia*, ie *Giardia* cysts were detected in their faeces. The cysts were round, non-refractile, and non-viable.

The time each faecal sample was screened for the presence of *Giardia* cysts was recorded as the day from inoculation. The results are presented in Figure 4.

From day 41 to day 73 all the mice tested negative ie $\log_{10}(1.0)$. They were then treated with flagyl for 1 week and reinoculated with 3.15×10^4 *G. muris* cysts in a 50 µl suspension. Faeces were collected from the mice and screened for the presence of *Giardia muris* cysts with the times recorded from the time of inoculation in days. The results are presented in Figure 5.

3.7.2. Group 2

8 juvenile Swiss albino mice were inoculated with *Giardia muris* cysts. They were determined to be positive for *Giardia muris* on day 5 after inoculation. The results are presented in Figure 6. From day 56, after the birth of baby mice, the amount of *G. muris* cysts per gram faeces collected from all the adult mice within the group increased. The adult mice were separated from the weaned baby mice and inoculated with flagyl.

3.7.3. Group 3

12 Swiss albino mice born to female mice positive for a *Giardia muris* infection. Initially cyst isolation was easy with no fatty tissue interfering with cyst flotation. From Day 49 (approximately) recovery of cysts was more difficult due to the amount of fatty tissue isolated on the interface containing the cysts. On Day 19 when no young mice were seen suckling they were then isolated from the parents. The results are presented in Figure 7.

3.7.4. Group 4

8 Swiss albino mice born to a female positive for *Giardia muris*. The time recorded in the day from birth. Results are presented in Figure 8.

3.7.5. Group 5

8 Swiss albino mice born to a female positive for *Giardia muris*. The time was recorded as the day from birth for when their faeces were screened for the presence of *Giardia muris* cysts. Results are presented in Figure 9.

3.7.6. Group 6

8 Swiss albino mice born to a female positive for *Giardia muris*. The time was recorded as the day from birth for when their faeces were screened for the presence of *Giardia muris* cysts. Results are presented in Figure 10.

3.7.7. Group 7

8 Swiss albino mice born to a female positive for *Giardia muris*. The time was recorded as the day from birth for when their faeces were screened for the presence of *Giardia muris* cysts. Results are presented in Figure 11.

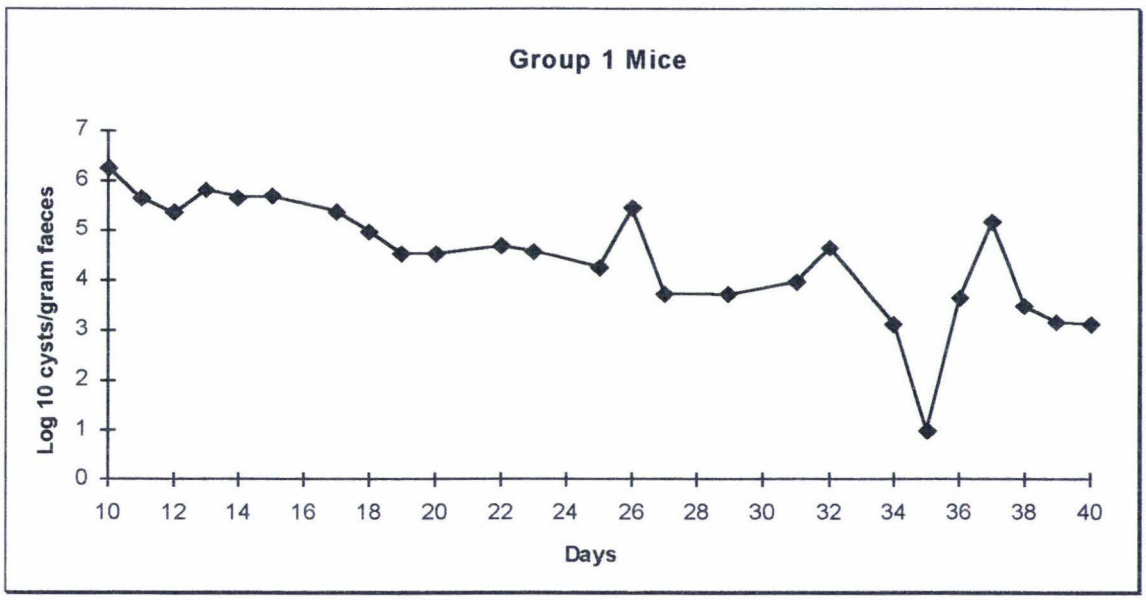


Figure 4: *Giardia muris* cysts isolated from fresh faeces of Swiss albino mice in group 1.

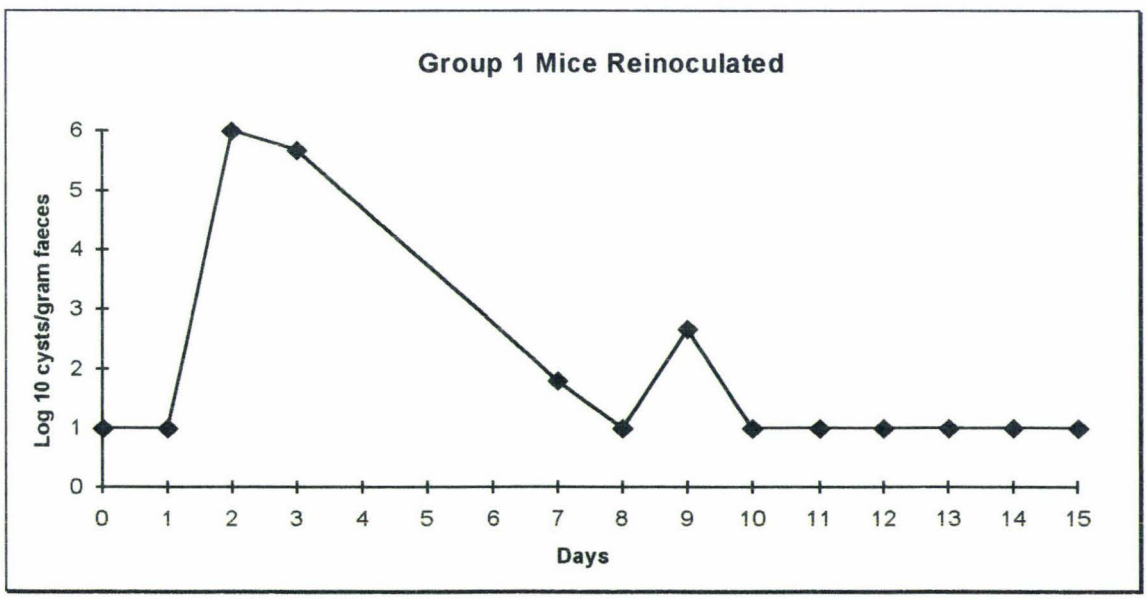


Figure 5: *Giardia muris* cysts isolated from fresh faeces of Swiss albino mice in group 1, after they had been treated with flagyl, tested negative for a *Giardia muris* infection and reinoculated with *G. muris* on Day 0 of this graph.

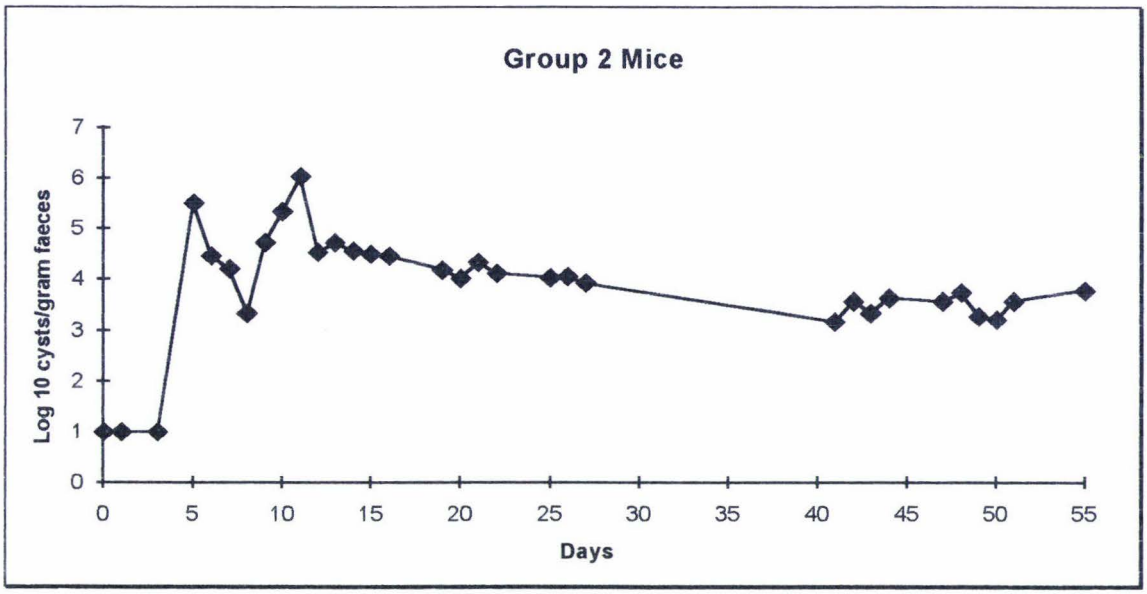


Figure 6: *Giardia muris* cysts isolated from fresh faeces of Swiss albino mice in group 2.

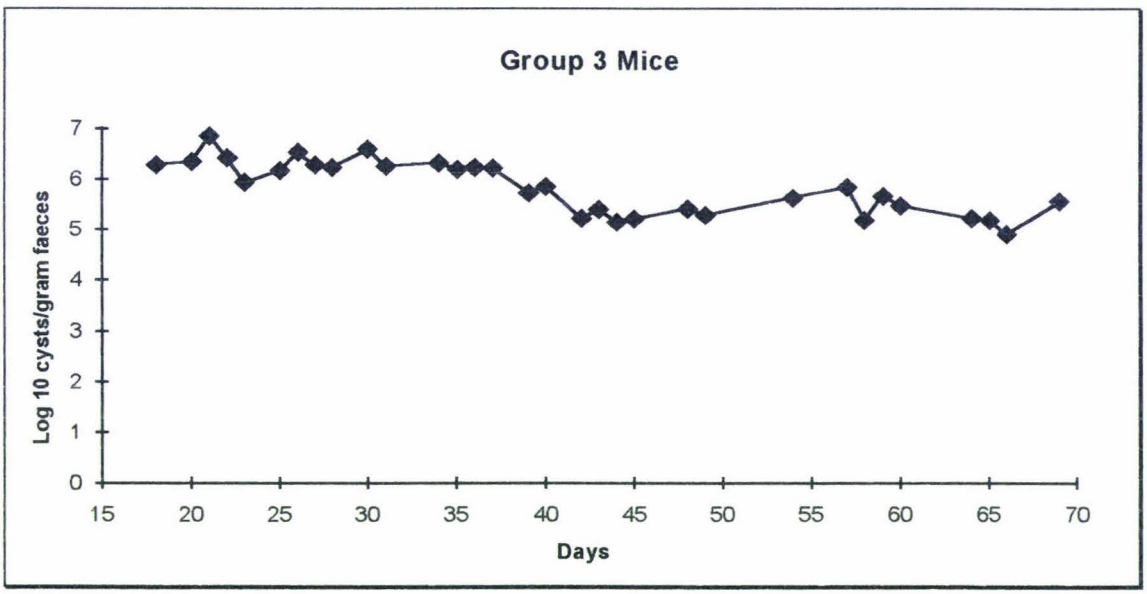


Figure 7: *Giardia muris* cysts isolated from fresh faeces of Swiss albino mice in group 3.

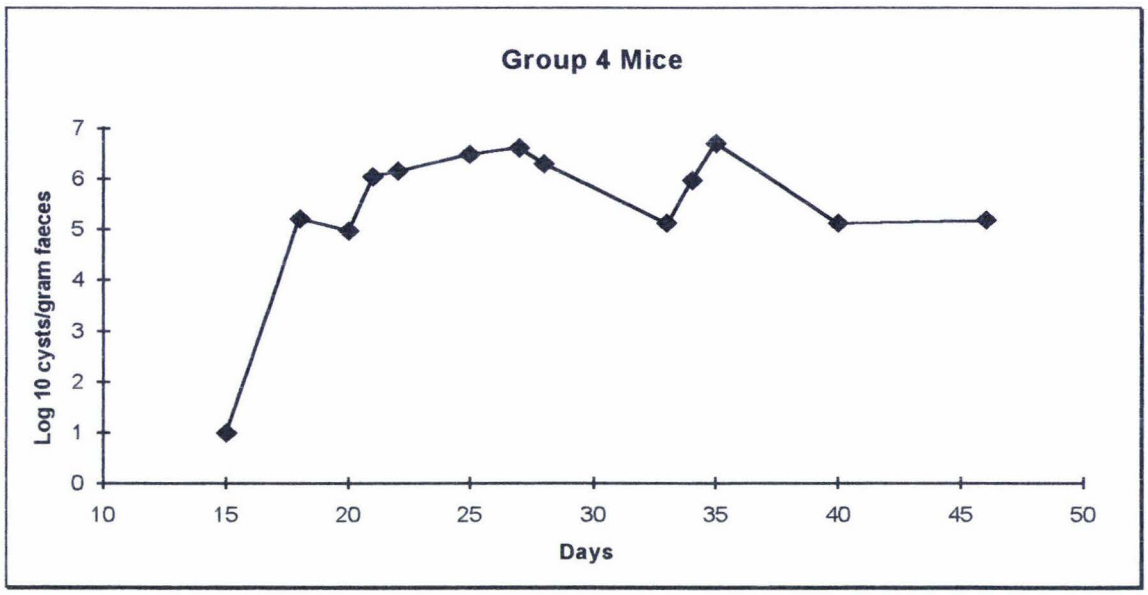


Figure 8: *Giardia muris* cysts isolated from fresh faeces of Swiss albino mice in group 4.

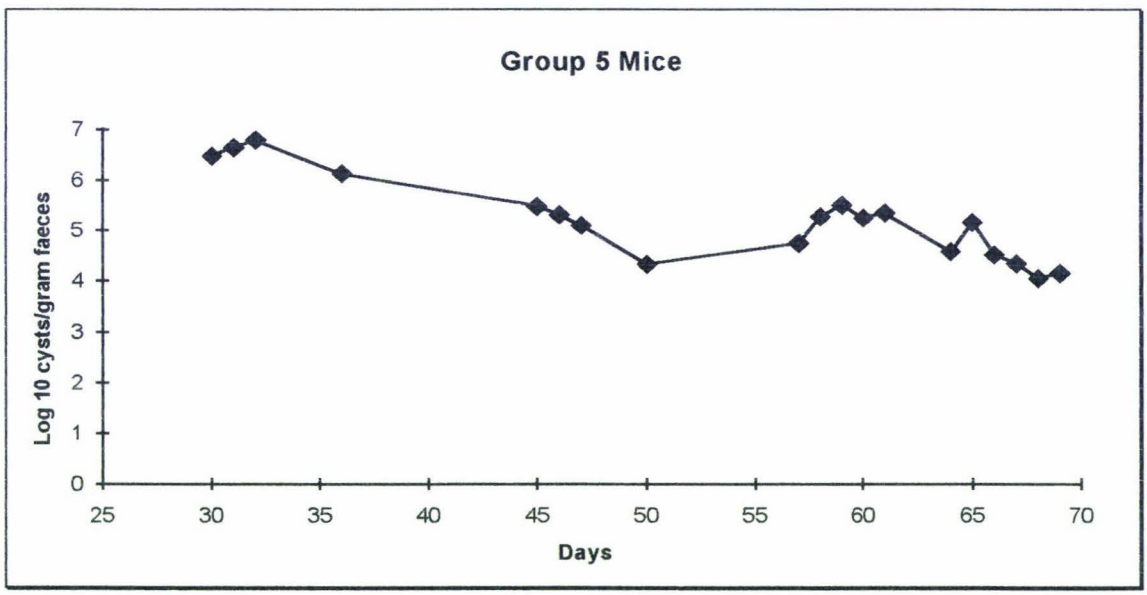


Figure 9: *Giardia muris* cysts isolated from fresh faeces of Swiss albino mice in group 5.

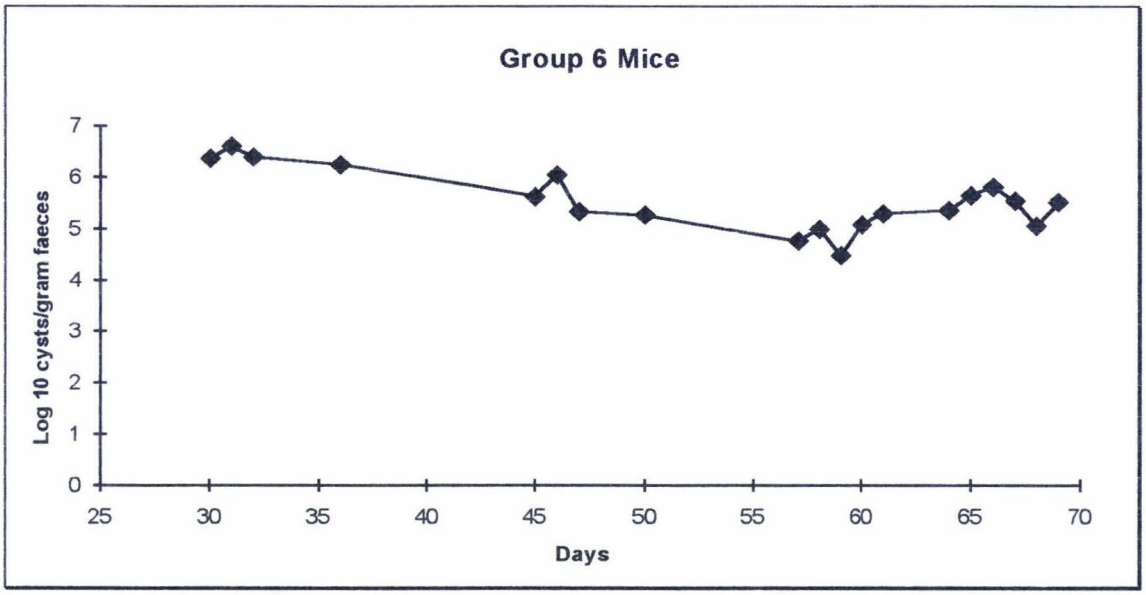


Figure 10: *Giardia muris* cysts isolated from fresh faeces of Swiss albino mice in group 6.

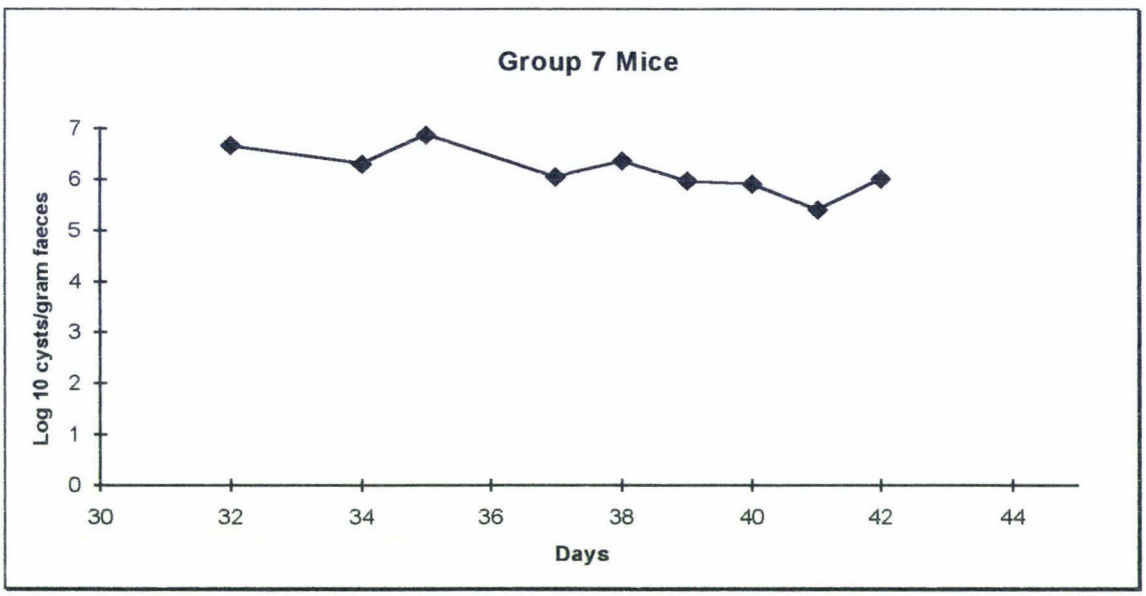


Figure 11: *Giardia muris* cysts isolated from fresh faeces of Swiss albino mice in group 7.

3.8. Establishing Viability

3.8.1. The method of Feely, 1986

Table 10: Data for establishing viability via Feely's Method, 1986

Stage	Average % Excysted	S.D. (±)
B	8.40	3.70
A	22.61	7.34
0 minutes	30.21	4.66
5 Minutes	38.72	6.42
15 minutes	46.63	7.72
30 minutes	50.73	6.93
1 Day	57.05	11.62

Key: B = a sample was taken before excystation induced
A = sample taken after acid or induction step

The rest of the time intervals commenced after excystation was induced and the cysts were washed and were incubating in the TY1-S-33 growth medium.

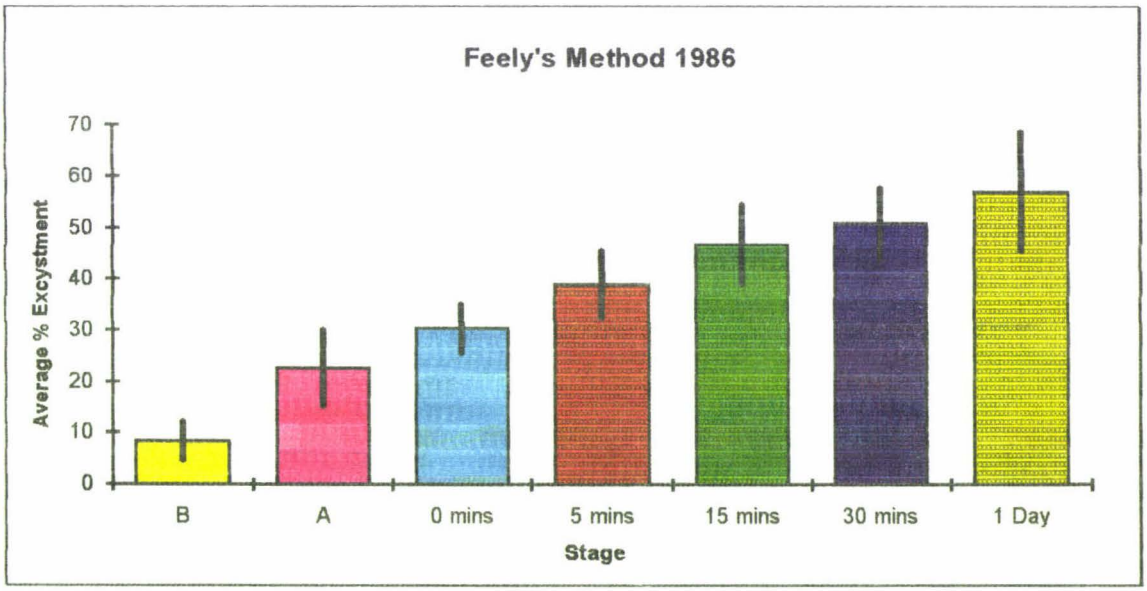


Figure 12: Histogram representing data in Table 10 - The Method of Feely, 1986.

Key: B = time before excystation induces; A = time after acid induction.
The rest of the time intervals are taken from the time the *Giardia* cysts were placed in TY1-S-33 growth medium. Vertical lines represent standard deviations.

3.8.2. The method of Feely, 1991

Table 11: Data for establishing viability via Feely's Method, 1991

Stage	Average % Excysted	S.D. (±)
B	14.04	3.37
A	9.36	3.92
0 minutes	28.90	6.21
5 Minutes	25.41	6.92
15 minutes	32.94	13.72
30 minutes	30.58	11.87
1 Day	46.79	18.37

Key: B = a sample was taken before excystation induced
A = sample taken after acid or induction step

The rest of the time intervals commenced after excystation was induced and the cysts were washed and were incubating in the TY1-S-33 growth medium.

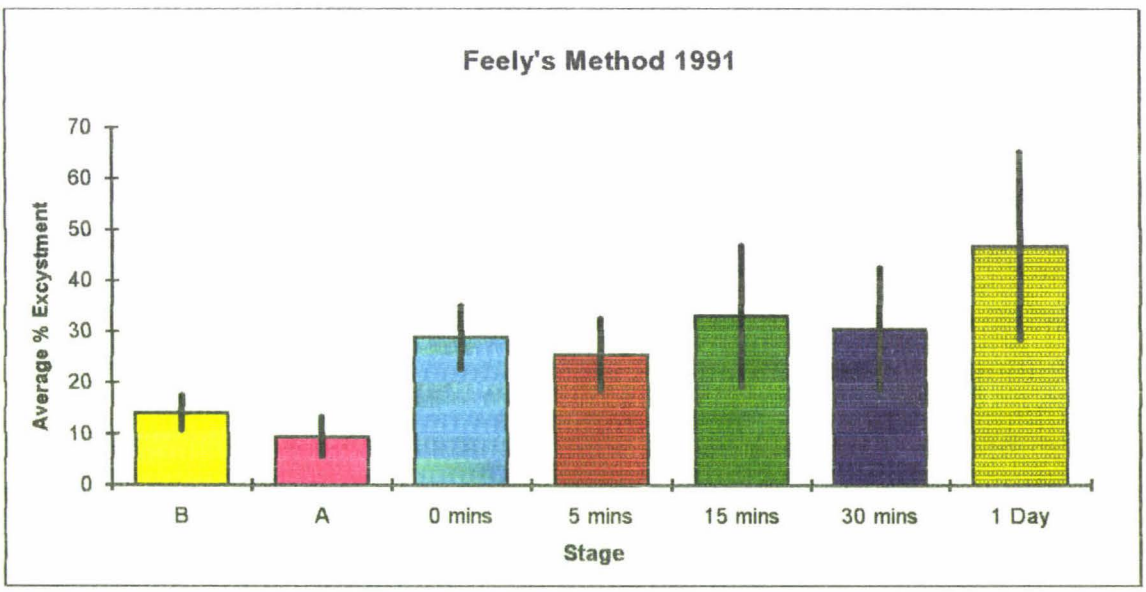


Figure 13: Histogram representing data in Table 11 - Feely's Method, 1991.

Key: B = time before excystation induces; A = time after acid induction.
The rest of the time intervals are taken from the time the *Giardia* cysts were placed in TY1-S-33 growth medium. Vertical lines represent standard deviations.

3.9. Environmental Effects on *Giardia* cyst Presence and Viability

3.9.1. *Giardia* cysts stored at -20°C

3.9.1.1. *G. intestinalis* cultured *in vitro*

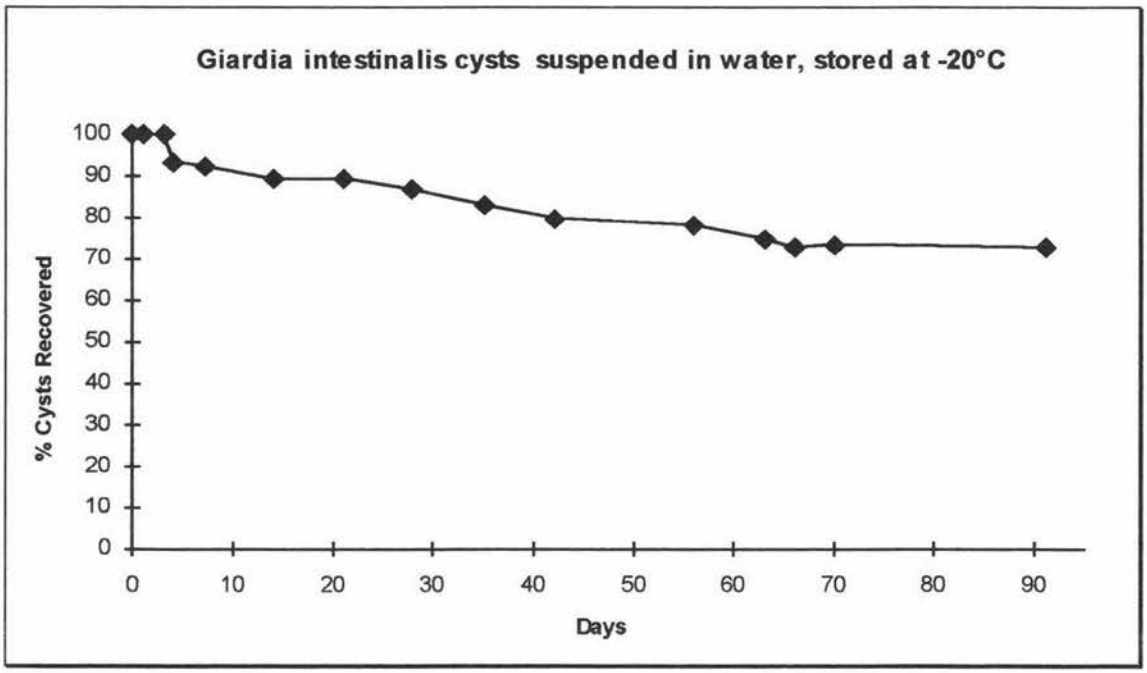


Figure 14: The % of recovered *Giardia intestinalis* cysts cultured *in vitro* according to the method of Schupp *et al* (1988), suspended in sterile milli-Q water and stored at -20°C. Viability was detected for one day in one sample and up to 63 days in another.

3.9.1.2. *G. muris* cultured in mice

3.9.1.2.1. *G. muris* cysts suspended in mouse faeces

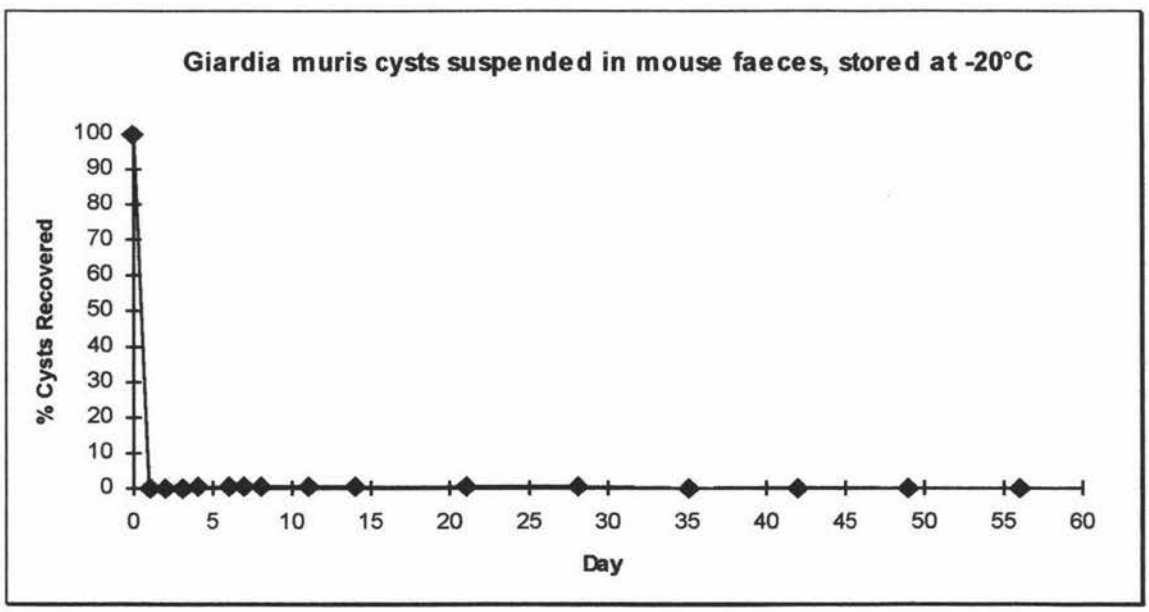


Figure 15: The % of recovered *Giardia muris* cysts suspended in mouse faeces, and stored at 4°C. Viability was detected up to day 28.

3.9.1.2.2. *G. muris* cysts suspended in water

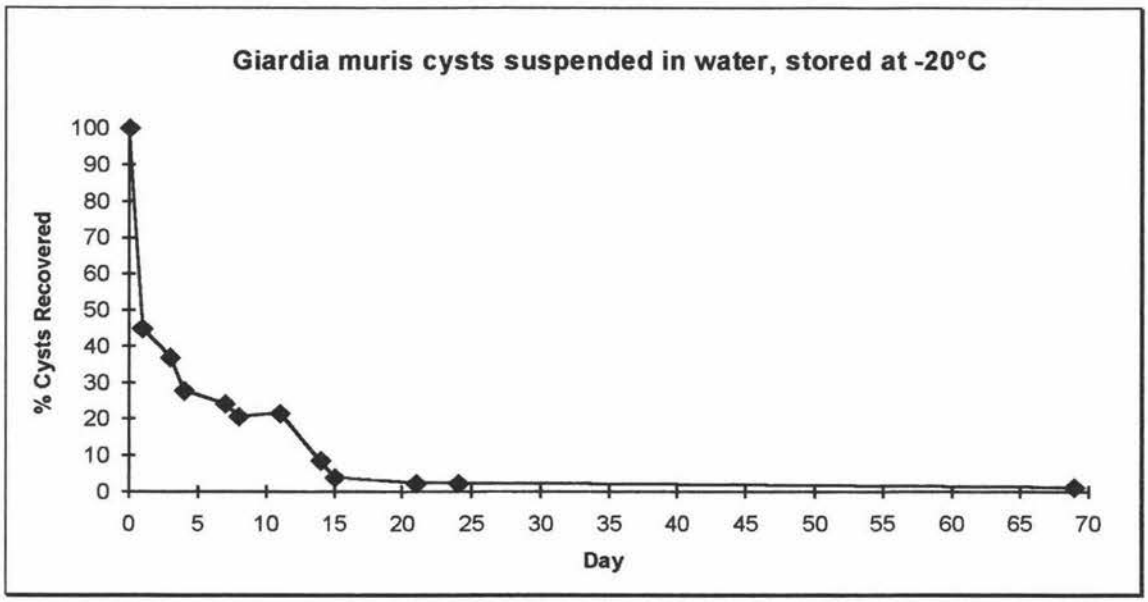


Figure 16: The % of recovered *Giardia muris* cysts isolated from mice faeces, their source, suspended in water and stored at 4°C. Viability was detected in different samples up to day 14 and day 69.

3.9.2. *Giardia* cysts stored at 4°C

3.9.2.1. *G. intestinalis* cultured *in vitro*

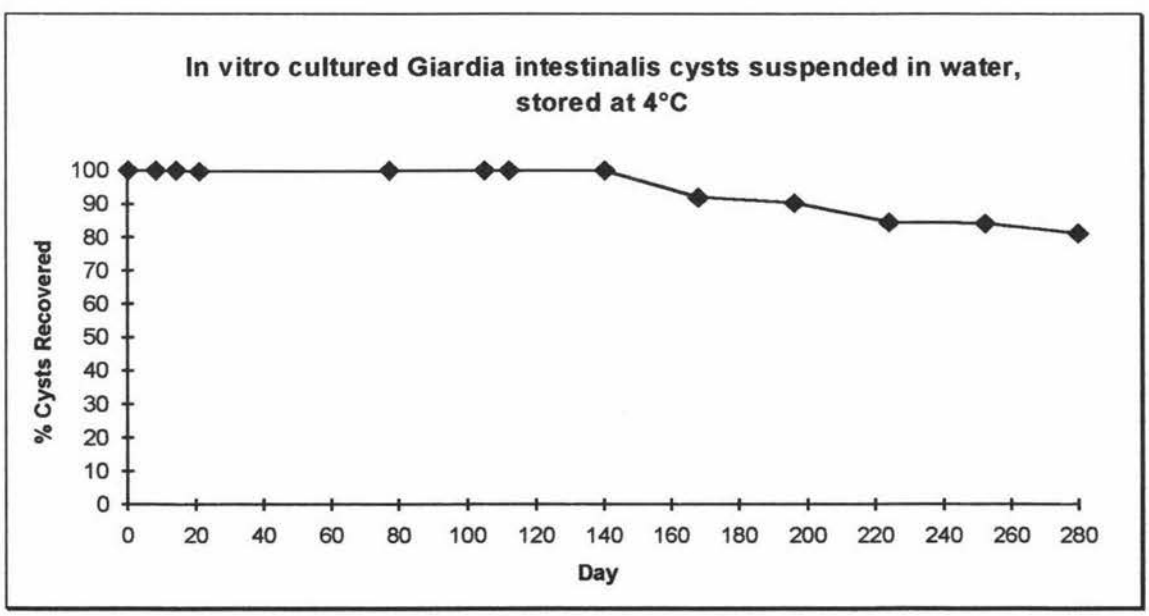


Figure 17: The % of recovered *Giardia intestinalis* cysts cultured *in vitro* according to the method of Schupp *et al* (1988), stored in sterile milli-Q water and incubated at 4°C. Viability was detected up to day 140.

3.9.2.2. *G. intestinalis* isolated from humans

3.9.2.2.1. *G. intestinalis* cysts suspended in human faeces.

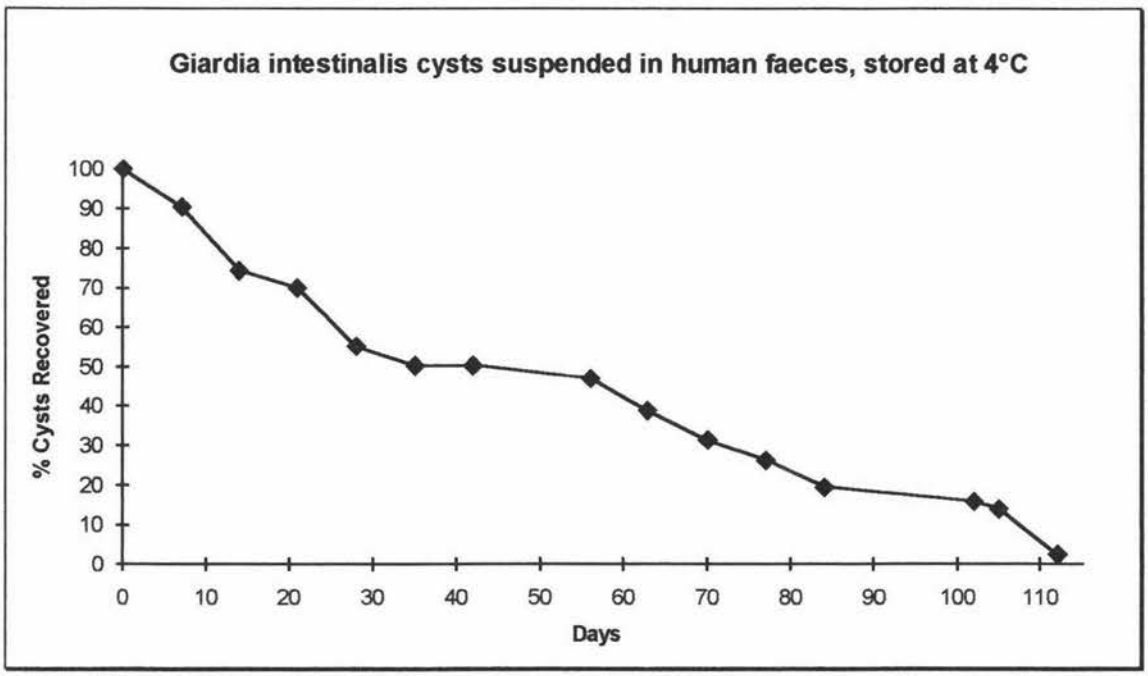


Figure 18: The % of recovered *Giardia intestinalis* cysts suspended in human faeces, their source, and stored at 4°C. Viability was detected up to day 63 in one sample.

3.9.2.2.2. *G. intestinalis* cysts suspended in water.

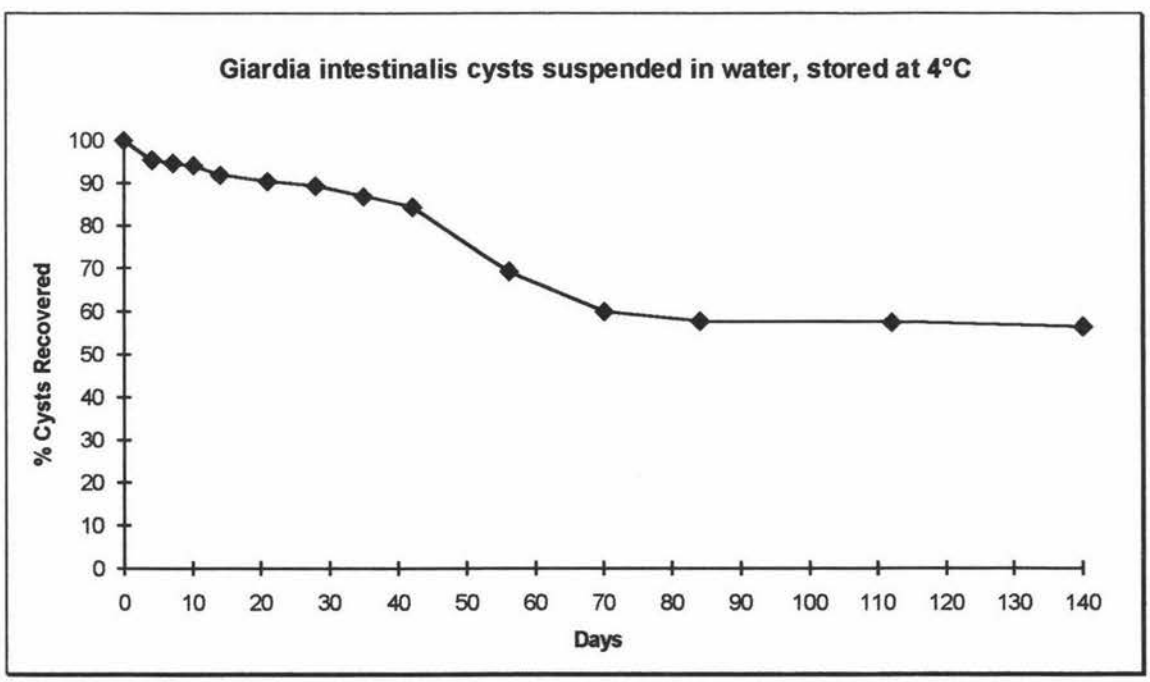


Figure 19: The % of recovered *Giardia intestinalis* cysts isolated from human faeces, suspended in sterile milli-Q water and stored at 4°C. Viability was detected up to day 63 in one sample.

3.9.2.3. *G. muris* isolated from mice

3.9.2.3.1. *G. muris* cysts suspended in mouse faeces.

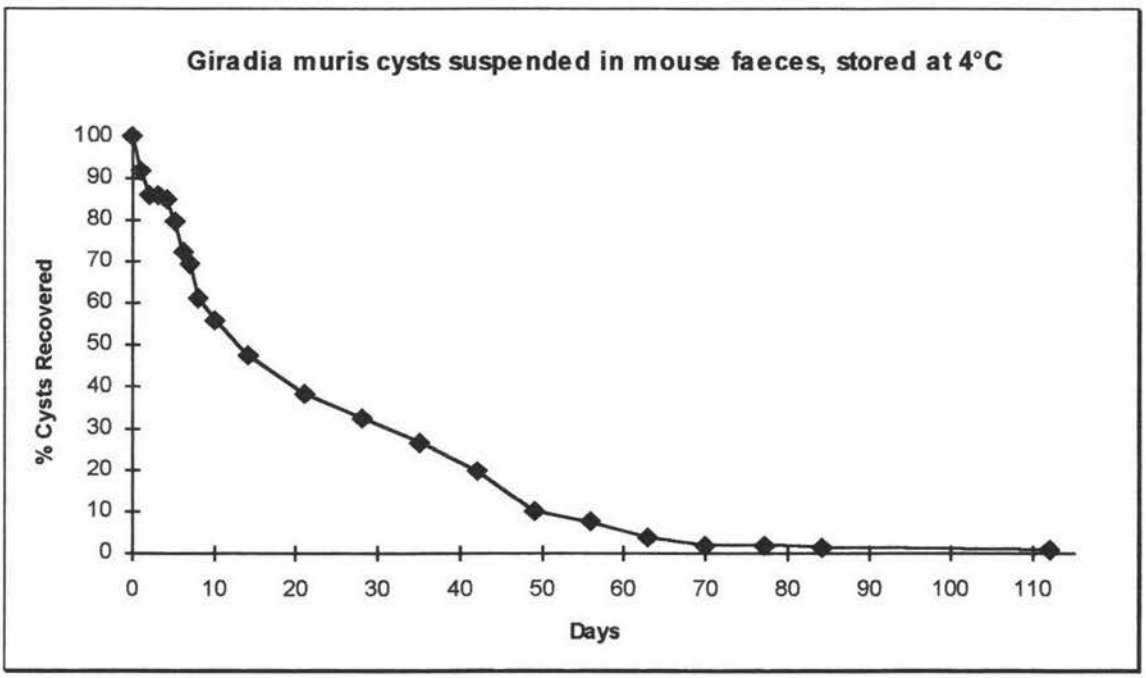


Figure 20: The % of recovered *Giardia muris* cysts suspended in mouse faeces, their source, and stored at 4°C. Viability was detected up to day 28, 49 and 63 in different samples.

3.9.2.3.2. *G. muris* cysts suspended water

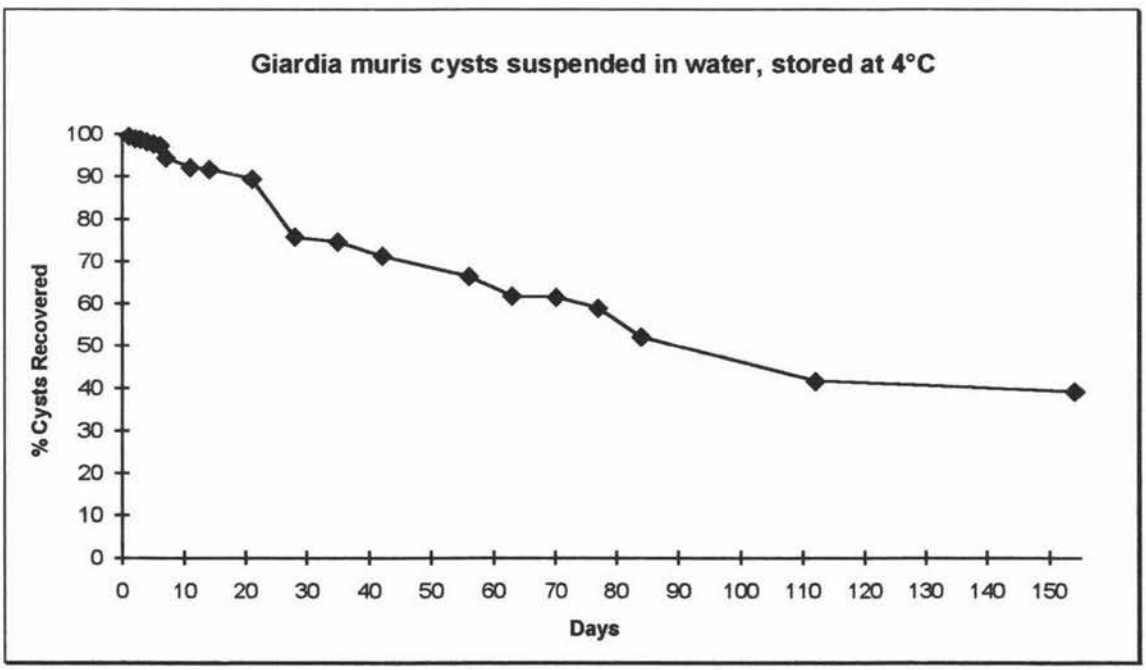


Figure 21: The % of recovered *Giardia muris* cysts isolated from mouse faeces, suspended water and stored at 4°C. Viability was detected up to day 42, 84 and 140 in different samples.

3.9.3. *Giardia* cysts stored at 20°C (room temperature)

3.9.3.1. *G. muris* cultured in mice

3.9.3.1.1. *G. muris* cysts suspended in mouse faeces.

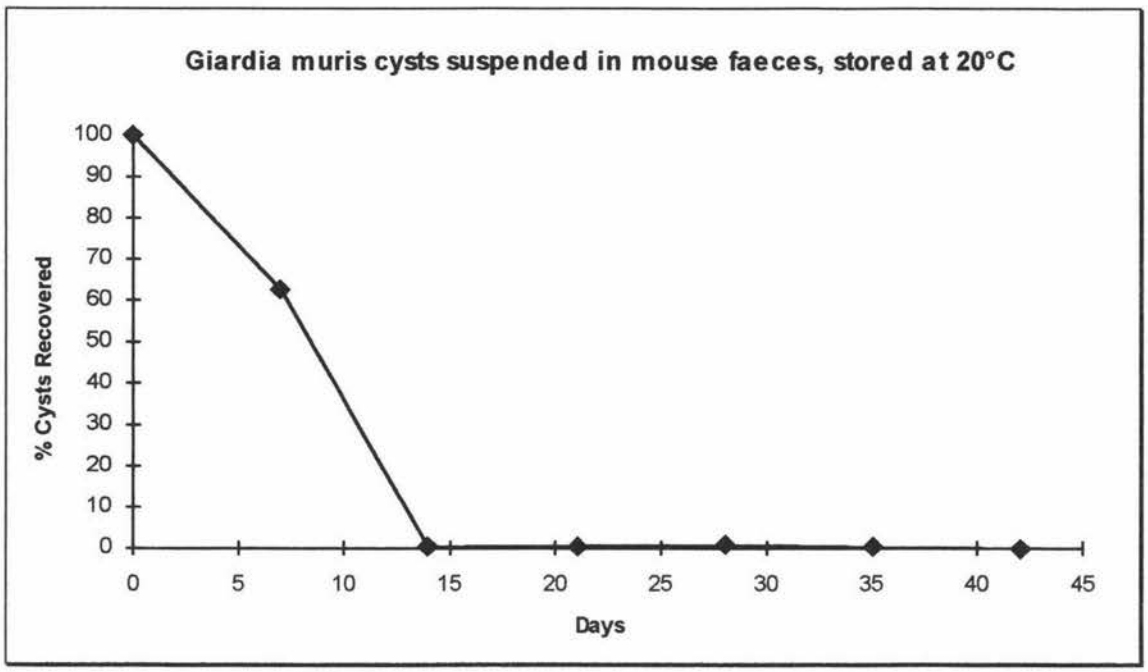


Figure 22: The % of recovered *Giardia muris* cysts suspended in mouse faeces and stored at 20°C. Viability was not detected after day 0.

3.9.3.1.2. *G. muris* cysts suspended in water

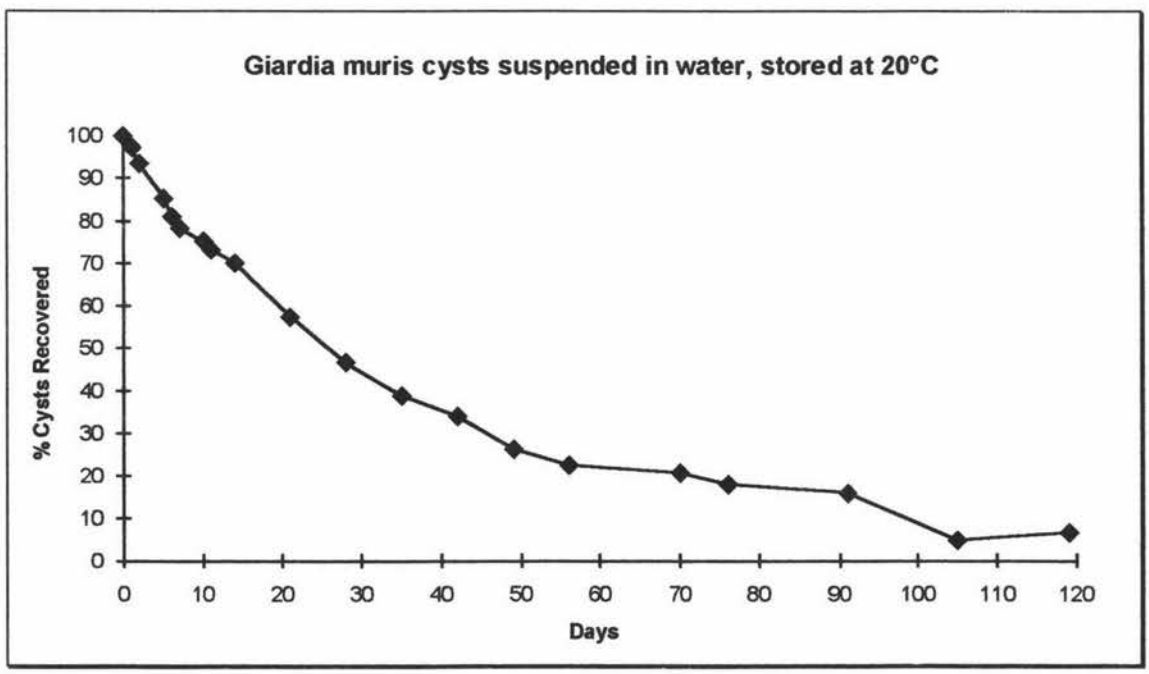


Figure 23: The % of recovered *Giardia muris* cysts isolated from mouse faeces, suspended in water and stored at 20°C. Viability was detected up to day 10, 21, 28 and 76 for different samples.

3.9.4. *Giardia* cysts left in sawdust for 24 Hours

Table 12: Data for recovery of *Giardia* cysts left in sawdust after 24 hours

Sample	Expected #/g	Detected #/g	Reduction %
1	6.41×10^5	0	>99.95
2	3.85×10^5	0	>99.96
3	5.29×10^5	0	>99.94
4	7.89×10^4	0	>99.58
5	5.79×10^5	500	>99.91
6	4.34×10^4	0	>99.19
7	2.40×10^4	2.0×10^3	>91.67
8	1.30×10^5	0	>99.75
9	3.61×10^3	0	>81.19
10	7.61×10^4	0	>79.03
11	1.96×10^3	0	>79.03
12	2.12×10^4	0	>90.76
13	8.51×10^4	0	>97.70

Only 2 out of 13 samples collected revealed any *Giardia* cysts at all. The faecal samples, when collected, were dry and brittle.

3.9.5. *Giardia* cysts stored at 37°C

3.9.5.1. *G. muris* cultured in mice

3.9.5.1.1. *G. muris* cysts suspended in mouse faeces

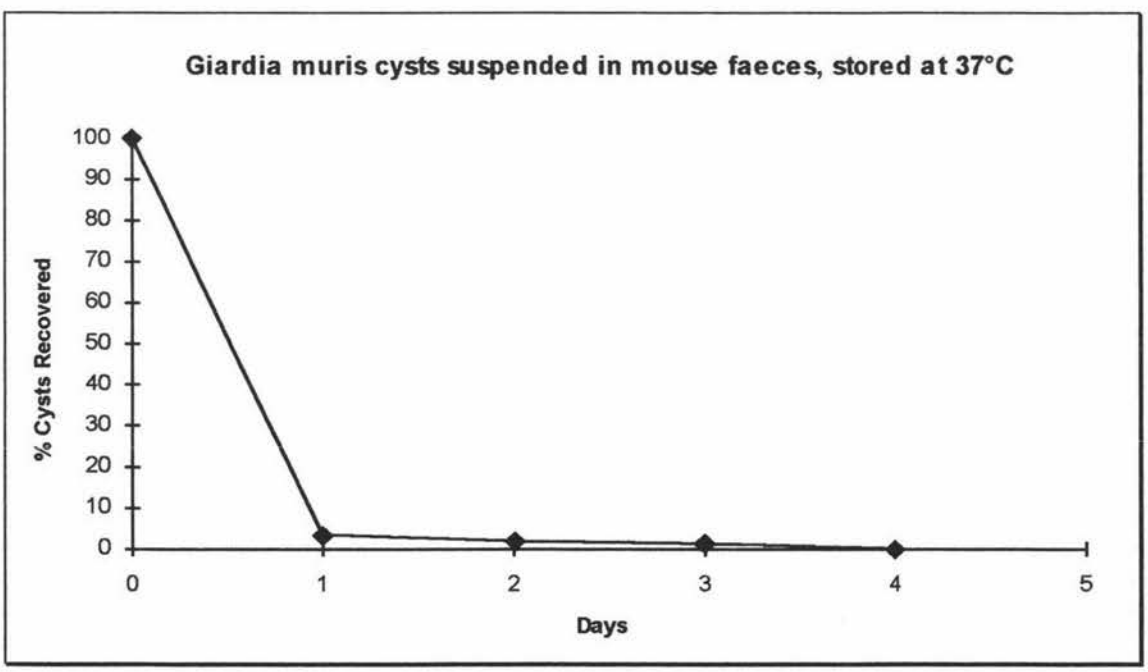


Figure 24: The % of recovered *Giardia muris* cysts suspended in mouse faeces, their source of origin, and stored at 37°C. When faeces dried, no cysts were detected. Viability was not detected after day 0 except once when it was detected up to day 4 in a faecal sample that did not dry out until day 6.

3.9.5.1.1. *G. muris* cysts suspended in water

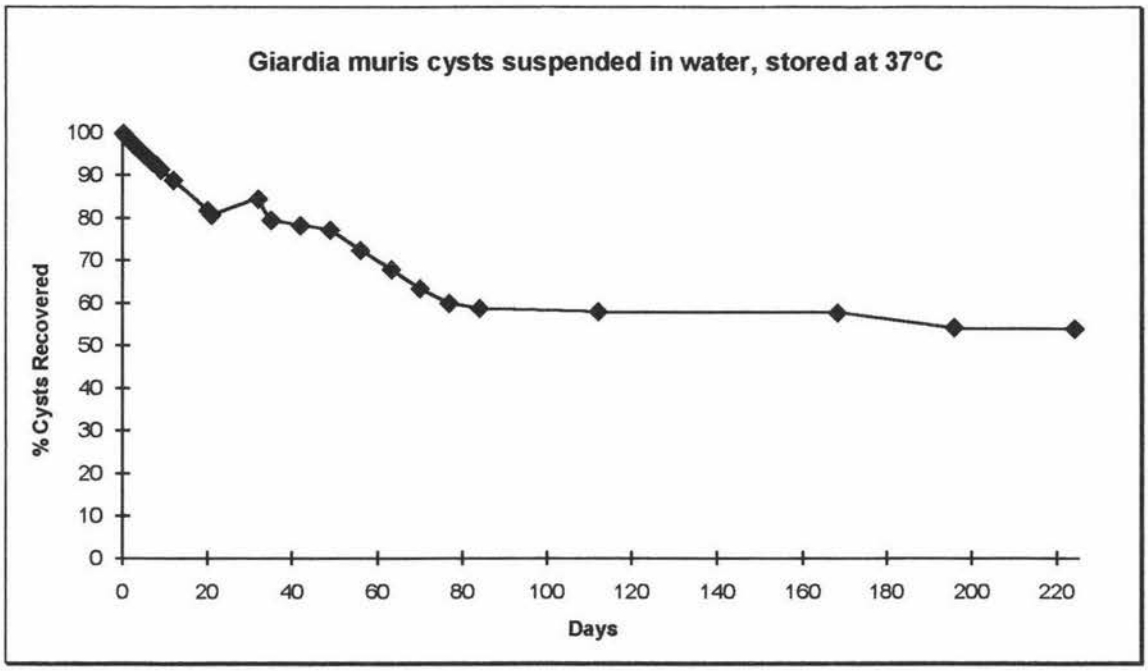


Figure 25: The % of recovered *Giardia muris* cysts isolated from mouse faeces, suspended in water and stored at 37°C. Viability was detected up to day 6.

3.10. Preservation of *Giardia* Cysts

The results are presented in the following figures and the data is tabulated in Appendix A.

3.10.1. PVA Fixation

3.10.1.1. *G. muris* cultured in mice

3.10.1.1.1. *G. muris* cysts suspended in mouse faeces

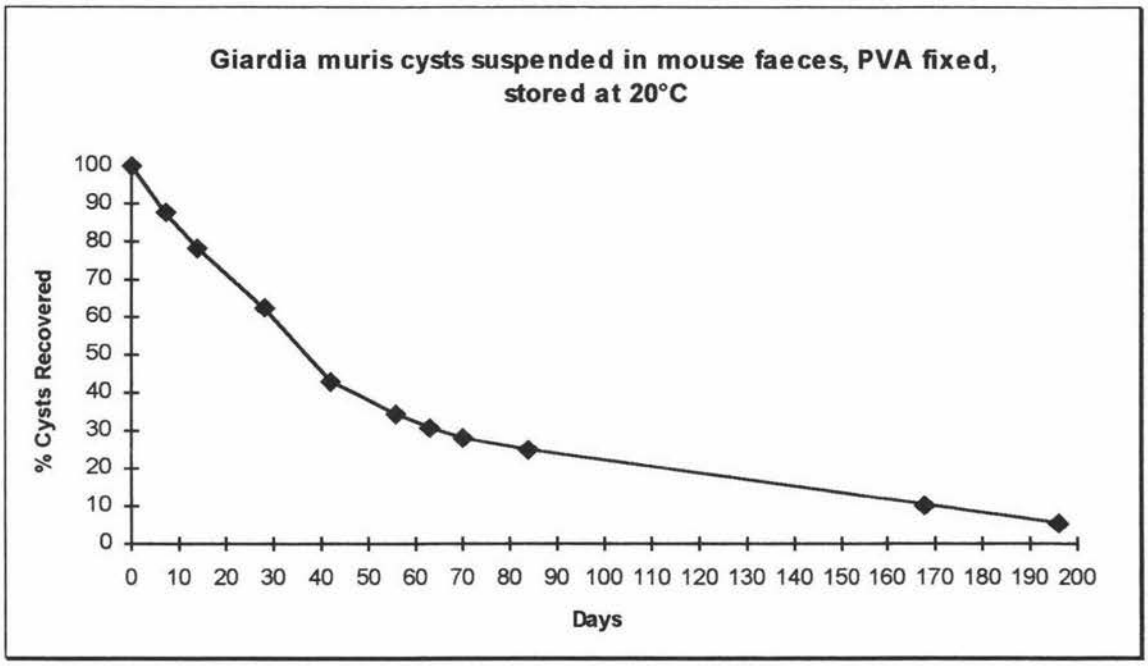


Figure 26: The % of recovered *Giardia muris* cysts suspended in mouse faeces, their source, fixed with PVA and stored at 20°C.

When *Giardia muris* cysts within faeces were fixed with PVA, on average only 1.72% of the cysts were recovered within 30 minutes of being exposed to PVA. The internal morphology of the *Giardia* cyst is disrupted with obvious shrinkage of the cytoplasm.

3.10.1.1.2. *G. muris* cysts suspended in solution.

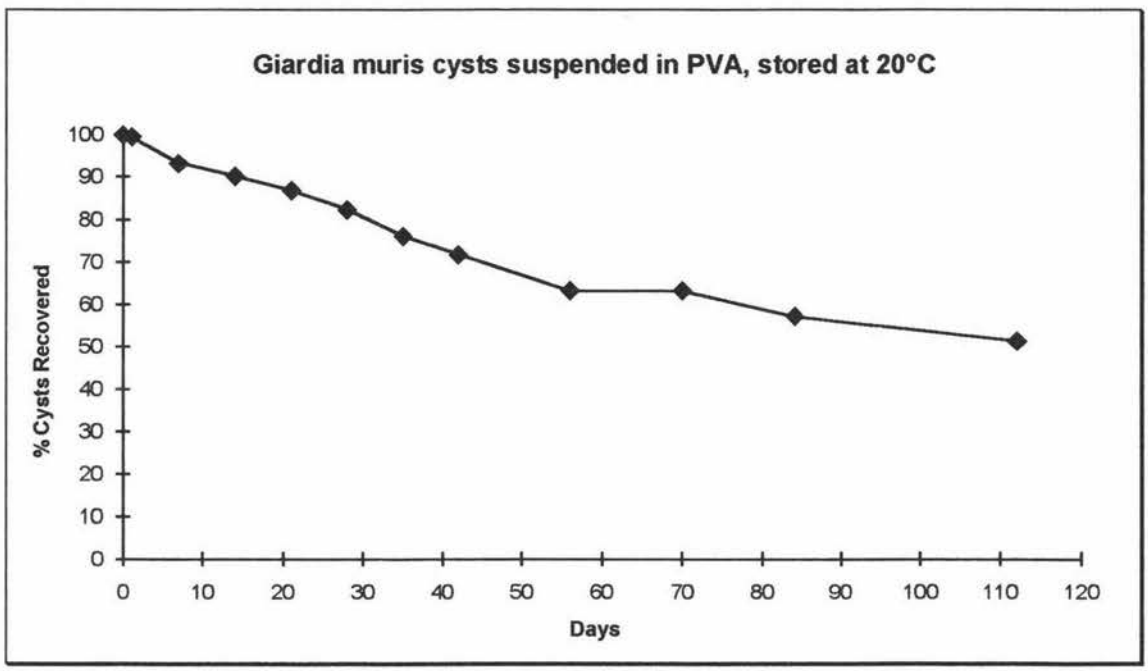


Figure 27: The % of recovered *Giardia muris* cysts isolated from mouse faeces, suspended in PVA and stored at 20°C.

When *Giardia muris* cysts isolated from mice faeces were fixed with PVA, on average only 44.85% of the cysts were recovered within 30 minutes of being exposed to PVA.

3.10.2. *Giardia* cysts stored in 10% formalin at 4°C

3.10.2.1. *G. intestinalis* cultured *in vitro*

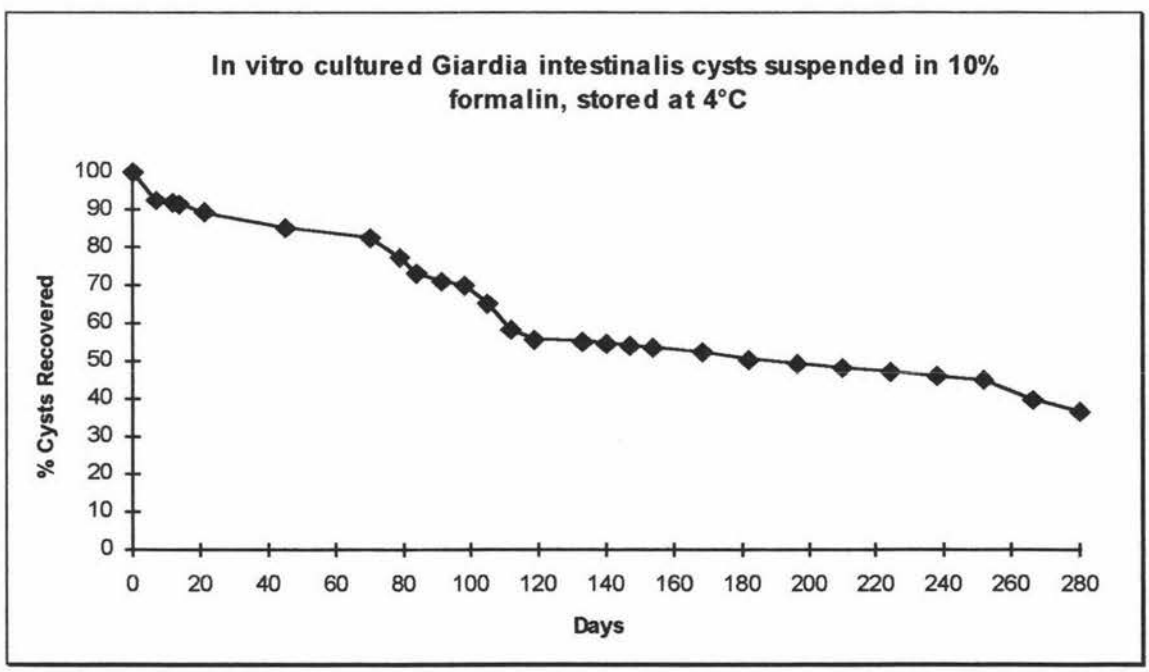


Figure 28: The % of recovered *Giardia intestinalis* cysts cultured *in vitro* according to the method of Schupp *et al* (1988), suspended in 10% formalin and stored at 4°C.

3.10.2.2. *G. intestinalis* isolated from humans

3.10.2.2.1. *G. intestinalis* cysts suspended in human faeces

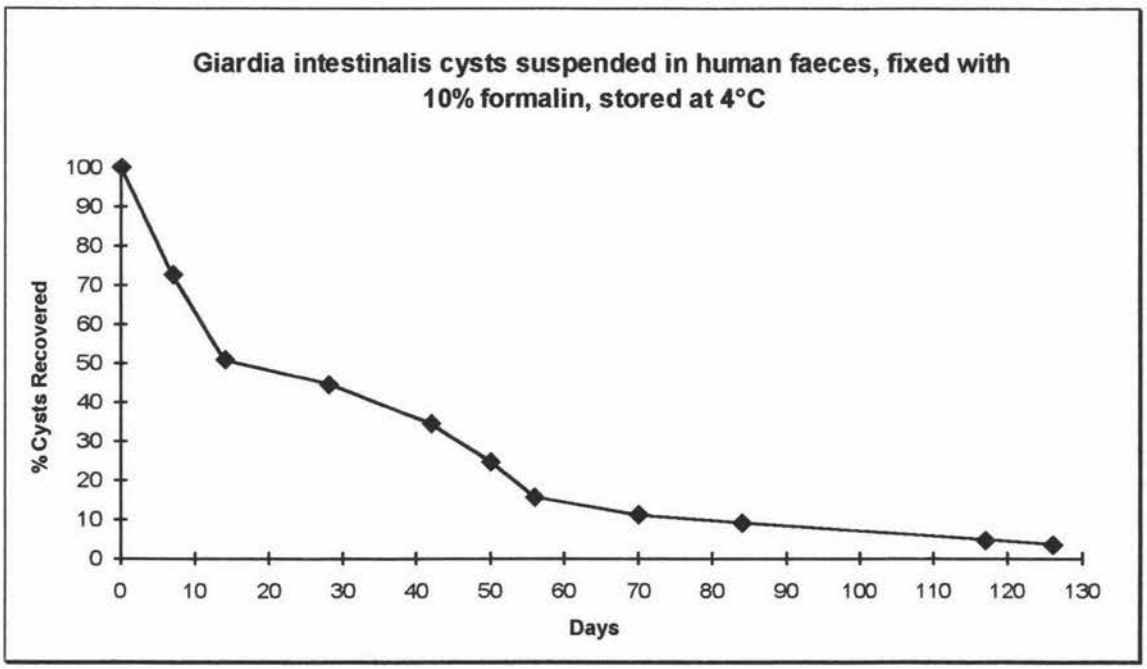


Figure 29: The % of recovered *Giardia intestinalis* cysts suspended in human faeces, their source, fixed in a final concentration of 10% formalin and stored at 4°C.

3.10.2.2.2. *G. intestinalis* cysts suspended in solution

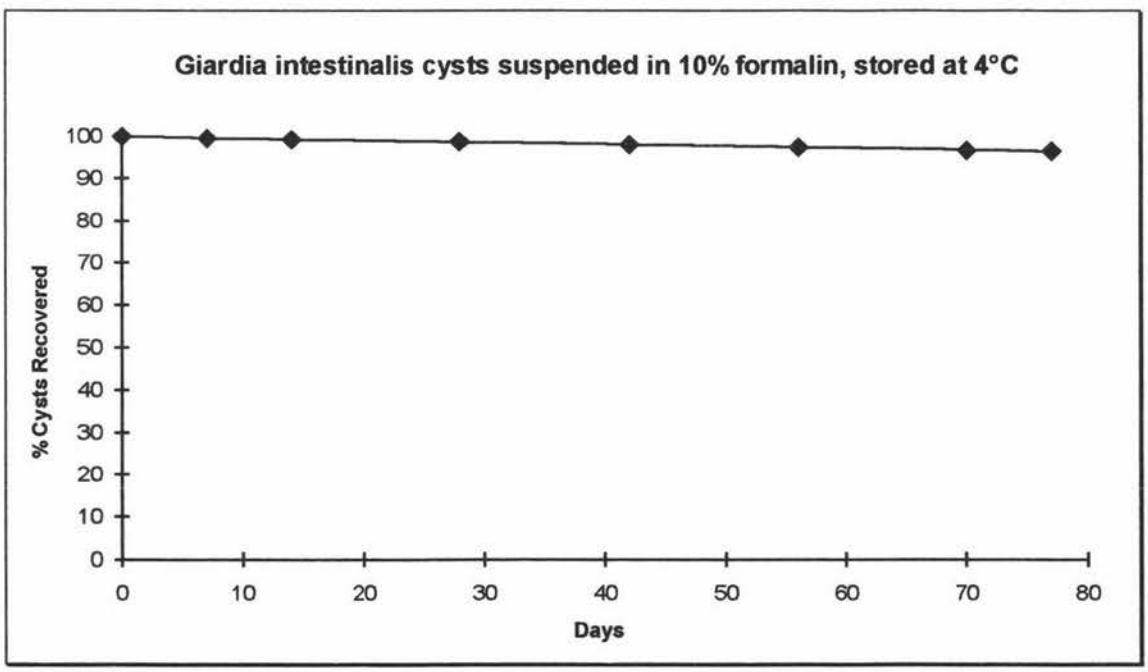


Figure 30: The % of recovered *Giardia intestinalis* cysts isolated from human faeces, suspended in 10% formalin and stored at 4°C.

3.10.2.3. *G. muris* cultured in mice

3.10.2.3.1. *G. muris* cysts suspended in mouse faeces

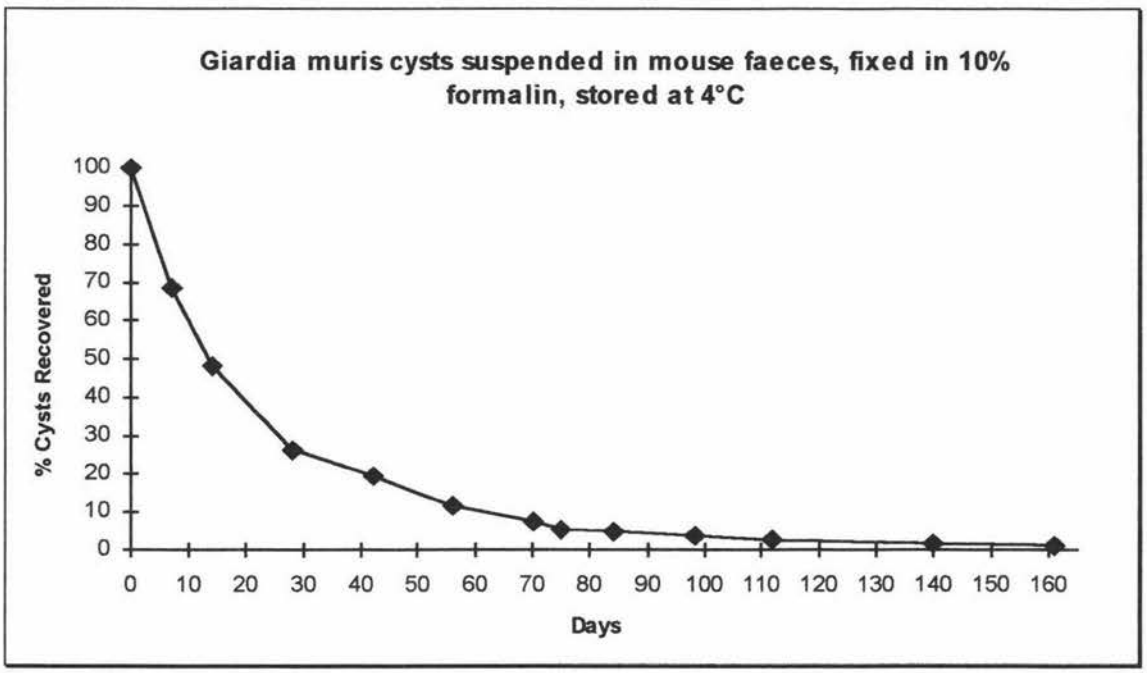


Figure 31: The % of recovered *Giardia muris* cysts suspended in mouse faeces, fixed in a final concentration of 10% formalin and stored at 4°C.

3.10.2.3.2. *G. muris* cysts suspended in solution.

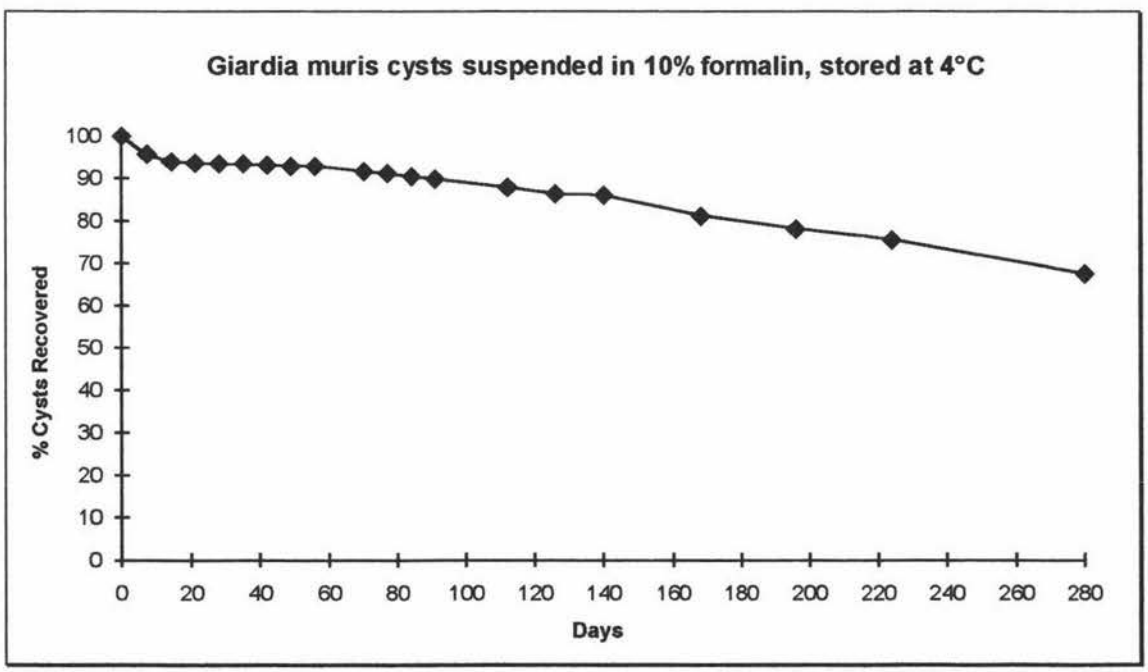


Figure 32: The % of recovered *Giardia muris* cysts suspended in 10% formalin and stored at 4°C.

3.10.3. 0.1% Sodium Dodecyl Sulphate (SDS)

3.10.3.1. *G. intestinalis* cultured *in vitro*

When the cysts were exposed to 0.1% SDS for 1-2 minutes, the trophozoites and incompletely formed cysts were disrupted so badly the remaining intact, non-viable cysts looked to be in a clear suspension ie no traces of disrupted cell or cyst membrane or organelles were visible.

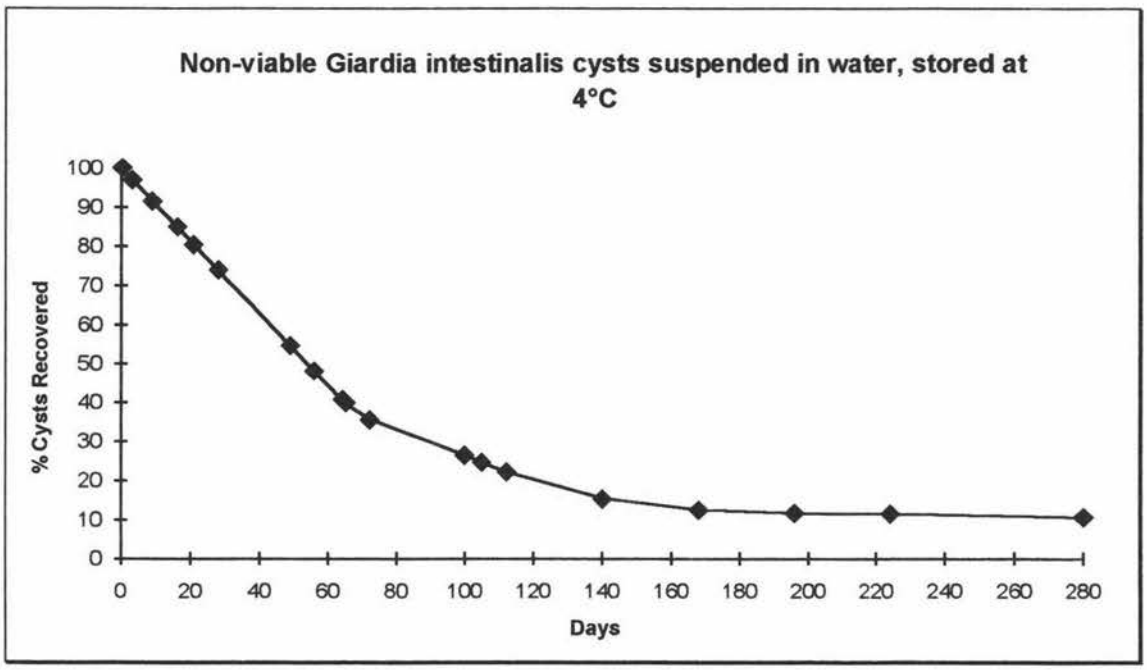


Figure 33: The % of recovered *Giardia intestinalis* cysts cultured *in vitro* according to the method of Schupp *et al* (1988), exposed to 0.1% S.D.S. then washed and suspended in sterile milli-Q water and stored at 4°C.

CHAPTER 4. DISCUSSION

4.1. Recovery of *In Vitro Giardia* Cysts from Trophozoites

Endeavours were made to separate *in vitro* cultured cysts from trophozoites using a range of methods. This was necessary was because with the excystment methods used, intact trophozoites and incompletely formed cysts were harvested along with completely formed cysts. As intact trophozoites are still present after being put through the Feely's 1986 excystment method, it may not be possible to differentiate between intact trophozoites harvested with cysts or those that were induced to excyst from viable cysts. If this were to happen the percent of viable cysts would be artificially elevated and could, at certain levels, result in viability being determined when in reality there are no viable cysts present! To avoid this Equation 1 (Section 2.9.1.) was used which did not include counts of totally excysted trophozoites but only counts of intact cysts, partially excysted trophozoites and empty cell walls.

The presence of dead trophozoites was undesirable for other reasons. Because of the process of cyst degradation, it was thought that enzymes could be released that could initiate further cyst degradation. This could artificially increase the rate of cyst degradation and shorten the term of cyst viability. This situation would not occur under conditions of cyst transmission via water as the cysts would not be exposed to decaying trophozoites. Also the presence of a large proportion of trophozoites can interfere with the counting of cysts. Lengthening of the time required to make a count increases fatigue and will lead to mistakes.

To destroy trophozoites sonication was used for varying times at different intensities. With a higher density of *Giardia* cysts, and therefore a higher density of *Giardia* trophozoites, present in suspension, a longer period of time was required to destroy all trophozoites present. With 10^5 cysts/ml in suspension, sonication at 5μ, 6μ and 7μ for only 1 minute destroyed all trophozoites that were present. When the density of cysts was increased to approximately 10^6/ml with a subsequent increase in the density of trophozoites, sonication at 6μ and 7μ required up to 1.5 minutes to destroy all cysts whereas sonication at 5μ for up to 2 minutes was not always successful in destroying all trophozoites, though only a few remaining trophozoites were ever seen. Sonication did not destroy incompletely formed cysts. The intent was to sonicate for as short a time as possible so as to minimise the destruction of viable completely formed cysts. Because extensive sonication may not destroy all incompletely formed cysts before destroying most of the complete and viable cysts present it is best to sonicate only to destroy trophozoites.

Lysis of trophozoites and incompletely formed cysts was attempted by incubation in double distilled water and milli-Q water at 4°C overnight (Section 2.2.4. and 3.1.2.). Up to 80% of all trophozoites present were lysed. It may be best to first incubate the cells in water overnight to reduce the number present and then sonicate if there is initially a very high density of cells present in harvested cultures, ie greater than 10^6 cysts/ml.

At no stage was it possible to separate *Giardia* cysts from trophozoites on the basis of their specific gravity, hence the need to destroy the trophozoites present.

The cyst suspensions treated with 0.1% SDS for 1-2 minutes were rendered non-viable, but gave a clean preparation of cysts with no incompletely formed cysts or trophozoites present. This was a quick easy method if used when the viability of the cysts was not a concern.

4.2. Techniques to Recover and Detect *Giardia* Cysts from Environmental and Laboratory Samples

The sheather-sucrose flotation method gave the highest recovery of *in vitro* cultured *Giardia intestinalis* cysts, 1.0 M sucrose flotation gave the lowest (see 3.2.1.). Using *G. intestinalis* from human faeces and *G. muris* from mouse faeces, sheather-sucrose flotation gave a higher recovery than 1.0 M sucrose flotation (see 3.2.2.). However, when isolating *Giardia* cysts from animal faeces, 1.0 M sucrose gave the highest recovery overall. It had the highest recovery from sheep, hens and cows while percoll-sucrose had the highest recovery of cysts from dog faeces. Sheather-sucrose always ranked third after 1 M sucrose and percoll-sucrose. Statistically there was not significant difference between any of the flotation methods compared, mainly due to the large error (standard deviation) determined for each method. Because of costs, ease of preparation of reagents and the simplicity of the method, the 1.0 M sucrose flotation was used for the rest of this study.

The Meridian™ brand Merifluor-*Giardia*, an indirect fluorescent stain, appeared to be the best overall and in particular for dogs, sheep and hens (see 2.3.4. and 3.2.5.) with the polyclonal antibody stain developed by J. Chan (Massey University) being best for cysts isolated from cattle faeces. It was expected that the polyclonal antibody would be more sensitive in all cases simply because it was not a monoclonal and was therefore directed at more than one antigen that would identify the presence of *Giardia* cysts. Meridian stated that the Meridian Hydrofluor kit would be as sensitive and as specific as the Meridian Merifluor kit but this was not found to be so.

The best possible combination for isolating and detecting *Giardia* cysts from animal faecal samples is to isolate *Giardia* cysts on a 1.0 M sucrose gradient and then stain with Meridian Merifluor-*Giardia* staining kit.

4.3. Comparison of Commercial Diagnostic Kits with Commonly Used Diagnostic Methods

Methods 1 to 3 (Section 2.4.) used 1.0 M sucrose flotation to concentrate cysts and three different stains. The Meridian Hydrofluor™ IFA - *combo* gave the best results with only 30% false negatives. The Meridian Merifluor™ DFA - *combo* kit was not expected to show any difference in performance when compared to the Meridian Hydrofluor IFA - *combo* even though the antibody used previously (IgM) was replaced by IgG. It was expected instead that fluorescence should be more intense (Meridian, pers. comm.).

For the Meridian Merifluor™ DFA - *combo* stain (methods 3 and 4) it appears that not concentrating the *Giardia* cysts gave better results ie only 20% false negatives compared to the 50% using 1.0 M sucrose flotation prior to staining.

Methods 4 and 5 did not involve *Giardia* cyst concentration, instead method 4 used a faecal suspension and method 5 required that faeces be fixed in PVA first, smeared on a glass slide and trichrome stained. The Meridian Merifluor™ DFA - *combo* stain, a specific stain, was better than the trichrome stain at detecting *Giardia* cysts. This may be due to the PVA fixative reducing the number of cysts present in faeces by a factor of 10^2-10^3 (see 3.10.1.).

Using the Cellabs - *Giardia* CELISA test kit, it was not necessary for *Giardia* cysts to be present in the faecal sample, as with the Prospect-65 kit. This kit detects antigens of molecular weights 30,000 and 60,000 which are major antigens shared by both the cyst and trophozoite form of *Giardia*. The colour reaction is measured visually or with a spectrophotometer making for a quick and easy interpretation, as with the Prospect-65 kit. This Cellab kit was better with only 10% false negatives (method 6), the best result gained from all the methods used.

It was expected the ELISA tests would be best as the presence of cysts were not required to give a positive result. Also the ELISA kits were the easiest tests to perform with the results read by eye using a colour card to indicate positive results.

The least satisfactory method used, giving 60% false negatives was method 1 using 1.0 M sucrose flotation and Lugols iodine to stain the cysts. Lugols iodine is a non-specific stain that stains *Giardia* cysts the same colour as surrounding debris. It is worthy of note that many, if not most, diagnostic laboratories in New Zealand use Lugols Iodine and not the other more specific methods described in 2.4. (Walker *et al*, 1991). Overall the best method of detection was to have a faecal suspension and test with the Cellab CELISA kit.

4.4. Sucrose Flotation Reviewed

The reason for not using the brakes during 1.0 M Sucrose flotation is so the interface where the cysts are suspended is not disturbed. This study found there was no significant difference between having the brake on and having the brake off during centrifugation. The results showed careful handling of tubes after centrifugation without brakes and taking care not to bump tubes could give up to a 74% recovery of cysts. On the other hand, use of the brake would save much time and the average recovery was only slightly lower than without the brakes.

For reasons of consistency (a smaller standard deviation) and speed, the brakes were used for the rest of this study.

4.5. New Zealand Animals Surveyed

This survey covered any animal samples that were donated by DSIR, Land Resources Division, and many other people donating samples in the interest of science. This was not intended to be an intensive study of individual animal species but just to give an idea of which animals in New Zealand, whether they be domestic or wild, could be potential reservoirs for *Giardia*. Marino *et al* (1992) looked at the prevalence of *Giardia* in possums, house mice and ship rats in various parts of the North Island of New Zealand. The results used for that publication were derived from this study. Neither the possums, whose behaviour had been intensely studied, or the mice were considered to have been infected by contaminated water and they would not pass faeces directly into water sources. It would be likely that the current level of infection (12.9% in possums, 25.3% in mice) was maintained within the population. 61% of the ship rats surveyed were also infected. Each animal has its own territorial range within which it tends to stay so individuals may not spread *Giardia* very long distances, but as populations tend to overlap from one area to another, one species could be considered a good source of *Giardia* throughout the island. It is important to remember when surveying animals species for a particular infection that there are many other species that could also be reservoirs for *Giardia*. One animal not surveyed in depth by this study is the duck, ie wild ducks that fly around New Zealand skies a particular times of the year, going from one water source to another, drinking and defecating into them. It would be of interest to determine in the future if the duck was a reservoir for human *Giardia* due to their behaviour. This study found none of the ducks surveyed were infected with *Giardia*, but only five were tested. Other aquatic birds of course may prove to be important reservoirs in line with the beaver of USA. The Great Blue Heron (*Ardea herodias*) could be a source of infection though the *Giardia* they harbour has been suggest to be a separate species (Erlandsen *et al*, 1990) from the three listed in the introduction of this study suggested by Filice, 1952 (section 1.1.3.)

Buret *et al* (1990) studied the prevalence of infection in sheep (17.7%) and cattle (10.4%). Infection was found to be significantly higher in the young of these animals (35.6% in lambs and 27.7% in calves). *Giardia* isolated from these sheep were successfully cultured in TY1-S-33 medium. Upon analysis of immunological, morphological and clinical symptoms of host animals, Buret *et al* (1990) postulated that domestic ruminants may be a reservoir for human infection and vice versa, classifying giardiasis as a zooanthroponotic disease. This is significant for dairy and beef farmers in particular who may be regularly exposed to transmission via faeces. In India calves aged one to six months old were surveyed, 51.6% tested positive for *Giardia* and calves three months old had the highest rate of infection (Deshpande and Shastri, 1981). Some calves were suffering from diarrhoea but responded to treatment with furazolidone compounds. This study found 7/32 (21.9%) of cattle surveyed on one farm were positive for *Giardia* but showed no clinical symptoms and appeared in good health.

Tonks *et al* (1991) surveyed dogs and cats in Palmerston North and Hamilton. In Palmerston North 7.7% of dogs and 6.7% of cats were infected with *Giardia* (n = 142 and 120 respectively). In Hamilton 24.7% of dogs and 2.8% of cats were infected with *Giardia* (n = 81 and 72 respectively). Being closely associated with people, these animals have in the past been indicated as possible sources of infection as discussed in the introduction (section 1.5.) so when people are treated for giardiasis is would be wise to also treat any household or working animals closely associated with infected people. Swan and Thompson (1986) surveyed dogs and cats in Perth, Western Australia. There was a high prevalence of infection with 21% of 333 dogs and 14% of 226 cats surveyed. Prevalence was higher in young animals. These animals were considered as possible sources of infection, especially in lower social-economic areas with over crowding of people and animals and poor sanitation.

Walker *et al* (1991) surveyed medical laboratories in New Zealand to collect information of the prevalence of giardiasis in people throughout this country and laboratory testing methodology. Though information was limited as giardiasis is not a notifiable disease, it did not support the hypothesis that water is the major route of transmission of giardiasis in New Zealand. The same has been stated for transmission in Australia (P. Boreham, pers. comm.).

When surveying animals for *Giardia*, it would be necessary to note whether the animals have a close association with water ways, humans or other animals to try and determine modes of transmission. This study has gone some way in determining which animals in New Zealand are infected by *Giardia*. Over the years several animals have been determined to harbour *Giardia intestinalis* type organisms. A small sample of these are budgerigars in Japan; Coyotes, beavers, raccoons, Cottontails and the Great Blue Heron in America, the Mongolian gerbil, goats and horses and the Ox (Davies and Hibler, 1979b; Erlandsen *et al*,

1990; Hirai *et al*, 1980; Wallis and Wallis, 1986; Of all the animals known to harbour *Giardia*, only beaver and mule deer have been shown to be possible reservoirs for human infection (Davies and Hibler, 1979). It is expected by this author that the number of animals determined to harbour animals would increase with and increase in the number and variety of animals surveyed for the presence of *Giardia*.

4.6. Methods Used to Differentiate Species

The *Giardia muris* cysts were stored in 10% formalin for more than 26 weeks before being measured. It was possible that the cysts had increased in specific gravity. As they cannot increase their dry weight content it is possible that they decreased their volume ie they shrunk, resulting in an increase in density.

Upon statistical analysis there was significant difference between the four groups of cysts whose dimensions were measured. There was significant difference between the *Giardia intestinalis* cysts used for Section 2.6.1. isolated from a single human and the other *G. intestinalis* cysts isolated from a variety of people. There was also significant difference between the cysts isolated from mice, ie the cysts used in the Meridian Merifluor™ - *Giardia* positive control and *G. muris* isolated from mice in the laboratory. Each population measured differed significantly from each other, even though they may have been of the same species. There are significant differences between populations of *Giardia intestinalis* when harvested from different human hosts which would be due to variation within the *Giardia* species. The assumption could be made that each time you collected *Giardia* cysts from a single host that there would be significant differences between each population sample therefore it would not be possible to differentiate between different species as it was not possible to establish a range within which the dimensions of a particular species was most likely to be.

4.7. Culturing *Giardia muris* in the Mouse

Using adult mice, previously free from *Giardia* infection, cultivation of *G. muris* was of limited success. As long as no young were born into the group there was a steady decrease in the density of the *Giardia* cysts shed in faeces over the weeks. Group 1 shed cysts for only 5 weeks. After being treated with Flagyl and reinoculated with *G. muris* cysts, reinfection lasted less than a week. These results indicated acquired resistance to recolonisation by *G. muris* in the gut. Group 2 mice while not having as quick a decrease in the density of cysts shed in their faeces, also had a slight increase in the amount of cysts shed once young had been born. This may indicate an impaired immune system in lactating females or that cysts are being reintroduced in very large quantities resulting in an increase in the number shed. It is possible that the baby mice act as in/out pipes with cysts going in

their mouths, straight out of their gut without colonising the upper intestines and back into the females intestinal tract when they clean their young stimulating them to pass faeces.

When attempts were made to isolate faeces from young born to females infected with *G. muris* it was found impossible to detect cysts in their faeces until they had been weaned, ie two to three weeks after birth. It was not possible to collect faeces from baby mice as anal stimulation was required. When cysts were collected from weaned mice by floating the cysts on 1.0 M sucrose gradient, the suspension was clear of debris. After a few weeks, however, there was an increase in the amount of fatty tissue shed in the faeces increasing the difficulty of collecting cysts from the interface. The fatty tissue would block the pipette used to suck up cysts in the interface and give cyst suspensions thick with undesirable debris. In Groups 3-7, ie mice born to females with a *G. muris* infection, there was initially a much greater density in the number of cysts shed in faeces and the density did not fall to zero in five weeks as it did with adult mice. Early infection by *G. muris* could lead to a life long infection, especially if the presence of young mice resulted in continued retention of the infection.

This study concluded that inoculating weaned mice that go on to reproduce, resulting in young mice shedding a high density of *G. muris* cysts in their faeces is the best way to cultivate *G. muris* in laboratory mice when large quantities of cysts are required for experimental work.

4.8. Establishing Viability

The two methods of Feely's were compared. The method of Feely, 1991, yielded 32.94% viability at 15 minutes., the time it was supposed to yield maximum excystation percent. There was no significant difference between the percent cysts excysted at 15 minutes and the percent obtained after 24 hours incubation. The method of Feely 1986 yielded 50.73% at 30 minutes, which was not significantly different from the percent obtained after 24 hours incubation. Neither yielded excystation greater than 90% that both papers talk about, which may be due to technical error or just to the fact that not all the cysts were viable and/or mature enough to excyst. Though the method of Feely 1991 was easier to prepare and quicker to perform, it did not yield as high a percent cyst excystment as did the method of Feely 1986. The percent cysts excysted after 30 minutes incubation using the method of Feely 1986 was significantly higher than the percent cysts excysted after 15 minutes using the method of Feely 1991. For this reason the method of Feely 1986 was used in the following experiments to determine viability as this author considered it to be the more sensitive method. The results for the Feely 1991 method yielded no significant difference in the percent cysts excysted no matter how long the cysts were incubated for after excystment was induced, ranging from zero time to 24 hours incubation. This would indicate that even 15 minutes was not necessary for maximum excystation to occur (Figure 12).

The easiest method by far to use to determine viability in *Giardia* not analysed in this study was the use of Nomarski optics. This requires very little preparation as the cysts only have to be placed on a glass slide in a drop of solution (water, TDW or PBS), a coverslip placed over the drop and cysts viewed under the microscope.

4.9. Environmental Effects on *Giardia* Cyst Presence and Viability

When *Giardia* cysts were stored at -20°C only *G. intestinalis* cysts cultured *in vitro* survived for any period of time and remained viable. These cysts were not exposed to the intestinal or other environmental effects that *G. muris* cysts cultured in mice would have been exposed to though whether this would have an effect on the results was not known. The *G. muris* cysts isolated on 1.0 M sucrose gradient, washed and suspended in water survived storage at -20°C for slightly longer than *G. muris* cysts suspended in faeces and water. It appears that the presence of faeces was detrimental to the survival of the cysts at -20°C.

Giardia cysts stored at 4°C also had a varied fate depending on whether they were exposed to faecal matter or not. For both *G. intestinalis* cysts suspended in human faeces and *G. muris* cysts suspended in mouse faeces and stored for 68 days, the percent of cysts recovered was significantly lower than for the cysts that were isolates free of almost all faecal material. *G. intestinalis* cysts cultured *in vitro*, and having never been exposed to the environment or to faecal matter, looked to be capable of surviving indefinitely by simple storage in sterile water at 4°C. The cysts suspended in faecal material may be degraded faster due to the presence of coliforms in the faeces which may act to degrade the cysts. Cysts isolated into water can remain viable for months and at remain present in high numbers. It may be that cysts that find themselves in running water eg a mountain stream, could last longer than when stored in a universal as they would be continuously washed with fresh clean water and they may remain viable for even longer periods of time. For the *G. intestinalis* cysts isolated from human faecal samples, the cysts suspension still contained a substantial amount of faecal material, however it seems that any reduction in the amount of faecal material present results in a higher percent cyst recovery over a period of time.

For *G. muris* cysts incubated at 20°C in the presence of water, the absence of faecal material again significantly extended the period of time the cysts remained detected and viable. For those cysts that were left in the faeces in sawdust (Section 2.10.4.) it appeared that the effects of the faeces drying out were enough to destroy all cysts detectable in all but two samples tested. As it was, in the samples that did have cysts detected, only one or two cysts were actually seen.

For the *G. muris* cysts in faeces at 37°C the results varied depending on whether or not the faeces dried out. As soon as the faeces appeared to be dry, no cysts were detected. For the

G. muris cysts stored in water, the absence/reduction of faecal material significantly extended the period of time the cysts remained detected though viability was only detected up to day 6 of storage.

It would appear that the most important factor when considering the survival of *Giardia* cysts in the environment is whether the cysts are suspended in water. Upon the water evaporating and exposing the cyst, cysts collapse leaving the cyst wall, axonemes and nucleus in relief (an observation made during this study).

The next important consideration is whether or not the cysts are suspended in clean water or exposed to faecal material. Cysts suspended in water free of faecal material were viable and detectable for a longer period than those cysts exposed to faecal material. Deng and Cliver (1992) found that the number and viability of *G. intestinalis* cysts mixed in waste, septic tank effluent both decreased 90% in a shorter period of time than control cysts suspended in PBS. They also found that increasing the proportion of swine manure slurry in mixed waste favoured the degradation of the cysts.

When considering the effect of temperature, it appears that warm temperatures resulted in a larger decrease in the percent of cysts surviving. When cysts were stored at -20°C the results varied. *G. intestinalis* cultured *in vitro* looked like they could survive for an indefinite period though their viability only lasted in one case one day, in another case 63 days. Cysts incubated at 4°C had the best survival rate with respect to viability and the length of time cysts were detected. De Regnier *et al*, 1989, suspended *G. muris* cysts within faecal pellets in lake, river, tap water and distilled water (stored at 4°C). During winter, with decreased temperatures, cysts in river and lake waters lasted as long as cysts stored at 4°C exposed to distilled water ie 56 to 84 days. This is compared to the results of this study with viability detected, using the method of Feely 1986, from 28 to 63 days. De Regnier *et al* (1989) compared the water quality parameters with their viability results and found that only decreased water temperatures (<10°C) was consistent with prolonged survival of *G. muris* in the different types of water compared.

Thus the crystal clear cold, clean mountain streams we have in New Zealand are ideal for the survival and transmission of viable *Giardia* cysts.

Analysis was done with ANOVA at 95% confidence levels.

4.10. Preservation of *Giardia* Cysts

When *Giardia muris* cysts in mice faeces or freshly isolated into water were exposed to PVA there was immediate destruction of a large proportion of the cysts present. Whether the cysts were suspended in faecal matter or not was not significant but the fact that up to 90% of the cysts are destroyed within 30 minutes of exposure to PVA is important. Especially when the method of fixing faeces with PVA for staining and microscopic

examination is commonly used in diagnostic laboratories throughout New Zealand. This means a high number of faecal may test negative for the presence of *Giardia* because the cysts present may have been destroyed by the PVA fixation method used. After 60 days of storage there was significant difference between the recovery of cysts suspended in mouse faeces and those cysts isolated from mouse faeces.

There was significant differences between the recovery of cysts fixed in 10% formalin. The recovery *G. intestinalis* cysts suspended in human faeces and *G. muris* cysts suspended in mouse faeces and fixed were both significantly different from cysts isolated from faecal material and fixed. The presence of faecal material interfered with the length of time *Giardia* cysts could be stored for with a significant decrease in the recovery of cysts from storage. It could be that a higher concentration of formalin was required to fix other organisms present that may destroy *Giardia* cysts directly through parasitism or indirectly due to metabolic by-products released into the environment. There was no significant difference in percent cyst recovery from storage between *G. intestinalis* cysts isolated from humans and *G. muris* cysts isolated from mice. There was significant difference between *G. intestinalis* cultured *in vitro* and *G. intestinalis* isolated from human faeces and suspended in 10% formalin. *G. intestinalis* cysts cultured *in vitro* did not survive as well as was expected when stored in 10% formalin. For this reason storage of *in vitro* cysts is only recommended for a few months.

The percent cysts recovery for *G. intestinalis* cultured *in vitro* was compared between storage in water, before and after exposure to 0.1% SDS, and in 10% formalin at 4°C. Only between storage in 10% formalin and treatment with SDS was there no significant difference. Therefore, if *in vitro* cysts were required to be stored it may be of use to destroy the trophozoites and incompletely formed cysts first and then fix in 10% formalin, otherwise storage in sterile milli-Q water is recommended as the percent of cyst recovery over long periods of time is significantly better.

Cryopreservation may be highly destructive but it allows for long term preservation (years as apposed to months) of viable cysts for future scientific research. Fixation in osmium tetroxide allowed for fixation of the cysts without distorting the internal morphology as was demonstrated with PVA fixation (Plate 3).

Overall, the least expensive method for storage was to isolate *Giardia* cysts from faecal material, wash the suspension so it was as free of faecal material as it could be and store at 4°C. This allowed for the long term survival of viable cysts.

APPENDIX A. DATA FOR THE RESULTS

Data for 3.1. Separation of *In Vitro Giardia* Cysts from Trophozoites by Sonication

Sonication of 10^5 cysts/ml

Table 13: Data for recovery of 10^5 *in vitro* cysts from trophozoites and incompletely formed cysts by sonicating a suspension at 5μ (27.5μ).

C_0 (cysts/ml)	ST (mins)	C_1 (cysts/ml)	% CR	IC	Av ± SD
1.14×10^5	1.0	1.04588×10^5	91.61	+	
1.13×10^5	1.0	1.04651×10^5	92.34	+	91.97 ± 0.52
1.14×10^5	1.5	6.9995×10^4	61.31	+	
1.13×10^5	1.5	8.7084×10^4	76.84	+	69.07 ± 10.98
1.14×10^5	2.0	6.5417×10^4	57.30	+	
1.13×10^5	2.0	8.2495×10^4	72.79	+	65.05 ± 10.96

Table 14: Data for recovery of 10^5 *in vitro* cysts from trophozoites and incompletely formed cysts by sonicating a suspension at 6μ (33μ).

C_0 (cysts/ml)	ST (mins)	C_1 (cysts/ml)	% CR	IC	Av ± SD
1.34×10^5	1.0	8.31×10^4	62.13	+	
1.41×10^5	1.0	1.05×10^5	74.04	+	68.08 ± 8.42
1.26×10^5	1.5	7.25×10^4	57.53	+	
1.41×10^5	1.5	8.17×10^4	57.82	+	57.68 ± 0.20
2.77×10^5	2.0	7.19×10^4	40.7	+	
1.41×10^5	2.0	6.29×10^4	44.54	+	42.62 ± 2.71

Table 15: Data for recovery of 10^5 *in vitro* cysts from trophozoites and incompletely formed cysts by sonicating a suspension at 7μ (38.5μ)

C_0 (cysts/ml)	ST (mins)	C_1 (cysts/ml)	% CR	IC	Av ± SD
1.26×10^5	1.0	7.7081×10^4	61.33	+	
1.07×10^5	1.0	8.4170×10^4	78.91	+	77.02 ± 14.07
1.26×10^5	1.5	8.04×10^4	63.98	+	
1.07×10^5	1.5	5.83×10^4	54.69	+	57.18 ± 16.43
1.26×10^5	2.0	6.17×10^4	49.07	+	
1.07×10^5	2.0	5.63×10^4	52.73	+	51.30 ± 17.97

Key for Tables 13-15:

C_0 = original concentration of *Giardia* cysts; ST = sonication time; mins = minutes; C_1 = final concentration of *Giardia* cysts; CR = cysts recovered after sonication; IC = presence or absence of incompletely formed *Giardia* cysts; Av = average of % CR; SD = standard deviation; μ = the movement peak-to-peak that is taking place at the transducer/probe interface, at 5μ the probe tip moves 27.5μ (5.5 x 5).

Sonication of 10^6 cysts/ml

Table 16: Data for recovery of 10^6 *in vitro* cysts from trophozoites and incompletely formed cysts by sonicating at 5μ (27.5μ).

C_0 (cysts/ml)	ST (mins)	C_1 (cysts/ml)	% CR	Tz	IC	Av ± SD
8.02×10^5	1.0	7.92×10^5	98.75	–	+	
9.95×10^5	1.0	8.55×10^5	85.85	+	+	92.30 ± 9.12
8.02×10^5	1.5	6.39×10^5	79.73	–	+	
9.95×10^5	1.5	6.46×10^5	64.88	+	+	72.31 ± 10.50
8.02×10^5	2.0	5.06×10^5	63.15	–	+	
9.95×10^5	2.0	52.54×10^5	52.78	+	+	57.97 ± 7.33

Table 17: Data for recovery of 10^6 *in vitro* cysts from trophozoites and incompletely formed cysts by sonicating at 6μ (33μ)

C_0 (cysts/ml)	ST (mins)	C_1 (cysts/ml)	% CR	Tz	IC	Av ± SD
1.13×10^6	1.0	7.55×10^5	67.07	+	+	
8.35×10^5	1.0	7.26×10^5	86.97	+	+	77.02 ± 14.07
1.13×10^6	1.5	5.13×10^5	45.56	–	+	
8.35×10^5	1.5	5.74×10^5	68.80	–	+	57.18 ± 16.43
1.13×10^6	2.0	4.34×10^5	38.59	–	+	
8.35×10^5	2.0	5.34×10^5	64.00	–	+	51.30 ± 17.97

Table 18: Data for recovery of 10^6 *in vitro* cysts from trophozoites and incompletely formed cysts by sonication at 7μ (38.5μ).

C_0 (cysts/ml)	ST (mins)	C_1 (cysts/ml)	% CR	Tz	IC	Av ± SD
6.92×10^5	1.0	6.01×10^5	86.93	+	+	
7.63×10^5	1.0	7.85×10^5	100.00	–	+	93.47 ± 9.24
6.92×10^5	1.5	4.45×10^5	64.40	–	+	
7.63×10^5	1.5	5.62×10^5	73.62	–	+	69.01 ± 6.52
6.92×10^5	2.0	3.74×10^5	54.10	–	+	
7.63×10^5	2.0	4.87×10^5	63.84	–	+	58.97 ± 6.89

Key for Tables 16-18:

C_0 = original concentration of *Giardia* cysts; ST = sonication time; mins = minutes; C_1 = final concentration of *Giardia* cysts; CR = cysts recovered after sonication; IC = presence or absence of incompletely formed *Giardia* cysts; Av = average of % CR; SD = standard deviation; Tz = presence or absence of *Giardia* trophozoites; μ = the movement peak-to-peak that is taking place at the transducer/probe interface, at 5μ the probe tip moves 27.5μ (5.5 x 5).

Data for 3.2. Techniques to Recover and Detect *Giardia* Cysts from Environmental and Laboratory Samples

Giardia cysts recovered from domestic animals

The results for the first 25 samples compared:

Sample	1 M Sucrose (C/g)	Percoll-sucrose (C/g)	Sheather-sucrose (C/g)	Zinc Sulphate (C/g)
1	0	0	243	0
2	128	0	0	5
3	185	2.34×10^3	0	0
4	196	0	353	5
5	210	0	0	0
6	246	740	289	0
7	256	1.58×10^3	0	0
8	295	379	0	0
9	361	0	0	0
10	400	0	0	14
11	454	344	0	6
12	634	1.27×10^3	1.31×10^3	13
13	746	1.49×10^3	0	203
14	761	190	5.22×10^3	32
15	781	0	0	4
16	800	0	0	0
17	856	183	0	4
18	895	895	0	0
19	2.61×10^3	3.48×10^3	869	25
20	4.02×10^3	0	250	0
21	4.54×10^3	1.94×10^3	126	0
22	5.00×10^3	1.00×10^4	1.00×10^4	3
23	5.42×10^3	933	2.09×10^3	125
24	2.65×10^4	1.10×10^3	1.34×10^4	0
25	2.53×10^5	1.82×10^5	1.91×10^5	87

Key: C/g = *Giardia* cysts/gram faecal sample

For the total 125 samples compared with 3 flotation mediums:

Dogs

Sample	Stain	Sucrose	Percoll-Sucrose	Sheather-Sucrose
1	Meridian-*Giardia*	**4020**	0	250
2	Meridian-*Giardia*	**26519**	1104	13442
3	Meridian-*Giardia*	761	190	**5217**
4	Meridian-*Giardia*	2608	**3478**	869
5	Meridian-*Giardia*	**252982**	181578	191290
6	Meridian-*Giardia*	5000	**10000**	**10000**
7	Meridian-*Giardia*	82478	**473166**	233194
8	Meridian-*Giardia*	25139	**310881**	232663
9	Meridian-*Giardia*	88	0	**859**
10	Meridian-*Giardia*	560	484	**835**
11	Meridian-*Combo*	**26341**	2512	0
12	Meridian-*Combo*	**27256**	18293	13278
13	Meridian-*Combo*	571	**16571**	760
14	Meridian-*Combo*	0	**869**	434
15	Meridian-*Combo*	228421	**318421**	146774
16	Meridian-*Combo*	0	**714**	357
17	Meridian-*Combo*	83898	**335600**	223105
18	Meridian-*Combo*	162	**308292**	251584
19	Meridian-*Combo*	0	0	**114**
20	Meridian-*Combo*	2298	**54666**	1985
21	Cellab-*Giardia*	**1005**	0	0
22	Cellab-*Giardia*	**5156**	736	1803
23	Cellab-*Giardia*	1714	**2285**	0
24	Cellab-*Giardia*	1739	0	**2173**
25	Cellab-*Giardia*	**100000**	7368	48709
26	Cellab-*Giardia*	**714**	0	**714**
27	Cellab-*Giardia*	67971	16583	**70626**
28	Cellab-*Giardia*	10573	40869	**90792**
29	Cellab-*Giardia*	59	143	**1661**
30	Cellab-*Giardia*	**3772**	2533	940
31	J Chan- PAb	**22613**	0	250
32	J Chan- PAb	**49723**	4051	12131
33	J Chan- PAb	**2285**	**2285**	869
34	J Chan- PAb	869	1739	**2608**
35	J Chan- PAb	**387368**	295263	198064
36	J Chan- PAb	5000	**6428**	1785
37	J Chan- PAb	85521	**506166**	260478
38	J Chan- PAb	13163	**327637**	304811
39	J Chan- PAb	29	0	**229**
40	J Chan- PAb	3301	**80400**	61
%Highest Recovery		**14/40 (35.0%)**	**17/40 (42.5%)**	**11/40 (27.5%)**

Sheep

Sample	Stain	Sucrose	Percoll-Sucrose	Sheather-Sucrose
1	Meridian-*Giardia*	0	0	**243**
2	Meridian-*Giardia*	634	**1269**	1311
3	Meridian-*Giardia*	185	**2340**	0
4	Meridian-*Giardia*	**361**	0	0
5	Meridian-*Giardia*	**454**	344	0
6	Meridian-*Giardia*	256	**1578**	0
7	Meridian-*Giardia*	**400**	0	0
8	Meridian-*Giardia*	**895**	**895**	0
9	Meridian-*Giardia*	**800**	0	0
10	Meridian-*Giardia*	246	**740**	289
11	Meridian-*Giardia*	**4537**	1944	126
12	Meridian-*Giardia*	59	93	**217**
13	Meridian-*Giardia*	**1737**	1413	0
14	Meridian-*Combo*	0	0	**243**
15	Meridian-*Combo*	**634**	0	163
16	Meridian-*Combo*	0	553	**3750**
17	Meridian-*Combo*	120	0	**535**
18	Meridian-*Combo*	151	**1379**	800
19	Meridian-*Combo*	133	**769**	0
20	Meridian-*Combo*	0	0	**500**
21	Meridian-*Combo*	0	0	**144**
22	Meridian-*Combo*	555	0	**1304**
23	Meridian-*Combo*	**295**	93	217
24	Meridian-*Combo*	1026	**1177**	58
25	Cellab-*Giardia*	**1269**	0	163
26	Cellab-*Giardia*	185	**1276**	0
27	Cellab-*Giardia*	**722**	0	0
28	Cellab-*Giardia*	**454**	0	400
29	Cellab-*Giardia*	256	**1052**	0
30	Cellab-*Giardia*	**666**	192	0
31	Cellab-*Giardia*	**2187**	0	0
32	Cellab-*Giardia*	**2068**	0	0
33	Cellab-*Giardia*	0	**277**	144
34	Cellab-*Giardia*	**3777**	0	0
35	Cellab-*Giardia*	59	93	**217**
36	Cellab-*Giardia*	671	**969**	58
37	J Chan - PAb	185	638	0
38	J Chan - PAb	120	**217**	0
39	J Chan - PAb	266	**384**	0
40	J Chan - PAb	**298**	0	0
41	J Chan - PAb	740	277	**1449**
42	J Chan - PAb	**9166**	666	126
43	J Chan - PAb	59	279	**652**
44	J Chan - PAb	**1184**	471	117
%Highest Recovery		**19/44 (43.18%)**	**14/44 (31.82%)**	**11/44 (25.00%)**

Cattle

Sample	Stain	Sucrose	Percoll-Sucrose	Sheather-Sucrose
1	Meridian-*Giardia*	**128**	0	0
2	Meridian-*Giardia*	196	0	**353**
3	Meridian-*Giardia*	**5422**	933	2093
4	Meridian-*Giardia*	**9477**	2867	2514
5	Meridian-*Giardia*	2690	**7800**	2218
6	Meridian-*Giardia*	**10440**	0	4161
7	Meridian-*Giardia*	**6113**	0	1529
8	Meridian-*Combo*	0	0	**114**
9	Meridian-*Combo*	533	0	**1627**
10	Meridian-*Combo*	**6176**	1729	771
11	Meridian-*Combo*	1563	743	**2654**
12	Meridian-*Combo*	**7860**	3801	4617
13	Meridian-*Combo*	**5886**	12	2103
14	Cellab-*Giardia*	**416**	0	0
15	Cellab-*Giardia*	**1714**	0	0
16	Cellab-*Giardia*	**5932**	0	852
17	Cellab-*Giardia*	**7652**	2776	2514
18	Cellab-*Giardia*	**2436**	875	2218
19	Cellab-*Giardia*	3060	3181	**4161**
20	Cellab-*Giardia*	**5170**	0	1529
21	J Chan-PAb	128	**208**	0
22	J Chan-PAb	**506**	0	88
23	J Chan-PAb	**13200**	1355	2015
24	J Chan-PAb	**9477**	1274	1085
25	J Chan-PAb	2218	525	**2298**
26	J Chan-PAb	**8340**	3925	6130
27	J Chan-PAb	**6471**	1864	2613
%Highest Recovery		**19/27 (40.74%)**	**2/27 (3.70%)**	**6/27 (7.41%)**
%Highest Recovery for all Animals	**125 Animals Tested**	**59/125 (47.20%)**	**38/125 (30.40%)**	**30/125 (24.00%)**

Hen

Sample	Stain	Sucrose	Percoll-Sucrose	Sheather-Sucrose
1	Meridian-*Giardia*	**781**	0	0
2	Meridian-*Giardia*	746	**1492**	0
3	Meridian-*Giardia*	295	**379**	0
4	Meridian-*Giardia*	**856**	183	0
5	Meridian-*Giardia*	**210**	0	0
6	Meridian-*Giardia*	51	0	**98**
7	Meridian-*Combo*	0	0	**277**
8	Meridian-*Combo*	0	**2315**	129
9	Meridian-*Combo*	**153**	0	98
10	Cellab-*Giardia*	**160**	0	0
11	Cellab-*Giardia*	**142**	0	0
12	Cellab-*Giardia*	0	**101**	0
13	J Chan- PAb	0	**2238**	0
14	J Chan- PAb	**153**	101	0
%Highest Recovery		**7 (50.0%)**	**5 (35.71%)**	**2 (14.29%)**

The detection of *Giardia* cysts with different stains

The following tables gives a comparison of stains used. Samples are listed in the order of animals first: dog, sheep, cattle and hen. Then they are listed in order of the flotation used to recover *Giardia* cysts from the faecal samples used in this study. The stain J Chan PAb is a polyclonal stain developed by Judy Chan of Massey University. The rest are IFT kits.

Dog

Sample	Flotation	Meridian™ Merifluor-*Giardia*	Meridian™ Hydrofluor-*Combo*	Cellab-*Giardia*	J Chan-PAb
1.	Sucrose	4020	**26341**	1005	22613
2	Sucrose	26519	27256	5156	**49723**
3	Sucrose	761	571	1714	**2285**
4	Sucrose	**2608**	0	1739	869
5	Sucrose	252982	228421	100000	**387368**
6	Sucrose	**5000**	0	714	**5000**
7	Sucrose	82478	83898	67971	**85521**
8	Sucrose	**25139**	162	10573	13163
9	Sucrose	**88**	0	59	29
10	Sucrose	560	2298	**3772**	3301
11	Percoll-Sucrose	0	**3768**	0	0
12	Percoll-Sucrose	1104	**18293**	736	4051
13	Percoll-Sucrose	190	**16571**	2285	2285
14	Percoll-Sucrose	**3478**	869	0	1739
15	Percoll-Sucrose	181578	**318421**	7368	295263
16	Percoll-Sucrose	**10000**	714	0	6428
17	Percoll-Sucrose	473166	335600	16583	**506166**
18	Percoll-Sucrose	310881	308292	40869	**327637**
19	Percoll-Sucrose	0	0	**143**	0
20	Percoll-Sucrose	484	54666	2533	**80400**
21	Sheather-Sucrose	**250**	0	0	**250**
22	Sheather-Sucrose	**13442**	13278	1803	12131
23	Sheather-Sucrose	**5217**	760	0	869
24	Sheather-Sucrose	869	434	2173	**2608**
25	Sheather-Sucrose	191290	146774	48709	**198064**
26	Sheather-Sucrose	**10000**	1785	714	1785
27	Sheather-Sucrose	233194	223105	70626	**260478**
28	Sheather-Sucrose	232663	251584	90792	**304811**
29	Sheather-Sucrose	859	114	**1661**	229
30	Sheather-Sucrose	835	**1985**	940	61
Total		**10/30**	**6/30**	**3/30**	**13/30**

Sheep

Sample	Flotation	Meridian™ Merifluor-*Giardia*	Meridian™ Hydrofluor-*Combo*	Cellab-*Giardia*	J Chan-PAb
1	Sucrose	634	634	**1269**	0
2	Sucrose	**185**	0	**185**	**185**
3	Sucrose	361	120	**722**	120
4	Sucrose	**454**	151	**454**	0
5	Sucrose	**256**	0	**256**	0
6	Sucrose	400	133	**666**	266
7	Sucrose	895	0	**2187**	298
8	Sucrose	800	0	**2068**	0
9	Sucrose	246	0	0	**740**
10	Sucrose	4537	555	3777	**9166**
11	Sucrose	59	**295**	59	59
12	Sucrose	**1737**	1026	671	1184
13	Percoll-Sucrose	**1269**	0	0	0
14	Percoll-Sucrose	**2340**	553	1276	638
15	Percoll-Sucrose	0	0	0	**217**
16	Percoll-Sucrose	344	**1379**	0	0
17	Percoll-Sucrose	**1578**	0	1052	0
18	Percoll-Sucrose	0	**769**	192	384
19	Percoll-Sucrose	**895**	0	0	0
20	Percoll-Sucrose	**740**	0	277	277
21	Percoll-Sucrose	**1944**	0	0	666
22	Percoll-Sucrose	93	93	93	**279**
23	Percoll-Sucrose	**1413**	1177	969	471
24	Sheather-Sucrose	**243**	**243**	0	0
25	Sheather-Sucrose	**1311**	163	163	0
26	Sheather-Sucrose	0	**3750**	0	0
27	Sheather-Sucrose	0	**535**	0	0
28	Sheather-Sucrose	0	**800**	400	0
29	Sheather-Sucrose	0	**500**	0	0
30	Sheather-Sucrose	289	144	144	**1449**
31	Sheather-Sucrose	126	**1304**	0	126
32	Sheather-Sucrose	217	217	217	**652**
33	Sheather-Sucrose	0	58	58	**117**
Total	**Sheather-Sucrose**	**13/33**	**9/33**	**8/33**	**8/33**

Cattle

Sample	Flotation	Meridian™ Merifluor-*Giardia*	Meridian™ Hydrofluor-*Combo*	Cellab-*Giardia*	J Chan-PAb
1	Sucrose	128	0	**416**	128
2	Sucrose	196	0	**1714**	506
3	Sucrose	5422	533	5932	**13200**
4	Sucrose	**9477**	6176	7652	**9477**
5	Sucrose	**2690**	1563	2436	2218
6	Sucrose	**10440**	7860	3060	8340
7	Sucrose	6113	5886	5170	**6471**
8	Percoll-Sucrose	0	0	0	**208**
9	Percoll-Sucrose	933	0	0	**1355**
10	Percoll-Sucrose	**2867**	1729	2776	1274
11	Percoll-Sucrose	**7800**	743	875	525
12	Percoll-Sucrose	0	3801	3181	**3925**
13	Percoll-Sucrose	0	12	0	**1864**
14	Sheather-Sucrose	0	**114**	0	0
15	Sheather-Sucrose	**353**	0	0	88
16	Sheather-Sucrose	**2093**	1627	852	2015
17	Sheather-Sucrose	**2514**	771	342	1085
18	Sheather-Sucrose	2218	**2654**	2100	2298
19	Sheather-Sucrose	4161	4617	3534	**5130**
20	Sheather-Sucrose	1529	2103	1720	**2613**
Total	**Sheather-Sucrose**	**8/20**	**2/20**	**2/20**	**9/20**

Hen

Sample	Flotation	Meridian™ Merifluor-*Giardia*	Meridian™ Hydrofluor-*Combo*	Cellab-*Giardia*	J Chan-PAb
1	Sucrose	**781**	0	0	0
2	Sucrose	**746**	0	0	0
3	Sucrose	**295**	0	160	0
4	Sucrose	**856**	0	142	0
5	Sucrose	**210**	0	0	0
6	Sucrose	51	**153**	0	**153**
7	Percoll-Sucrose	1492	0	0	**2238**
8	Percoll-Sucrose	**379**	0	0	0
9	Percoll-Sucrose	0	**2315**	0	0
10	Percoll-Sucrose	0	0	**101**	**101**
11	Sheather-Sucrose	0	**277**	0	0
12	Sheather-Sucrose	0	**129**	0	0
13	Sheather-Sucrose	**98**	**98**	0	0
Total	**Sheather-Sucrose**	**7/13**	**5/13**	**1/13**	**3/13**
Overall % Highest Number Stained		**38/96 (39.6%)**	**22/96 (22.9%)**	**14/96 (14.6%)**	**33/96 (34.4%)**

Data for 3.5. New Zealand Animals Surveyed

Blackbird

Location	Date(s)	Animals tested	Number positive	Percent positive
Dannevirke	15,16/8/91	2	0	00.00
Dannevirke	20/9/91	1	0	00.00
Dannevirke	4/12/91	1	1	100.00
Dannevirke	29-31/1/91	4	2	50.00
Total Blackbirds		**8**	**3**	**37.50%**

Cattle

Location	Date(s)	Animals tested	Number positive	Percent positive
Dannevirke	12/90	9	0	00.00
Dannevirke	11/4/91	11	3	27.27
Dannevirke	10/5/91	5	4	80.00
Dannevirke	23/1/92	5	0	00.00
Westland	13,25/8/91	2	0	00.00
Total Cattle		**32**	**7**	**21.88%**

Chaffinch

Location	Date(s)	Animals tested	Number positive	Percent positive
Dannevirke	15/8/91	1	0	00.00
Dannevirke	19/9/91	2	1	50.00
Total Chaffinch		**3**	**1**	**33.33%**

Cockatiel

Location	Date(s)	Animals tested	Number positive	Percent positive
Mt Stewart	13/9/91	1	0	00.00

Deer

Location	Date(s)	Animals tested	Number positive	Percent positive
Westland	16,20/8/91	2	1	50.00

Dog

Location	Date(s)	Animals tested	Number positive	Percent positive
Dannevirke[1]	12/90	3	3	100.00
Dannevirke	7/3/91	3	2	66.67
Dannevirke	11/4/91	4	4	100.00
Dannevirke	10/5/91	4	4	100.00
Dannevirke	13/6/91	4	4	100.00
Dannevirke	6/12/91	4	1	25.00
Dannevirke	23/1/92	4	4	100.00
Napier		5	2	40.00
Mt Stewart[2]	23/8/91	1	0	00.00
Mt Stewart	13/9/91	1	0	00.00
Total Dogs		**10**	**6**	**60.00%**

Duck

Location	Date(s)	Animals tested	Number positive	Percent positive
Massey	3/90	3	0	00.00
Lake Wairarapa	23/6/90	1	0	00.00
Westland	16/8/91	1	0	00.00
Total Ducks		**5**	**0**	**00.00%**

Ferret

Location	Date(s)	Animals tested	Number positive	Percent positive
Dannevirke	11/7/91	1	0	00.00

Goat

Location	Date(s)	Animals tested	Number positive	Percent positive
Mt Stewart	20/8/91	2	1	50.00
Mt Stewart	13/9/91	1	0	00.00
Total Goats		**3**	**1**	**33.33%**

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[1] Note that some dogs tested negative at some times, assumed all positive.

[2] Only one dog that tested negative.

Hedgehog

Location	Date(s)	Animals tested	Number positive	Percent positive
Dannevirke	4/12/91	1	0	00.00
Dannevirke	29-31/1/92	2	1	50.00
Total Hedgehogs		**3**	**1**	**33.33%**

Hedge-Sparrow

Location	Date(s)	Animals tested	Number positive	Percent positive
Dannevirke	30-31/1/92	1	0	00.00
Dannevirke	30/4/92	1	0	00.00
Total Hedge-Sparrows		**2**	**0**	**00.00%**

Hen

Location	Date(s)	Animals tested	Number positive	Percent positive
Dannevirke[3]	12/90	9	3	00.00
Dannevirke	7/3/91	4	2	50.00
Dannevirke	11/4/91	5	3	60.00
Dannevirke	10/5/91	6	1	11.11
Dannevirke	13/6/91	6	1	11.11
Mt Stewart[4]	20/8/91	5	0	00.00
Mt Stewart	13/9/91	1	0	00.00
Total Hens		**16**	**3**	**28.75%**

Horse

Location	Date(s)	Animals tested	Number positive	Percent positive
Palmerston North	28/8/91	1	1	100.00

Magpie

Location	Date(s)	Animals tested	Number positive	Percent positive
Dannevirke	31/1/92	1	1	100.00

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[3] Total animals 10 in the coop. Do not which individual animals are tested, so do not know if all where positive and which positive animals did not test positive ie false negatives.

[4] Total animals unknown in coop, all tested, tested negative.

Mouse

Location	Date(s)	Animals tested	Number positive	Percent positive
Hutt River	7-8/3/90	7	0	00.00
Hutt River	9/3/90	16	0	00.00
Hutt River	15-16/3/90	5	0	00.00
Hutt River	20-21/3/90	4	0	00.00
Hutt River	28-29/3/90	6	0	00.00
Wainui Golf Course	5-6/4/90	8	0	00.00
Wainui Golf Course	11-20/4/90	9	3	33.33
Wainui Golf Course	10-11/4/90	7	5	71.42
Orongorongo Valley	28/5/90	9	4	44.44
Orongorongo Valley	28-30/8/90	68	18	26.47
Orongorongo Valley	30/5/90	14	4	28.57
Orongorongo Valley	11/90	24	13	54.17
Orongorongo Valley	26-28/11/91	25	5	20.00
Dannevirke	29-30/5/91	9	3	33.33
Dannevirke	10-12/7/91	5	1	20.00
Dannevirke	14/8/91	4	0	00.00
Dannevirke	18-20/9/91	3	0	00.00
Dannevirke	4-6/12/91	1	0	00.00
Dannevirke	28-30/4/92	7	4	57.14
Belmont	18/3/90	2	0	00.00
Manawatu River	6-7/6/90	6	1	16.67
Holes Creek, Silverstream		6	2	33.33
Miramar, Auckland	9/11/90	2	0	00.00
Hastings	23/11/90	19	1	5.26
Total Mice		**263**	**60**	**22.81%**

Possum

Location	Date(s)	Animals tested	Number positive	Percent positive
Orongorongo Valley		34	2	5.88
Orongorongo Valley	2/10/90	24	5	20.83
Orongorongo Valley	12/2/91	23	5	21.74
Dannevirke	28-30/5/91	7	1	14.29
Dannevirke	10/7/91	11	6	54.55
Dannevirke	14-15/8/91	9	0	00.00
Dannevirke	18-20/9/91	5	0	00.00
Dannevirke	4-6/12/91	1	0	00.00
Dannevirke	28,29/4/92	3	1	33.33
Hastings	21/6/90	9	1	11.11
Otaki	22-23/8/90	2	0	00.00
Auckland	6-7/11/90	15	2	13.33
Hawkes Bay	21-22/11/90	3	0	00.00
Tinui	5/2/91	21	5	23.81
Hastings	5-7/6/91	11	0	00.00
Westland	16,20/8/91	2	1	50.00
Mt Stewart	13/9/91	1	0	00.00
Total Possums		**177**	**28**	**15.81%**

Pukeko

Location	Date(s)	Animals tested	Number positive	Percent positive
Lake Wairarapa	23/6/91	1	0	00.00

Rabbit

Location	Date(s)	Animals tested	Number positive	Percent positive
Dannevirke	31/1/92	1	1	100.00
Dannevirke	end 3/92	1	0	00.00
Total Rabbits		**2**	**1**	**50.00%**

Rat

Location	Date(s)	Animals tested	Number positive	Percent positive
Orongorongo Valley	28/5/90	10	7	70.00
Orongorongo Valley	30-31/5/90	10	5	50.00
Orongorongo Valley	28/8/90	24	19	79.2
Orongorongo Valley	5/7/91	7	5	71.4
Orongorongo Valley	26-28/11/91	9	7	77.78
Dannevirke	31/5/91	2	0	00.00
Dannevirke	12/7/91	1	0	00.00
Dannevirke	18/9/91	1	0	00.00
Dannevirke	4-6/12/91	3	2	66.67
Dannevirke	30/1/92	1	0	00.00
Dannevirke	28/3/92	2	1	50.00
Manawatu River	6/6/90	1	0	00.00
Taita	16-17/8/90	5	4	80.00
Auckland	2-9/11/90	10	6	60.00
Hastings	23/11/90	1	1	100.00
Lake Papatongo, Ohau	7-8/8/91	7	2	28.57
Total Rats		**94**	**59**	**62.77%**

Sheep

Location	Date(s)	Animals tested	Number positive	Percent positive
Dannevirke	12/90	23	8	34.78
Dannevirke	7/3/91	16	7	43.75
Dannevirke	11/4/91	14	4	28.57
Dannevirke	10/5/91	14	4	28.57
Dannevirke	13/6/91	3	1	33.33
Dannevirke	23/1/92	6	0	00.00
Mt Stewart	20/8/91	3	0	00.00
Mt Stewart	13/9/91	1	0	00.00
Total Sheep		**80**	**24**	**30.00%**

Sparrow

Location	Date(s)	Animals tested	Number positive	Percent positive
Dannevirke	5/12/91	2	0	00.00
Dannevirke	30-31/1/92	10	0	00.00
Dannevirke	30/4/92	5	1	20.00
Total Sparrows		**17**	**1**	**5.88%**

Swan

Location	Date(s)	Animals tested	Number positive	Percent positive
Lake Wairarapa	23/691	4	0	00.00

Thrush

Location	Date(s)	Animals tested	Number positive	Percent positive
Dannevirke	5/12/91	1	1	100.00
Dannevirke	29-30/1/92	2	2	100.00
Total		**3**	**3**	**100.00%**

Turkey

Location	Date(s)	Animals tested	Number positive	Percent positive
Dannevirke	28/3/91	3	0	00.00
Dannevirke	5/12/91	2	0	00.00
Dannevirke	end 3/92	1	0	00.00
Total		**6**	**0**	**00.00%**

Data for 3.7. Culturing *Giardia muris* in mice

Group 1

Days	Log10	Cysts/g faeces	Comments
3		Positive	all type two
10	6.27	1.88×10^{6}	
11	5.66	1.37×10^{5}	
12	5.36	2.31×10^{5}	
13	5.83	3.83×10^{5}	
14	5.68	4.82×10^{5}	
15	5.69	4.86×10^{5}	
17	5.39	2.48×10^{5}	
18	4.98	9.66×10^{4}	
19	4.54	3.43×10^{4}	
20	4.53	3.37×10^{4}	
22	4.69	4.87×10^{4}	
23	4.59	3.91×10^{4}	
25	4.27	1.86×10^{4}	
26	5.44	2.75×10^{5}	
27	3.74	5.49×10^{3}	
29	3.72	5.17×10^{3}	
31	3.97	9.35×10^{3}	
32	4.67	5.65×10^{4}	
34	3.14	1.37×10^{3}	
35	1.0	0	
36	3.64	4.33×10^{3}	
37	5.16	1.45×10^{5}	mainly type 2
38	3.47	2.98×10^{3}	
39	3.16	1.44×10^{3}	
40	3.12	1.32×10^{3}	

Reinoculation of Group 1

Days	Log_{10}	Cysts/g faeces	Comments
0	1.0	0	
1	1.0	0	
2	6.0	Positive	All type 2
3	5.68	4.81×10^{5}	
7	1.79	61	
8	1.00	0	
9	2.66	454	
10	1.00	0	
13	1.00	0	
15	1.00	0	

Group 2

Days	Log_{10}	Cysts/g faeces	Comments
0	1.0	Inoculate	
1	1.0	Negative	
3	1.0	Negative	
5	5.51	3.24×10^5	All type 2
6	4.45	2.82×10^4	
7	4.21	1.63×10^4	
8	3.33	2.14×10^3	
9	4.72	5.29×10^4	
10	5.33	2.16×10^5	
11	6.04	1.10×10^6	
12	4.54	3.49×10^4	
13	4.72	5.23×104	
14	4.55	3.55×10^4	
15	4.50	3.17×10^4	
16	4.46	2.88×10^4	
19	4.19	1.53×10^4	
20	4.01	1.03×10^4	
21	4.33	2.13×10^4	
22	4.12	1.32×10^4	
25	4.04	1.09×10^4	
26	4.05	1.13×10^4	
27	3.92	8.28×10^3	
41	3.17	1.47×10^3	4 mice are negative
42	3.58	3.82×10^3	
43	3.35	2.22×10^3	
44	3.64	5.50×10^3/g	
47	3.57	3.72×10^3	
48	3.73	5.36×10^3	
49	3.28	1.89×10^3	
50	3.21	1.62×10^3	
51	3.58	3.77×10^3	
55	3.79	6.2×10^3	

Group 3

Day	Log_{10}	Cysts/g faeces	Comment
18	6.29	1.93×10^6	some babes still suckling
20	6.35	2.26×10^6	
21	6.85	7.04×10^6	
22	6.42	2.63×10^6	
23	5.94	8.71×10^5	
25	6.18	1.52×10^6	
26	6.53	3.35×10^6	
27	6.28	1.89×10^6	
28	6.25	1.79×10^6	
30	6.59	3.85×10^6	
31	6.26	1.80×10^6	
34	6.32	2.07×10^6	
35	6.20	1.60×10^6	
36	6.24	1.72×10^6	
37	6.22	1.66×10^6	
39	5.74	5.50×10^5	
40	5.86	7.20×10^5	
42	5.23	1.68×10^5	
43	5.40	3.51×10^5	
44	5.15	1.41×10^5	
45	5.21	1.62×10^5	
48	5.42	2.64×10^5/g	
49	5.29	1.96×10^5	
54	5.64	4.36×10^5	
57	5.84	6.86×10^5	
58	5.20	1.58×10^5	
59	5.68	4.81×10^5	Only 3 type 1 viewed.
60	5.48	3.05×10^5	Less than 10% type 2
64	5.23	1.70×10^5	
65	5.20	1.56×10^5	
66	4.91	8.12×10^4	
69	5.56	3.65×10^5	

Group 4

Day	Log_{10}	Cysts/g faeces	Comments
15	1.00	0.00	
18	5.21	$1.61 \pm 0.03\times10^5$	
20	4.97	$9.32 \pm 0.17\times10^4$	
21	6.06	$1.16 \pm 0.01\times10^6$	
22	6.15	$1.42 \pm 0.03\times10^6$	
25	6.49	$3.10 \pm 0.04\times10^6$	
27	6.61	$4.04 \pm 0.05\times10^6$	
28	6.30	$2.01 \pm 0.02\times10^6$	
33	5.12	1.33×10^5	
34	5.96	9.17×10^5	
35	6.71	5.17×10^6	
40	5.12	1.32×10^5	
46	5.18	1.50×10^5	

Group 5

Day	Log_{10}	Cysts/g faeces	Comments
30	6.47	$2.96 \pm 0.04 \times 10^6$	
31	6.65	$4.42 \pm 0.08 \times 10^6$	
32	6.78	$5.97 \pm 0.11 \times 10^6$	
36	6.13	$1.36 \pm 0.04 \times 10^6$	
45	5.49	$3.07 \pm 0.10 \times 10^5$	
46	5.32	$2.10 \pm 0.11 \times 10^5$	
47	5.10	$1.25 \pm 0.04 \times 10^5$	
50	4.33	$2.13 \pm 0.43 \times 10^4$	
57	4.75	$5.60 \pm 0.32 \times 10^4$	mucous
58	5.28	$1.92 \pm 0.04 \times 10^5$	
59	5.51	$3.21 \pm 0.07 \times 10^5$	
60	5.25	$1.76 \pm 0.07 \times 10^5$	
61	5.36	$2.30 \pm 0.14 \times 10^5$	
64	4.57	$3.70 \pm 0.35 \times 10^4$	
65	5.17	$1.49 \pm 0.03 \times 10^5$	
66	4.52	$3.33 \pm 0.36 \times 10^4$	
67	4.35	$2.26 \pm 0.23 \times 10^4$	
68	4.06	$1.16 \pm 0.03 \times 10^4$	
69	4.15	$1.40 \pm 0.08 \times 10^4$	

Group 6

Day	Log_{10}	Cysts/g faeces	Comments
30	6.37	$2.34 \pm 0.04 \times 10^6$	
31	6.60	$3.95 \pm 0.05 \times 10^6$	
32	6.40	$2.52 \pm 0.03 \times 10^6$	
36	6.25	$1.79 \pm 0.03 \times 10^6$	mucous
45	5.64	$4.36 \pm 0.08 \times 10^5$	
46	6.05	$1.13 \pm 0.01 \times 10^6$	
47	5.34	$2.21 \pm 0.06 \times 10^5$	
50	5.27	$1.85 \pm 0.05 \times 10^5$	
57	4.77	$5.91 \pm 0.22 \times 10^4$	
58	5.01	$1.03 \pm 0.02 \times 10^5$	
59	4.49	$3.06 \pm 0.17 \times 10^4$	
60	5.08	$1.21 \pm 0.03 \times 10^5$	
61	5.30	$2.01 \pm 0.06 \times 10^5$	
64	5.36	$2.29 \pm 0.04 \times 10^5$	
65	5.65	$4.49 \pm 0.09 \times 10^5$	
66	5.82	$6.66 \pm 0.07 \times 10^5$	
67	5.54	$3.44 \pm 0.09 \times 10^5$	
68	5.07	$1.18 \pm 0.03 \times 10^5$	
69	5.52	$3.29 \pm 0.08 \times 10^5$	

Group 7

Day	Log_{10}	Cysts/g faeces	Comments
32	6.66	$4.62 \pm 0.08 \times 10^6$	
34	6.31	$2.02 \pm 0.03 \times 10^6$	
35	6.88	$7.59 \pm 0.17 \times 10^6$	
37	6.05	$1.12 \pm 0.32 \times 10^6$	
38	6.37	$2.36 \pm 0.06 \times 10^6$	
39	5.97	$9.27 \pm 0.53 \times 10^5$	mucous
40	5.92	$8.33 \pm 0.33 \times 10^5$	
41	5.41	$2.57 \pm 0.11 \times 10^5$	
42	6.01	$1.03 \pm 0.05 \times 10^6$	

Data for 3.9. Environmental Effects on *Giardia* Cyst Presence and Viability

Giardia cysts stored at -20°C

3.9.1.1		3.9.1.2.1.		3.9.1.2.2	
Day	% Cysts†	Day	% Cysts	Day	% Cysts
0	100 ± 3.27	0	100 ±6.29	0	100 ± 3.36
1	100 ± 3.43	2	0.31± 0.03	1	44.82 ± 36.14
3	100 ± 5.29	4	0.4 ± 0.95	3	37.00 ± 9.68
4	93.56 ± 9.11	6	0.47 ± 0.94	4	27.89 ± 14.97
7	92.58 ± 10.64	7	0.60 ± 1.31	7	24.22 ± 12.75
14	89.42 ± 14.97	8	0.40 ± 0.96	8	20.73 ± 11.93
21	89.53 ± 11.97	11	0.61 ± 1.00	11	21.69 ± 11.58
28	87.00 ± 12.73	14	0.41 ± 0.95	14	8.54 ± 3.83
35	83.26 ± 15.19	21	0.42 ± 0.95	15	3.82 ± 1.07
42	80.0 ± 16.97	28	0.57 ± 0.88	21	2.41 ± 1.41
56	78.36 ± 16.46	35	0.03 ± 0.07	24	2.41 ± 1.41
63	75.10 ± 15.42	42	0.27 ± 0.42	69	1.30 ± 1.83
66	72.87 ± 16.51	49	0.05 ± 0.03		
70	73.67 ± 12.49	56	0		
91	73.00 ± 2.33				

† % Cysts is the Average ± Standard Deviation.

Giardia cysts stored at 4°C

3.9.2.1

Day	% Recovery
0	100 ± 6.86
8	100 ± 3.42
14	100.00 ± 2.99
21	99.79 ± 2.85
77	100.00 ± 3.21
105	100.00 ± 2.18
112	100.00 ± 0.59
140	100.00 ± 3.78
168	92.04 ± 1.02
196	90.30 ± 1.32
224	84.62 ± 3.28
252	84.33 ± 1.15
280	81.11 ± 1.23

3.9.2.2.1.

Day	% Recovery
0	100 ± 5.76
7	90.49 ± 26.30
14	74.53 ± 25.27
21	70.06 ± 17.24
28	55.25 ± 30.69
35	503 ± 37.82
42	50.49 ± 1.38
56	47.15 ± 29.76
63	38.81 ± 30.27
70	31.41 ± 23.56
77	26.40 ± 24.71
84	19.63 ± 27.76
102	15.88 ± 32.36
105	14.09 ± 19.92
112	2.56 ± 3.61

3.9.2.2.2

Day	% Recovery
0	100 ± 3.01
4	95.65 ± 6.15
7	94.68 ± 7.53
10	94.30 ± 8.07
14	92.01 ± 11.30
21	90.50 ± 13.44
28	89.40 ± 14.99
35	87.00 ± 8.38
42	84.53 ± 9.63
56	69.26 ± 17.34
70	60.06 ± 18.81
84	57.75 ± 17.46
112	57.62 ± 12.28
140	56.42 ± 11.06

3.9.2.3.1.

Day	% Recovery
0	100 ± 6.18
1	91.70 ± 7.51
2	85.92 ± 13.83
3	86.11 ± 14.42
4	85.27 ± 15.55
5	79.92 ± 14.73
6	72.56 ± 13.15
7	69.77 ± 14.82
8	61.20± 15.77
10	56.10 ± 16.97
14	47.75 ± 21.22
21	38.53 ± 18.06
28	32.65 ± 15.40
35	26.96 ± 17.78
42	19.85 ± 17.74
49	10.08 ± 10.81
56	7.56 ± 10.37
63	3.78 ± 5.32
70	2.02 ± 4.44
77	2.12 ± 4.74
84	1.50 ± 3.35
112	0.93 ± 2.08

3.9.2.3.2.

Day	% Recovered
0	100 ± 5.25
1	99.50 ± 1.00
2	99.15 ± 1.70
3	98.73 ± 2.55
4	98.30 ± 3.40
5	97.88 ± 4.25
6	97.39 ± 5.06
7	94.46± 6.43
11	92.28 ± 8.48
14	91.67 ± 11.23
21	89.56 ± 17.07
28	75.81 ± 21.26
35	74.74 ± 17.24
42	71.36 ± 16.81
56	66.50 ± 19.09
63	61.92 ± 19.68
70	61.63 ± 24.42
77	59.06 ± 22.22
84	52.20 ± 22.22
112	41.72 ± 25.37
154	39.24 ± 4.96

Giardia cysts stored at 4°C

3.9.3.1.1.		3.9.3.1.2.	
Day	% Cysts	Day	% Cysts
0	100 ± 3.58	0	100 ± 3.25
7	62.68 ± 2.52	1	97.39 ± 3.10
14	0.26 ± 0.40	2	93.43 ± 5.46
21	0.69 ± 0.82	5	85.57 ± 14.29
28	0.79 ± 1.08	6	81.17 ± 16.46
35	0.29 ± 0.57	7	78.36 ± 19.30
42	0.00	10	75.33 ± 20.93
		11	73.51 ± 21.19
		14	70.10 ± 22.87
		21	57.55 ± 31.34
		28	46.65 ± 31.74
		35	38.92 ± 33.31
		42	34.03 ± 31.59
		49	26.44 ± 27.16
		56	22.54 ± 27.66
		70	20.73 ± 25.84
		76	18.12 ± 24.91
		91	16.05 ± 22.28
		105	4.85 ± 13.31
		119	6.80 ± 13.34

Giardia cysts stored at 37°C

3.9.5.1.1.		3.9.5.1.2.	
Day	% Cysts	Day	% Cysts
0	100 ± 6.98	0	100 ± 6.02
1	3.55 ± 1.90	1	98.96 ± 3.00
2	2.06 ± 2.72	2	97.92 ± 2.40
3	1.35 ± 2.71	3	96.87 ± 2.80
4	0.15 ± 0.66	4	95.83 ± 7.87
		5	94.96 ± 3.70
		6	94.09 ± 4.09
		7	93.23 ± 5.09
		8	92.36 ± 1.20
		9	91.49 ± 5.04
		12	88.89 ± 7.73
		20	81.94 ± 7.41
		21	80.78 ± 4.17
		32	84.72 ± 2.31
		35	79.55 ± 5.56
		42	78.43 ± 5.09
		49	77.31 ± 3.70
		56	72.68 ± 1.85
		63	68.06 ± 3.98
		70	63.43 ± 4.63
		77	60.02 ± 7.87
		84	58.80 ± 3.70
		112	58.04 ± 5.56
		168	57.87 ± 5.09
		196	54.17 ± 2.78
		224	54.01 ± 1.99

Data for 3.10. Preservation of *Giardia* Cysts

Giardia cysts stored in PVA at 20°C

3.10.1.1.1.

Day	% Cysts
0	100 ± 10.71
7	87.75 ± 9.84
14	78.33 ± 15.47
28	62.45 ± 30.76
42	43.25 ± 29.62
56	34.31 ± 30.20
63	31.08 ± 29.92
70	28.14 ± 29.58
84	25.16 ± 26.84
168	10.49 ± 7.15
196	5.69 ± 2.32

3.10.1.1.2.

Day	% Cysts
0	100 ± 3.17
1	99.44 ± 2.00
7	93.35 ± 4.09
14	90.20 ± 5.78
21	86.89 ± 7.78
28	82.53 ± 10.11
35	76.17 ± 16.13
42	71.99 ± 17.39
56	63.23 ± 18.98
70	63.23 ± 18.98
84	57.21 ± 21.41
112	51.41 ± 20.76

Giardia cysts stored in10% formalin at 4°C

3.10.2.1

Day	% Cysts
0	100 ± 2.85
7	92.41 ± 9.04
12	91.98 ± 8.43
14	91.81 ± 8.19
21	89.20 ± 10.18
45	85.23 ± 9.98
70	82.53 ± 7.71
79	77.18 ± 10.15
84	72.89 ± 13.36
91	71.16 ± 11.79
98	69.84 ± 9.67
105	65.42 ± 9.79
112	58.16 ± 12.50
119	55.83 ± 7.79
133	55.34 ± 7.54
140	54.77 ± 6.75
147	54.21 ± 5.94
154	53.64 ± 5.14
168	52.51 ± 3.54
182	50.57 ± 0.80
196	49.37 ± 0.90
210	48.17 ± 2.60
224	47.34 ± 3.76
238	45.97 ± 5.71
252	45.00 ± 5.59
266	40.00 ± 2.00
280	36.67 ± 1.57

3.10.2.2.1.

Day	% Cysts
0	100 ± 7.54
7	72.67 ± 15.92
14	50.83 ± 31.54
28	44.65 ± 29.97
42	34.63 ± 21.04
50	24.72 ± 17.82
56	15.70 ± 11.49
70	11.19 ± 8.12
84	9.13 ± 0.72
117	4.84 ± 6.01
126	3.75 ± 0.37

3.10.2.2.2.

Day	% Cysts
0	100 ± 1.17
7	99.68 ± 0.16
14	99.36 ± 0.33
28	98.72 ± 0.66
42	98.08 ± 0.98
56	97.44 ± 1.31
70	96.80 ± 1.63
77	96.47 ± 1.80

3.10.2.3.1.

Day	% Cysts
0	100 ± 2.27
7	68.54 ± 19.19
14	48.28 ± 22.71
28	26.60 ± 25.44
42	19.53 ± 17.83
56	11.74 ± 11.28
70	7.59 ± 2.13
75	5.48 ± 1.79
84	4.93 ± 1.18
98	3.77 ± 1.12
112	2.62 ± 1.49
140	1.83 ± 1.42
161	1.23 ± 1.38

3.1.2.3.2.

Day	% Cysts
0	100 ± 3.40
7	95.69 ± 4.99
14	94.02 ± 6.97
21	93.69 ± 7.29
28	93.56 ± 7.28
35	93.41 ± 7.26
42	93.27 ± 7.25
49	93.13 ± 7.24
56	93.00 ± 7.27
70	91.80 ± 6.96
77	91.18 ± 7.05
84	90.57 ± 7.27
91	89.96 ± 7.61
112	87.90 ± 18.82
126	86.53 ± 9.97
140	86.21 ± 9.65
168	81.31 ± 14.19
196	78.28 ± 17.21
224	75.67 ± 20.45
280	67.61 ± 26.45

Giardia cysts exposed to SDS, stored in water at 4°C

3.10.3.1.

Day	% Cysts
0	100.00 ± 4.61
3	97.23 ± 1.20
9	91.67 ± 3.59
16	85.19 ± 6.38
21	80.56 ± 8.37
28	74.05 ± 11.11
49	54.67 ± 19.57
56	48.20 ± 22.37
64	40.80 ± 25.56
65	39.87 ± 25.96
72	35.64 ± 25.58
100	26.49 ± 13.40
105	24.71 ± 11.07
112	22.23 ± 8.24
140	15.50 ± 1.41
168	12.60 ± 0.94
196	11.77 ± 0.81
224	11.64 ± 0.50
280	10.65 ± 0.29

APPENDIX B. REAGENT FORMULATION

ELUTING SOLUTION

Ingredients to prepare:	**0.1% v/v**
S.D.S. Stock	100 ml
1% Tween 80 solution	100 ml
Make to a final volume with PBS	1000 ml

FORMALDEHYDE (FORMALIN) SOLUTIONS

Ingredients to prepare:	**7.4% (20%)**	**3.7 % (10%)**
Formaldehyde solution, 37%	20 ml	10 ml
Distilled Water	80 ml	90 ml

10% formalin is equivalent to 3.7% Formaldehyde solution.

1 N HYDROCHOLORIC ACID (HCl)

Ingredients to prepare:	**50 ml**	**100 ml**	**500 ml**	**1000 ml**
Conc. HCl, 11.6N	4.31 ml	8.62 ml	43.1 ml	86.2ml
Dilute to a final volume of (with deionised distilled H_2O)	50 ml	100 ml	500 ml	1000 ml

5 N HYDROCHLORIC ACID (HCl)

Ingredients to prepare:	**50 ml**	**100 ml**	**500 ml**	**1000 ml**
Conc. HCl, 11.6N	21.55 ml	43.1 ml	215.5 ml	431.0 ml
Dilute to a final volume of (with deionised distilled H_2O)	50 ml	100 ml	500 ml	1000 ml

HANKS BALANCED SALT SOLUTION (HBSS)

Solution A

1. $NaCl$ 160 g
   KCl 8 g
   $MgSO_4 . 7H_2O$ 2 g
   $MgCl_2 . 6H_2O$ 2 g
   Distilled water 800 ml

2. $CaCl_2$ 2.8 g
   Distilled water 100 ml

Mix solutions 1 and 2 and make up to a final volume of 1L with distilled water. Add 2 ml chloroform and store at 4°C.

Solution B

$Na_2HPO_4 . 12H_2O$ 3.04 g

KH_2PO_4	1.2 g
Glucose	20.0 g
Distilled Water	800 ml

Mix solution B with 100 ml of 0.4% phenol red in NaOH and make up to 1000 ml with distilled water. Add 2 ml chloroform at store at 4°C.

To make up Working Strength HBSS:

Solution A	100 ml
Solution B	100 ml
Distilled water	800 ml

Final volume is 1L. This solution can be sterilised either by membrane filtration or autoclaving at 121°C for 15 minutes.

LUGOL'S IODINE SOLUTION

Ingredients to prepare:	**Amount**
Potassium Iodide	10 g
Powdered Iodine	5 g
Deionised distilled H_2O	100 ml

The potassium iodide must first be dissolved in the deionised distilled H_2O. After dissolving the potassium iodide, the powdered iodine is added slowly with continual stirring until it is dissolved. This solution is filtered through filter paper into tightly stoppered brown bottle. This solution should be dated when prepared as it is good for only three weeks.

0.15 M NaCl SOLUTION

Ingredients to prepare:			
NaCl (M. W. 58.5)	0.43875 g	0.8775 g	4.3875 g
Distilled water to make up required volume to:	50 ml	100 ml	500 ml

PERCOLL SOLUTIONS

Ingredients to prepare:	**20%**	**40%**	**70%**
Stock Percoll (Pharmacia™) sp.gr. 1.130 ± 0.05	20 ml	40 ml	70 ml
P.B.S.	80 ml	60 ml	30 ml
Total Volume	100 ml	100ml	100ml

PERCOLL SOLUTIONS

Ingredients to prepare:	**sp. gr. 1.05**	**sp. gr. 1.095**
Percoll, stock sp. gr. 1.125	40 ml	80 ml
0.15 M NaCl to make up the final volume to:	100 ml	100 ml

PERCOLL-SUCROSE SOLUTION sp. gr. 1.09-1.10

Ingredients to prepare:	**200 ml**	**400 ml**
Stock percoll (Pharmacia™) sp. gr. 1.130 ± 0.05	90 ml	180 ml
2.5-M Sucrose	20 ml	40 ml
Deionised distilled water	90 ml	180 ml

0.1 M PHOSPHATE BUFFER, pH 7.2†

Solution A: 27.6 g $NaH_2PO_4.H_2O$/1000 ml Solution

Solution B: 71.7 g $Na_2HPO_4.12\ H_2O$/1000 ml Solution

Ingredients to prepare:	**50 ml**	**100 ml**	**200 ml**	**500 ml**	**1000 ml**
Solution A	7 ml	14 ml	28 ml	70 ml	140 ml
Solution B	18 ml	36 ml	72 ml	180 ml	360 ml
0.01% Tween 20 in distilled H_2O	25 ml	50 ml	100 ml	250 ml	500 ml

10X PHOSPHATE BUFFERED SALINE

Ingredients to prepare:	**Amount**
NaCl	85.0 g
KH_2PO_4	2.0 g
$Na_2HPO_4.12\ H_2O$	29.0 g
KCl	2.0 g
Deionised Distilled H_2O	1000 ml final volume

To 900 ml deionised distilled water add the ingredients above and mix until the salts are dissolved.

Adjust the pH, if necessary, to 7.4.

Adjust the final volume of the 10X phosphate buffered saline to 1000 ml with additional deionised distilled H_2O.

Store at 4°C.

1X PHOSPHATE BUFFERED SALINE

Ingredients to prepare:	**Amount**
10X Phosphate Buffered Saline	100 ml
Deionised Distilled H_2O	900 ml

Adjust the pH to 7.4 with 1N NaOH or 1N HCl, as required.

Check to make sure the final volume is 1000 ml.

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† Based on Schaefer *et al* 1986.

SODIUM DODECYL SULPHATE (SDS) STOCK SOLUTION

Ingredients to prepare:	**1% W/V**
Sodium Dodecyl Sulphate	1 g
Distilled Water to make up to	100 ml

SHEATHER-SUCROSE, sp. gr.

Ingredients to prepare:	**Amount**
Sucrose	500 g
Phenol	6.5 g
Deionised Distilled H_2O	320 ml

Add the solid ingredients to water and mix with a magnetic stirrer over heat, store at 4°C.

Final volume is 600 - 700 ml.

0.5 M AQUEOUS SUCROSE ($C_{12}H_{22}O_{11}$) SOLUTION, sp. gr. 1.13

Make a 50:50 dilution of 1.0-M sucrose with sterile milli-q water

1.0 M AQUEOUS SUCROSE ($C_{12}H_{22}O_{11}$) SOLUTION, sp. gr. 1.13

Ingredients to prepare:	**100 ml**	**500 ml**
Sucrose	34.23 g	171.15 g
Dilute with deionised distilled H_2O to get final volume	100 ml	500 ml

2.0-M AQUEOUS SUCROSE ($C_{12}H_{22}O_{11}$) SOLUTION, sp. gr. 1.13

Ingredients to prepare:	**100 ml**	**500 ml**
Sucrose	68.46 g	342.30 g
Dilute with deionised distilled H_2O to get final volume	100 ml	500 ml

2.5-M AQUEOUS SUCROSE ($C_{12}H_{22}O_{11}$) SOLUTION, sp. gr. 1.13

Ingredients to prepare:	**100 ml**
Sucrose	85.575 g
Dilute with deionised distilled H_2O to get final volume	100 ml

ZINC SULPHATE SOLUTION (sp. gr. 1.18)

Ingredients to prepare:	**33%**
Zinc Sulphate crystals	330 g
Dilute with deionised distilled H_2O to get final volume	670 ml

Check specific gravity with a hydrometer and adjust with Zinc sulphate or water as necessary.

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