THE OCCURENCE OF *GIARDIA* IN CATS AND DOGS IN NEW ZEALAND AND SUBSEQUENT ISOLATION AND DIFFERENTIATION OF STRAINS.

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ABSTRACT.

Giardiasis, a debilitating diarrhoea that affects many people every year is caused by the ubiquitous protozoan parasite *Giardia intestinalis* (syn *lamblia, duodenalis*). This parasite infects and causes disease in birds and animals as well as man and has no known host specificity. Dogs and cats are some of the animals infected by *Giardia* and due to their close association with man, may be carrier sources of human giardiasis.

In an attempt to discover a relationship between man and these animals, a survey of the level of *Giardia* infection in cats and dogs in both Hamilton and Palmerston North, New Zealand, was undertaken. Percentages of 25% and 8% for dogs and 3% and 7% for cats respectively were obtained. Statistically the level of infection in Hamilton was higher than that of Palmerston North. In both cities the sex and breed of the animals showed no correlation to infection although animals less than 3 years old were more likely to be infected. Clinical manifestations of giardiasis were observed but did not significantly correlate with the presence of *Giardia* and were not necessarily caused by the *Giardia* when present.

To further enhance the relationship hypothesized it was attempted to culture the *Giardia* from the cats and dogs and relate them to cultured human isolates. Our attempts were unsuccessful and from 91 samples only 8 human strains from 5 geographical areas were isolated. These isolations were made by both *in vitro* and *in vivo* techniques that both yielded 7% sample to culture success.

The isolated *Giardia* strains plus a control culture, Bris/83/HEPU/106 supplied by Boreham, Australia, were compared by growth rate and sodium dodecylsuphate polyacrylamide gel electrophoresis (SDS-PAGE). Both these tests showed the similarity of these strains. The average growth rate was 0.09 ± 0.01 hours^{-1} and no strain varied from the statistical mean. In relation to the total protein banding patterns measured by SDS-PAGE, the isolates varied, at most, by one or two bands.

An isolate of *G. muris* extracted from a naturally infected mouse and a human isolate that was extracted from an experimentally infected mouse were also compared by SDS-PAGE to a cultured isolate. The results showed many different bands between all three samples and suggests that an adaption, or selection, of the *Giardia* must take place when it is cultured. If this is so, then perhaps the emphasis put on strain variation of cultured *Giardia* trophozoites is to be questioned.

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INTRODUCTION.

Giardia intestinalis (syn *lamblia, duodenalis*) is a ubiquitous protozoan parasite regarded as the most common intestinal parasite in man in the United States of America, United Kingdom, Australia and probably the world. It causes a disease, primarily in the young, which is in the form of a debilitating diarrhoea often with complications of steatorrhoea and mucosal damage. *Giardia* is also found in a wide range of other animals including household pets. The possibility of these animals providing a source of *Giardia* for human infection is, as yet, unknown although quite feasible.

For this reason a survey of the occurence of *Giardia* in cats and dogs was carried out within two cities in New Zealand. To accomplish this, faecal samples were obtained from pets, breeding kennels and from stray animals, thus attempting to acurately assess the level of infection within the whole community. In correlation to this ideal we also determined the sensitvity and specificity, or in other words the acuracy, of our diagnostic test. The need to do this is highlighted by the intermittency of cyst shedding that occurs during infection.

Following the survey the cysts retrieved from the positive cats and dogs, plus symptomatic samples from humans, were used to establish cultures of *Giardia* in bile supplemented TY1-S-33 medium. This was done using the latest *in vitro* method of excystation as well as an *in vivo* method using suckling mice.

Cultures of *Giardia* were then compared via their growth rates in bile supplemented TY1-S-33 and by their total protein banding patterns as determined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). The aim of this was to determine if any relationship between the *Giardia* of cats, dogs and humans existed and if strain variations occured between geographical regions.

Chapter 1.

HISTORICAL PERSPECTIVE OF *GIARDIA* AND GIARDIASIS.

1.1 The Organism

1.11 Biology.

Giardia has a diphasic life cycle consisting of a trophozoite stage and a cyst stage

Trophozoites are bilaterally symmetrical organisms shaped rather like a pear, i.e. anteriorly broad and rounded, posteriorly bluntly pointed. In size they measure 12-15μm x 6-10μm. Trophozoites have 4 pairs of flagella and attach to the microvilli of the duodenum and jejunum by a ventral sucking disc. Highly distinctive of the organism are two laterally placed large nuclei with central karyosomes, which, with the positioning of the median bodies, give the trophozoite the appearance of a face (Filice 1952). The trophozoite is responsible for the pathogenesis of disease.

Cysts are ellipsoid and uniform, 9-13μm x 7-10μm and consist typically of 4 nuclei, intracytoplasmic axonemes and median bodies encased within a hyaline cyst wall. The cyst forms during passage through the jejunum (Lambert 1970; Burke 1977), and being the mechanism of transmission and survival, is the predominate form found in the faeces. Trophozoites may occasionally be seen in the faeces if the stool is very diarrhoeic. The mechanism of encystment is, as yet, unknown.

1.12 Taxonomy.

The genus *Giardia* is a group of flagellate parasites, members of which inhabit the gastrointestinal tract of frogs, reptiles, birds and mammals. They were first discovered by Antony Van Leuwehoek in 1681 when in a letter to the Royal Society of London he described, from his own diarrhoeic stool, an organism that was longer than it was broad, with a flat "belly" and, which was "..furnisht with sundry little paws". This description is taken as being that of *Giardia* (Dobell 1926,1932).

This group of organisms belong in the phylum Protozoa and subphylum Sarcomastigophorea. From there they can be classified as class Zoomastigophorea, order Diplomonadida (Wenyon 1926), family Hexamitidae (Kent 1880), subfamily Octomitinae (Kent 1880), and genus *Giardia* (Kunstter 1882).

Most authors agree with this classification up to this point, although some in the East still refer to it by the genus name of *Lamblia*, after Blanchard (1888) who suggested this name to commemorate the organisms "rediscoverer", Lambl (1859) (Dobell 1926). The biggest problem however arises over the giving of specific, or trivial names to organisms in the genus *Giardia*. Hegner (1922,1926[a b]) expressed his belief in "rigid host specificity" for *Giardia* and gave a new name for each new *Giardia* found. Kulda and Nohynkova (1978) revealed that there are 49 species of *Giardia* recorded in the literature in this manner.

The most significant work on taxonomy probably belongs to Filice (1952). In it he exhaustively sets out to establish three species of *Giardia* upon morphometric characteristics of the median bodies only. The three species are; *G.duodenalis*, characterized by two "claw hammer" shaped median bodies; *G.muris*, with two small circular centrally located median bodies; and *G.agils*, with two tear drop like median bodies. Among these species Filice proposes, "races" or strains. For *G.duodenalis* he suggests ten such "races", including 'lamblia' from man (cf Kofoid and Christiansen 1915),'simoni' from the rat (Lavier 1924) and 'microti' from *Microtus californicus* (Kofoid and Christiansen 1915).

At present there is still no unity on the subject with authors using a variety of different names for what could be the same strain. This report has determined to use the name *G.intestinalis* put foreward by Boreham *et al* (1984) for both man and animals. Clearly, much work is still to be done in this area.

1.2 Disease.

1.21 History.

Early in history most authors were of the opinion that *Giardia* was simply a commensal, living harmlessly within the small intestine of man, animals and birds. Some early authors however, did not agree with this opinion and expressed the likelihood of *Giardia* being pathogenic. This veiw is now widely accepted.

Indications of the pathogenic nature of *Giardia* were observed in 1881 by Grassi. He associated cases of diarrhoea in man with infection of *Giardia lamblia* . Similar associations are reported by the following authors Grassi and Schewiakoff (1888), Moritz and Holzl (1892). In his paper of Intestinal parasites of man, Wenyon (1915) documents an observation of *Giardia* infection and disease in "two or three persons". He states "...there occur at intervals attacks of diarrhoea with the passage of mucus in which *Lamblia* are to be found in enormous numbers...the occurence of repeated attacks of this nature with a certain degree of abdominal uneasiness and the passage of such extraordinary numbers of the flagellates, especially in the mucus leads me to suspect that sometimes, at any rate, *Lamblia intestinalis* may produce sufficient irratation of the small intestine to justify us in regarding it as pathogenic." To back up this claim, he records his observations of the invasion of the "tubular glands" of the small intestine of the rabbit as proof of the pathogenesis of the organism.

More evidence as to the relationship between *Giardia intestinalis* infection and diarrhoea came with reports, such as that of Miller(1926) who noted that *Giardia* infection in children was symptomatically very similar to coeliac disease. Coeliac disease is a total, or subtotal, villous atrophy normally HLA-A1 and B8 associated. Children with this disease show a virtual absence of villi, thickening of the lamina propria and various other mucosal abnormalities as well as diarrhoea. Typically, the disease is centered around the jejunum and recedes when the patient changes to a gluten free diet (Bateson and Bouchier 1981).

In 1939 Veghelyi (1939, 1940), made the association of malabsorbtion of fats to *Giardia* infection, and enhanced the causal association by showing that when treated with quinacrine, symptoms departed upon eradication of the parasites. This cure came about by the work of Brumpt (1935) who discovered that mice infected with *Giardia muris* were cured by an oral dose of quinacrine. This was used by Galli-Valerio in 1937 to demonstrate that quinacrine also cured man of *Giardia* infection. Many other workers record similar findings (Morrison and Swalm 1939; Love and Taylor 1940; Voltrina 1941; Hartman and Kyser 1941).

Although evidence was strong for the association between *Giardia* and disease, evidence was also available for the contrary position. For example, Boeck (1927) applied statistics to conclude that *Giardia* was a mere commensal.

Rendtorff (1954, 1978) and Rendtorff and Holt (1954[a] [b]) echoed well the sentiment of the day, with their study on parasite transmission in man. At this time *Entamoeba histolytica* was the big problem and the work undertaken by them was to expose human volunteers to a variety of 'non pathogenic' protozoa including *Giardia*, in the hope of relating their findings to *Entamoeba histolytica*. The reason for using *Giardia* was that "..at that time it *(Giardia)* was not generally believed to be an invasive pathogenic parasite of man." He goes on to say that in the 1950's *Giardia* was thought to cause the occasional case of diarrhoea in children but due to it being so commonplace, and so lacking in clinical symptomology in adults, it was considered non pathogenic. The real

paradox is that their studies seemed to prove this theory right. None of the men used in this study showed any serious pathological effects and the gastrointestinal complaints that did arise could not be comprehensively attributed to *Giardia*.

Now it is a widely accepted that *Giardia* are pathogenic, particularily since the first report of an epidemic outbreak of giardiasis (Moore *et al* 1969). In this instance, reports of a characteristic malodourous diarrhoeal illness were placed by a number of people who had holidayed in Aspen, Colorado during the ski season of 1965-66. Out of some 1094 skiers, 11.3% developed characteristic symptoms, all of which reported, on pathological examination, the absence of all other pathogens apart from *Giardia intestinalis*. It is interesting to note that this epidemic also proved the popular hypothesis of the time that said "giardiasis is spread through water". The source of *Giardia* cysts in this outbreak was linked to defective sewage pipes draining into two wells supplying part of the city's water supply. Although, post season, the water was tested for presence of cysts and found negative, sewage contamination from the defective pipes was found in the well water and hence the possibility of transmission of *Giardia* cysts from the pipes was high.

Since that time, many waterborne outbreaks have been reported. In the United States alone, twenty-two isolated outbreaks of giardiasis occurred between the eleven years from Aspen 1966 to July 1977 (Craun 1978). Cases range from five in an outbreak in Grand Co., Tennessee in 1973, to 4800 in Rome, New York in 1974. All of these outbreaks were associated with disease indicating the pathogenicity of *Giardia*.

1.22 Symptomology

Many surveys and reports have been done since the realization of giardiasis causing symptomatic disease. Many of these have quantified the accompaning complaints. The table following is a summary of complaints listed by a number of various authors.

Table 1.1 Percentage of confirmed cases of giardiasis showing the major signs and symptoms.

Literature Sources

Signs and Symptoms	Moore *et al.* 1969	Walzer *et al.* 1971	Shaw *et al.* 1977	Lopez *et al.* 1980	Chester *et al.* 1985	
	Acute	Acute	Acute	Acute	Acute	Chronic
Diarrhoea	83.9	71.9	92	86.4	70	55
Constipation	-	-	-	-	22	39
Abdominal cramps	76.7	59.4	70	80.5	63	66
Flatulence	-	59.4	6	58.4	78	79
Foul stools	87.5	52.2	-	-	-	-
Bloating	67.5	-	9	55.2	81	74
Malaise / fatigue	80.3	87.5	20	-	78	71
Weight loss	73.2	-	13	-	56	45
Anorexia	60.4	56.2	2	64.9	-	-
Nausea	58.8	59.4	58	14.3	63	66

Clinical giardiasis begins as an acute disease. This stage starts with the onset of parasites in the faeces following a prepatent period of 9-22 days (Rendtorff 1954, 1978; Walzer *et al* 1971; Brodsky *et al* 1974).The acute disease mimics that of many other pathogens such as *Campylobacter*, bacillary dysentry, enterotoxigenic *E.coli*, food poisoning etc.(Wolfe 1984), nevertheless, the characteristic foul smelling stools in absence of blood or cellular exudate is typical of giardiasis. The acute stage lasts from 2 weeks to 2 months (Brodsky *et al* 1974).

After resolving spontaneously, the acute infection may then develop into either an asymptomatic carrier stage or a subacute and chronic phase. This latter stage affects about one third of patients (Veazie 1969). Chronic disease is characterized by recurrent, intermittent bouts, of loose foul stools with a general feeling of being "all gassed up" (Wolfe 1984). This stage of the disease can be confused with peptic ulcers, hiatal hernia, biliary tract disorders etc.

Asymptomatic patients show no sign of disease although upon jejunal biopsy examination, mucosal damage and a mild degree of malabsorbtion of lipids is found to occur (Barbieri *et al* 1970).

In some patients the acute stage never resolves and the patients end up with a severe malabsorbtion syndrome with pronounced weight loss and steatorrhoea.

The literature also records observations of *Giardia* causing megaloblastic anaemia (Buckley 1986), arthritis (Barton *et al* 1986) and psychiatric manifestations (Khan 1974). This latter disorder included severe behaviour changes and altered EEG readings in children infested with *Giardia*. These disorders came right after treatment and eradication of the parasites. Khan suggested that these observations were similar to the meningoencephalitis caused by *Entamoeba histolytica*. This assumption has been discredited by Isaacs and Warhurst (1975) who indicate that the behavioural disturbances are more likely to be caused by the diarrhoea itself, as in coeliac disease (McNeish and Anderson

1974), or due to side effects associated with the treatment with metronidazole.

1.23 Animal Disease.

Many different animals have been surveyed for *Giardia*, and the effect of the infection evaluated. For example Craige (1948) attributed *Giardia* infection to deaths in puppies. Budgerigars show clinical signs of diarrhoea and depression when infected with *Giardia* (Hiral *et al* 1980) and kid goats in New Zealand have symptoms of watery diarrhoea and decreased growth (Sutherland 1982; Sutherland and Clarkson 1984). In this latter case, however, *Cryptosporidium* was also found and may have contributed markedly to the clinical signs. Cats are also affected, although, clinical illness was not acknowledged in these animals until 1976 (Brightman and Slouka 1976).

As with humans, animal disease is predominately found in the young. For example Bemrick (1961) notes that the prevelance of giardiasis in dogs is higher among younger animals.

Symptoms that can be observed objectively, are found in animal disease to be similar to those found in humans.

1.24 Pathogenesis

As *Giardia* are found in the small intestine, and this is not readily accessible in humans, the finding of an animal model was necessary for evaluating the pathogenesis of giardiasis. In 1976 Roberts-Thomson *et al* (1976[a]) published the succesful infection of a mouse colony with a *G.muris* type organism from a naturally infected hamster. Although this model does not use a *G.intestinalis* type organism, it has been useful in giving insight into human diaease.

From the *G.muris* model (Roberts-Thomson *et al* 1976[a]) it has been determined that the mid jejunmum is the primary zone of infection in giardiasis. During infection it is found that the mucosa of the jejunum under goes morphological changes such as a reduction in the villus:crypt ratio, microvillus deformation or blunting and an increase of inflamatory cells in the lamina propria. Blunting is a consequence of T-lymphocytes in the gut mucosa (Roberts-Thomson and Mitchell 1978). When human biopsies are done, similar changes are seen (Ament 1972; Ament and Rubin 1972). In one case villus structure had an abnormal configuration for a period of 10 years, until diagnosis and treatment of giardiasis. Following eradication of the parasite normal villus structure returned (Ament and Rubin 1972).

The mechanism of *Giardia* pathogenicity is still incompletely understood. Many theories have arisen throughout the years, including the hypothesis that the sheer intensity of the infection provides a physical barrier to absorbtion (Speghelyi 1940). Others have proposed changes in the pH (Morecki and Parke 1967), competition, toxin production, bile salt decongigation (Smith *et al* 1981, 1982; Solomons 1982) are possible mechanisms. Reports have also mentioned the ability of *Giardia* to invade tissues (Brandborg *et al* 1967; Suteu and Corman 1974; Saha and Ghosh 1977), but the significance of this is unknown. Recent work implies that malabsorbtion is partly due to the blunting of the villi and a decrease in the villus:crypt ratio which combine to produce a marked reduction in the surface area available for absorbtion (Kirkpatrick 1986). Also, evidence in mice infected with *Giardia* suggests that the replacing of columnar enterocytes with cuboidal cells and the increase in sloughing off has some role in malabsorbtion (Anand *et al* 1982).

In his work on the mouse model, Roberts-Thomson *et al* (1976[b]) noticed that mice rechallenged with *G.muris* were 'immune'. Since then the mouse model has been used to show the immunological mechanism. Owen *et al* (1981) showed phagocytosis occured in Peyer's patch epithelium and this is enhanced by opsonization (Radulescu and Meyer 1981). IgA and IgG are made in response to giardial infection (Heyworth 1986) and it is found that expulsion of parasites occurs with the appearance and increase in

secreted IgA (Snider and Underdown 1986). These latter workers also found anti-giardia IgG and IgA in serum up to 10 weeks after clearance of the infection. Natural killer cells are found not to be immportant in parasite elimination (Heyworth *et al* 1986)

The immune response is important in controlling giardiasis as the high prevalence of giardiasis among immunocompromised patients suggests (Hermans *et al* 1976; Gillon and Fergusson 1984). In humans, 80% of individuals make a humoral response to *Giardia* infection (Smith 1984). However the cell mediated response is also very important, particularly the mononuclear leucocytes, and in particular the monocyte fraction (Smith 1984). In *in vitro* experiments, these cells exhibit spontaneous killing of trophozoites. Other experiments have demonstrated that antibody dependent cellular cytotoxicity is also effective against *Giardia* (Smith *et al* 1983).

1.25 Prevalence.

The prevalence of *Giardia* in man varies from 1-30% worldwide depending totally upon local sanitary standards (WHO 1980; Kulda and Nohynkova 1968). All authors agree that the parasite is most prevalent among children between one and five years old. A prevalence rate of between 50-70% among children in this age bracket was recorded by Kulda and Nohynkova (1968). Prevalence rates in travellers have also been compiled, particularly among those going to the Soviet Union where 79% of travellers come down with *Giardia* (Anderson *et al* 1972). For a comparison of prevelances in man see table 2.1.

Table 1.2 Prevalence of *Giardia intestinalis* in human populations

AUTHOR	AREA	AGE	PERCENTAGE[a]	METHOD
Al-Issa *et al* 1986	Iraq	primary children	21.8%	stool *examination*
Nasilowska 1983	Poland	total population	5.0%	stool examination
Nasilowska 1984	Poland	total population	4.6%	stool examination
Lall 1985	Port Blair, Andaman and Nicobar Isl.	total population	30.0%	stool examination
Walia *et al* 1986	Ropar dist. Punjab India	children (6yr ≥6 yr	35.1% 73.1-87.1%	stool examination
Develaux *et al* 1986	6 districts Niger	7-13 yrs	3.5-16.7%	stool examination
Chochillon *et al* 1986	France	men women boys girls	1.25% 0.45% 2.80% 3.70%	stool examination
Giugliano *et al* 1986	Manaus, Brazil	total population	16.4%	stool examination
Miotti *et al* 1986	Arizona Indian reserve Baltimore city Panama rural Peru urban	adults children adults children adults children	44.0% 8 yrs[b] 18.0% NF[c] 48.0% 9-20 yrs[b] 46.0% 6 mths[b]	Solid phase ELISA

a Percentage of population with stools containing *Giardia* cysts or showing positive antibodies to *Giardia intestinalis* when tested with solid phase enzyme linked immunosorbent assay

b Age of children reaching adult levels of antibodies to *Giardia intestinalis*.

c NF = not found

1.26 Diagnosis.

Primarily diagnosis is made upon identification of *Giardia* cysts in a microscopic examination of a faecal specimen. During infection, cysts are shed intermittantly and it is advisable that three separate samples should be submitted for the greatest possibility of identifying an infection (Wolfe 1984). Many different techniques are available for stool examination and differences in sensitivity and specificity have been compared (Thornton *et al* 1983; Zimmer and Burrington 1986). Tests involve examining the stool specimen either fresh or preserved, stained or unstained, direct or concentrated. Steele and McDermott (1977) report greatest success with the method of formal ether sedimentation in compliance with the work of Baughn and Morales (1971). The latter work of Zimmer and Burrington (1986) concludes that $ZnSO_4$ concentration with interferance or phase microscopy yields the best results.

Even after three stool examinations the results may still be falsely negative. Examination of intestinal fluid can then aid diagnosis. Failing that, a duodenal biopsy from the area known as the 'ligament of Treitz' (Kamath and Murugasa 1974) should reveal the parasites (Ament 1972).

Serological tests for determination of *Giardia* infection are available but as of yet are not widely used. Two general approaches are made. Firstly, the testing of serum for antibodies to *Giardia* by ELISA or Immunoflourescence (Visvesvera *et al* 1980; Winieka *et al* 1984). Secondly by detecting *Giardia* antigen in the faeces by counter immunoelectrophoresis (Smith *et al* 1981; Craft and Nelson 1982; Vinyak *et al* 1985), or by ELISA (Ungar *et al* 1984; Green *et al* 1985).

1.27 Treatment.

Wenyon (1915) addressed himself to the problem of treatment siting the case of a man doubly infected with *Entamoeba histolytica* and *Giardia intestinalis*. Treatment with emetine cured the man of the *Entamoeba* infection but had no effect on the *Giardia*. Subsequent treatment with βnapthol reduced the infection, but not for long. The "drastic treatment" used for *Ankylostoma duodenale* was also tried but had no effect.

The answer to Wenyons problem did not arrive until Brumpt (1935) observed the effect of quinacrine on *Giardia*. Since then quinacrine (Atabrine[R1]) has become the "drug of choice" for treatment of giardial infections. Cure rates using quinacrine were initially low (Galli-Valerio 1937), but are now reported at rates between 90-95% (Wolfe 1975;Craft *et al* 1981). Complications with this drug involve some detestable side effects, including nausea, vomitting and bitter taste which are particularly not well tolerated by children (Craft *et al* 1981). Another problem is that it produces a reversable toxic psychosis in 1.5% of patients and thus it is recommended that it should not be administered to patients with a psychotic history (Wolfe 1984).

Metronidazole (Flagyl[R]) is the next most widely used drug. It was introduced in 1961 (Schneider 1961) and is reported to have cure rates of 85-90% (Wolfe 1984). Metronidazole is better tolerated (Kavousi 1979), but has the annoying drawback that it is potentially carcinogenic. However Finegold (1980) after comparing the effect of metronidazole in causing lung tumours in mice and its mutagenic effect on some bacteria, with a retrospective study on recipient humans, concluded, that the claims of carcinogenic activity were unfounded. However, metronidazole should still not be prescribed during pregnancy except in life threatening circumstances.

1 [R] : registered trademark.

Other drugs to be used include Furazolidone (Furoxone^{R}) with a cure rate of 80% (Bassily 1970; Wolfe 1979; Craft *et al* 1981) and Tinadazole (Fasigyn^{R}) which is used widely in Europe with a cure rate of 86-93% (Anderson *et al* 1972, Jokipii 1979). To a lesser extent, the drugs Paromycin (Humatin^{R}), Ornidazole (Tiberal^{R}),and Nimorazole are also used. Of all the drugs mentioned only paromycin is recommended as safe for use during pregnancy.

1.3 Transmission.

As *Giardia* is a gastrointestinal pathogen it is obvious that transmission occurs via the faecal oral route. This can be achieved either directly or indirectly. Only a few *Giardia* cysts are required to be ingested for infection to take place. The work of Rendtorff (1954) demonstrates this in that they found that ten cysts were able to initiate an infection.

1.31 Direct Transmission.

Person to person transmission is a well documented means of *Giardia* transmission, particularly among instituitionalized persons such as children (Ormiston *et al* 1942; Brown 1948; Black *et al* 1977; Woo and Patterson 1986) and the mentally and physically impaired (Yoeli *et al* 1972). The emphasis upon these persons is their lower level of personal hygiene.

Veneral transmission is also very significant among the adult population. Homosexual men are particularly so afflicted although it is also found frequently among heterosexual men and women. The problem, as such, arises due to promiscuity and practices of oral and anal eroticism (Owen *et al* 1979).

Direct transmission may also be important through pets, although this is yet to be confirmed. Dogs could easily be a source, particularly since dogs practice anal licking as a social function and also show affection to their owners by licking their faces. Cats are also a possibility, particularly cats that have litter boxes inside. Another source could be budgerigars which are often allowed to fly freely inside the house. Budgies are found to host a species of *Giardia* not unlike those in humans (Box 1981). Animal resevoirs will be discussed in greater detail later in this chapter.

1.32 Indirect Transmission.

A considerable number of reports occur in the literature on work that has been done on waterborne transmission of giardiasis. The first water implicated outbreak was documented by Moore *et al* in 1969. It is reportedly the most significant means of *Giardia* spread and has been implicated for transmission in travellars to Leningrad (Anderson *et al* 1972; Johnson 1972), trampers (Craun 1979), epidemics (Craun 1979), and in normal endemic transfer (Wright *et al* 1977; Frost and Harter 1980).

Transmission of *Giardia* by food was recognized early this centuary by Musgrave (1922) who noticed that *Giardia* "spores" could be transmitted through vegetables, cereals and other foods. Later, Conroy (1960) reported an outbreak of giardisis with good circumstantial evidence implicating a Christmas Pudding. Osterholm *et al* (1981) also document a case of a foodborne outbreak of giardiasis. Many authors have also shown the presence of cysts in a variety of foods (Malstrandrea and Micarelli 1968; Kasprzak *et al* 1981). Experimentally, starved mice can be infected with *Giardia* by feeding them pellets that have been contaminated with a few trophozoites (Carneri and Trane 1978).
Beer has been tested experimentally for cyst survival and the possibility of being a source of *Giardia* transmission. Results show it is ill-favoured for cyst survival and not likely to be a reservoir (Kuhn and Owades 1985).

Transmission of *Giardia* cysts by flies and cockroaches is feasible according to Wenyon and O'conner (1917), and Macfie (1922). Rendtorff (1954) also experimented with flies in his parasite transmission studies but had negative results. This could, however, have been due to a "low infective" strain of *Giardia* being used (Rendtorff 1978). Kasprzak and Majewska (1981) caught 308 flies and 50 cockroaches from the air and examined them for *Giardia*. They found no cysts in or on the flies, but four of the cockroaches contained cysts indicating a possible source of transmission.

Another possible mode of indirect transmission is suggested by Schuman *et al* (1982). They report a case of giardiasis in a doctor, apparently due to inhalation of dust contaminated by moist faeces on the plaster cast he was removing.

1.33 Animal Resevoirs and Host Specificity.

As previously mentioned the boundaries of *Giardia* host specificity are unknown. Filice (1952) accounted for this lack of knowledge in giving the condition for his three species that it "..is possible that there are physiologically distinct species among forms that are similar morphologically". It is most likely that, as with organisms like as trichomonads, the genus *Giardia* may be comprised of both very host specific and less host specific species (Kulda and Nohynkova 1968). Knowledge of *Giardia* specificity is needed for determination of animal resevoirs. However at present our knowledge of animal hosts dictates our knowledge of host specificity.

The mechanism for determining animal hosts is primarily by cross transmission experiments. The first such study was carried out by Perroncito (1888). His studies, and those of other early workers, however failed to recognize the possibility of naturally occuring host infections (Simon 1922). Cross transmission studies thus need to be carefully monitored for it to be of any value. Grant and Woo (1978) prescribes a standard method for cross transmission experiments in which strict adherance to, would, in his opinion, give conclusive proof of an animal host. Woo and Patterson (1986) following this pattern found that they were unable to produce experimental infection in either SPF mice, adult rats, hamsters, cats, dogs, kittens or puppies with human source *Giardia* cysts. This latter work is in opposition to the work of Davies and Hibler (1979). In their experiments, human source *Giardia* cysts were unable to infect hamsters, domestic rabbits, laboratory mice or deer mice yet the same cysts were able to infect the laboratory rat, gerbils, guinea pigs, beaver, dogs, sheep, and pronghorn antelope. Some of the "susceptable" animals only ever passed cysts for one or two days which is not conclusively indicative of infection (Woo 1984). Most significantly Davies and Hibler also reported that cysts from naturally infected beaver and deer mice were infective for human volunteers.

Many others have found success in cross transmission experiments. Hill *et al* (1983) succeeded in using axenically cultured trophozoites to infect suckling mice not older than 14 days old. In these mice the life cycle was completed and the cysts produced were infective for other mice. Cysts from human sources have also been used to produce infections in rats (Sehgal *et al* 1976; Anand *et al* 1980,1982), mice (Vinayak *et al* 1979; Craft 1982), mongolian gerbils (Belosevic *et al* 1983) dogs (Padchenko and Stolyarchuk 1969; Hewlett *et al* 1982), and rabbits (Schleinitz *et al* 1983).

Upon epidemiological evidence it would seem that human and non human primates share susceptibility to the same strains of *Giardia*. This was demonmstrated by an outbreak of giardiasis among two gibbons, two chimpanzees, a gorilla, an orangutan and three zoo attendants (Center for Disease Control 1979). Cats and dogs have also been implicated as a source of human disease (Davies and Hibler 1979).

At present, beaver carry heavy epidemiological evidence as the "source" of *Giardia* in some waterborne outbreaks (Davies and Hibler 1979; Dykes *et al* 1980). In the Camas outbreak, the source of cysts was traced to beaver droppings in the watershed (Dykes *et al* 1980). Beaver are found to be infected with *Giardia* as neonates and may remain infected for many years. Throughout this time they are found to excrete *Giardia* cysts, and if this is around a water supply intake pipe, it could very possibly be a source of infection (Monzingo Jr. 1985)

1.4 *In Vitro* Cultivation.

1.41 Cultivation.

Methods of cultivation of most intestinal protozoan parasites have existed since the early part of this century (Dobell and Laidlaw 1926), *Giardia* was not succesfully and convincingly cultured untill 1960 (Karapetyan 1960). This was not for the lack of trying! In 1927 Chatterjee reported trophozoites surviving for up to 5 weeks in faeces diluted with normal saline and containing live trichomonads . Poindexter (1931) kept *Giardia* alive for 19 days in a liver extract medium (Boeck-Drbohlavs medium) yet this latter experiment was unable to be repeated (Filice 1952).

Karapetyan (1960) used a complex medium containing a yeast of the genus *Candida* and chick fibroblasts. In this medium *Giardia* were maintained for 7 months. Following cultivations by Karapetyan required no fibroblasts (Karapetyan 1962). Axenic culture was not discovered until 1970 when Meyer (1970) cultured *Giardia* in modified Karapetyans medium with the addition of yeast extract and which had previously had a yeast grown in it within a dialysis sac.

Culture of *Giardia intestinalis* was not truly axenic until the development of HSP-2 (Meyer 1976). HSP-2 initially contained human serum, and was later modified by Visvesvera *et al* (1977) to contain either rabbit or bovine serum and redesignated HSP-3. The next break

came in 1979 with Gillin and Diamond (1979) reporting succesfull growth of *Giardia* in Diamonds TPS-1 medium (Diamond 1968) and TY1-S-33 medium (Diamond *et al* 1978), with the noteworthy addition of L-cysteine monohydrochloride. TPS-1 was modified and used by Visvesvera (1980). TY1-S-33 was modified in 1983 with the addition of bovine bile (Farthing *et al* 1983; Keister 1983). TPS-1 is a richer medium but because of the scarcity of many of the essential ingredients, TY1-S-33 is the medium of choice (Kasprzak and Majewska 1985)

The role of serum is very importatant in the medium, particularly for attachment (Gillin and Reiner 1982) however it is possible to grow *Giardia* in serum free medium (Weiden 1983).

The above culture methods are for *G.duodenalis* type organisms. *G.muris* and *G.agilis* type organisms have not yet been successfully cultured (Meyer and Radulescu 1984).

1.42 Isolation of Giardia.

Giardia trophozoites from human sources can be recovered from duodenal aspirates and biopsies or from excystation of cysts. *Giardia* from animals can be recovered using the previous methods, or by sacrificing the animal and elucidating the trophozoites in high concentration by scraping the small intestine.

1.42.1 Duodenal Aspirates and Biopsies.

This is an ideal means due to the availablity of viable trophozoites. This method involves a little discomfort to the patient, but once retrieved, it is an easy matter of concentraion of the trophozoites by centrifugation at 600-1000xg. This is followed by innoculation into fresh medium and incubation at 37°C (Kasprzak and Majewska 1985; Gordts *et al* 1984,1986).

1.42.2 Excystment

Excystment is first mentioned by Chatterjee (1927) who inoculated cysts of *Giardia* and subsequently cultured motile trophozoites. Poindexter (1931) also got his trophozoites from a cyst source. This latter work came after the publication of Hegner (1927) whose experimentation showed that digestive juices were unneccessary and excystation only required moisture and a temperature of 37°C. Later workers agree indirectly with Hegner, yet all report experiments in which excystation is maximal when cysts are passed through acid similar to gastric pH (Bingham and Meyer 1979; Bingham *et al* 1979).

Bingham and Meyer (1979) report a method in which cysts are initially incubated in HCl at pH 2. The cysts are left in acid for 20 minutes at 37_oC and then washed and resuspended in HSP-3 medium. A maximum exystment rate of 50.9 ± 4.0% was reported using this method .

Rice and Schaeffer (1981) adapted the previous method to give more consistent results. In their method, pure acid is replaced by an induction solution of hanks balanced salts solution supplemented with L-cysteine HCl and glutathione, HCl at pH 2, and sodium bicarbonate. Incubation in this solution is for 20-30 minutes. This is followed by a wash step and incubation in an excystment medium consisting of Tyrodes solution supplemented with trypsin and adjusted to pH 8 with sodium bicarbonate. With this method, excystments of 40-95% are obtained.

The latest excystment method is that of Feely (1986). Fresh hanks balanced salts solution is adjusted to pH 7.2 with sodium bicarbonate and then to pH 2 with 1N HCl. The cysts are incubated for 20-30 minutes, washed and then left to excyst in modified TY1-S-33. Feely reports excystments of 90-96%.

Most times, excystation does occur, however the developing of cultures from these excysted trophozoites is difficult. Many methods occur in the literature particularly aimed at this problem. These include increasing the L-cysteine percentage to 0.2% (Kasprzak and Majewska 1983[a] [b]), and

addition of infant hamster liver (Smith *et al* 1982[b]). Another method involves transfering the cysts to a 1:2 dilution of fresh bovine bile in Tyrodes solution for the "wash step". After 30 minutes incubation in this medium, the cysts are then incubated in TY1-S-33 supplemented with extra bile (Isaac-Renton *et al* 1986).

Another method of excystation is by using animals such as gerbils and experimentally infecting them with cysts (Wallis and Wallis 1986). Trophozoites are then cultured from scrapings of the intestine after killing of the animals. This method, works well, although, as could be expected *G.muris* type organisms which are infective to gerbils were unable to be cultured.

1.43 Use of Cultures.

Axenic cultures have been used for studies of metaboloism (Lindmark and Jarroll 1984), attachment (Erlandsen and Feely 1984; Gillin 1984), immunology (Smith 1984), drug sensitivity (Smith 1982) and strain determination.

1.43.1 Giardia Strains.

Traditionally differention of strains of protozoa is done on morphometric grounds. This involves comparing average distances between major features. In *Giardia* this includes the length and width of the organism, the size of the median bodies, distances between the karyosomes and distances between the flagella, etc.(Grant and Woo 1978; Bertram *et al* 1984[a]). Presently work is being done on determining strains via antigenic analysis such as Enzyme Linked ImmunoSorbent Assay (ELISA) (Moore *et al* 1982; Smith *et al* 1982), High Performance Liquid Chromotography (HPLC) (Moore *et al* 1982), Poly Acrylamide Gel Electrophoresis (PAGE) (Moore *et al* 1982; Smith *et al* 1982), ImmunoElectroPhoresis (IEP), and Crossed IEP, (Smith *et al* 1982[a]). Or by Isoenzyme analysis (Bertram *et al* 1984[a b]; Korman *et al* 1986), DNA

restriction endonuclease banding patterns (Nash *et al* 1985) or Excretory-secretory products (Nash *et al* 1983; Nash and Keister 1985).

These latter methods compare with the morphological approach in that only subtle differences can be detected. However, even though the differences are small, the authors of these methods report that *Giardia* can be grouped by their respective procedure (Nash and Keister 1985; Bertram *et al* 1984; Meloni : personal communication).

Chapter 2.

SURVEY OF THE OCCURENCE OF *GIARDIA INTESTINALIS* WITHIN CATS AND DOGS IN THE CITIES OF PALMERSTON NORTH AND HAMILTON

2.1 Introduction.

Giardia is found in both cats and dogs as it is in most animals. In general it does not cause a symptomatic disease although sometimes infection may correlate to a clinical illness (Brightman and Slonka 1976; Barlough 1979). The main reason for study of the occurence of *Giardia* in cats and dogs is because of *Giardia*'s questionable host specificity. The infection of cats and dogs could provide a resevoir of infection for human disease (Schantz 1983). Surveys may thus indicate whether these animals could be resevoir hosts (Swan and Thompson 1986). The aim of this study was to determine the occurence of *Giardia* in the cat and dog populations within Hamilton and Palmerston North and simultaneously use any *Giardia* isolated in excystation and strain differentiation experiments described later in this thesis.

2.2 Materials and Methods.

2.21 Materials.

Pottels.- 75 ml specimen containers (Gibco N°3002).

1M sucrose solution.

Zeiss phase contrast microscope. No. D 7082.

Haereus Christ Labofuge GL.

2.22 Sampling.

Swan and Thompson (1986) in evaluating the prevalence of *Giardia* of cats and dogs in Perth, obtained faecal specimens from three distinct subpopulations within the community. Samples were obtained from animals in refuge shelters, breeding kennels and from animals kept as pets. This method of sampling was an attempt to find the level of *Giardia* within the whole community. The arrangement of our survey was similar to the above workers.

Pets were sampled from boarding kennels and catteries with samples of dogs in Palmerston North also obtained from hospitalized animals at a veterinary clinic. Breeding Animals, distinct from pets, in high density populations were sampled from selected breeders. Refuge Shelter Animals from the dog pound and S.P.C.A. animal shelters provided specimens from stray and unwanted animals which, according to Swan and Thompson (1986), are usually stressed and living on a lower plane of nutrition.

Animals from refuge shelters and boarding kennels were not examined more than a week after the arrival of the animal. This was to limit the possibility of detecting infections picked up at the institutions.

The survey attempted to get a cross section of the whole community. However, due to the use of boarding kennels, which were restricted in number and whom not all people of every socio-economic grouping use, the true level of *Giardia* in the community may have been missed.

Faecal specimens from animals were collected in pottels with the aid of wooden spatulas. As well as the specimen, information regarding the age, sex, breed and form of the faeces was collected. The area of where the animals within Palmerston North were from was also obtained to determine the distribution of animals being examined and to discover if there was any clustering of cases. This latter information was unavailable in Hamilton.

2.23 Testing.

Many methods of faecal examination are reported within the literature, all with different effectivenesses (Thornton *et al* 1983; Zimmer and Burrington 1986). This laboratory decided upon a modification of the sucrose flotation technique described by Roberts-Thomson *et al* (1976). Although this method may not be as sensitive as zinc sulphate concentration, it provided the extra ability of having cysts already available for the excystation experiments described later in Chapter 3.

The method used is outlined in figure 2.1.

Figure 2.1 Method of Sucrose Floatation.

Sample in pottel

↓

Add water and mix

↓

Decant water fraction into 10 ml plastic test tube

↓

Centrifuge @ 1000 x g

↓

Discard supernatant

↓

Resuspend pellet and layer on 3 ml
of 1 M sucrose in a test tube

↓

Centrifuge @ 400 x g

↓

Remove water fraction to another test tube
and centrifuge @ 1000 x g

↓

Discard supernatant

↓

Resuspend pellet in a drop of water and veiw
@ 400 x with phase contrast microscopy

2.24 Sensitivity and Specificity.

In the literature, many reports mention in detail the intermittency of *Giardia* cyst excretion. Individuals are also shown to have differing excretion patterns (Danciger and Lopez 1975; Burke 1976; Kirkpatrick 1986). Danciger and Lopez (1975) recorded patients as either high, low or mixed excreters. This suggests that a diagnostic test for giardiasis must be able to detect the infection when very low numbers of cysts are present. For this reason the sensitivity and specificity of our sucrose flotation test was measured.

Sensitivity is described as the ability of a test to give a positive result when an animal is diseased. A highly sensitive test will have very few false negative results.

Specificity is described as the ability of a test to give a negative result when an animal is not diseased. A highly specific test will have very few false positive results.
(Blackmore and Harris 1979).

The method used was as follows.

1. A sample of dog faeces, already determined *Giardia* positive, was purified using the sucrose flotation technique, modified by the use of 0.85 M sucrose instead of 1 M sucrose. The cysts were then washed well in distilled water by resuspension and centrifugation until the supernatant was clear. Once cleaned the number of cysts per ml was determined via an haemocytometer.

2. Twenty 5 gram samples from four previously determined *Giardia* negative cat and dog faeces were prepared in 75 ml pottels.

3. Known numbers of cysts were added to the twenty samples by a person unrelated to the experiment. This was done to satisfy any possible bias.

4. The standard sucrose flotation technique described previously was performed on all samples and the results aligned with the addition of cysts or water.

2.3 Results.

2.31 Sensitivity and Specificity.

The number of cysts added were varied to give an overall picture of the cyst recovery ability. At 5 x 10^4 / 5 g faeces, most were detected. Below this there was no difference between the number of cysts present and the percentage isolation. Overall 7 out of 20 were detected as positive of which one was not correlated to the addition of cysts. The results can be seen in tables 2.1 and 2.2.

Table 2.1 Analysis of Sensitvity and Specificity results of Sucrose Floatation Method of Faecal examination. Part I

Innoculation	N° of samples	N° of *Giardia* detected	Percent detection
Water only	5	1	20%
1 x 10^2 cysts	5	2	40%
1 x 10^3 cysts	3	1	33%
5 x 10^3 cysts	4	1	25%
5 x 10^4 cysts	3	2	67%

Table 2.2 Analysis of Sensitvity and Specificity results of Sucrose Floatation Method of Faecal examination. Part II

	Cysts added	Cysts not added	Total
Test +ve	6	1	7
Test -ve	9	4	13
Total	15	5	20

Sensitivity of the test can be determined as the percent detection for each value of cysts added (table 2.1) or as a whole, i.e.

Sensitivity = 6 ÷ 15 = 0.4 or 40%
with a False negative rate = 1 ÷ sensitivity = 60%

Specificity can be determined also.

Specificity = 4 ÷ 5 = 0.8 or 80%
with a False positive rate = 1 ÷ specifictiy = 20%

These sensitivity results indicate that this method of faecal examination will detect at least 40 % of all specimens submitted, in which *Giardia* cysts are in concentration of 5×10^4 cysts / 5 gram of faecal material or less. Above this level of cysts the method should detect 100% as the result of 5×10^4 / 5g indicates.

The specificity value of 80 % implies that in every 100 positive isolates, 20 would be falsely so. However if this should occur during the survey, all isolates are reexamined for later experiments and any false positive results would be resolved.

This experiment reveals that our test is not very sensitive at levels below 5 x 10^4 cysts / 5 g faeces. However there is no comparison test that has been done that proves that this is unusual to other diagnostic tests.

2.32 Survey Data.

The occurence of *Giardia* within dogs in Palmerston North was determined during autumn (March to June) 1987. A total of 142 samples were taken in this time of which 11 or 8 ± 0.2 % were positive for *Giardia*. Most of the positive samples came from the refuge shelter animals (72%). The distribution of animals within the separate subpopulations was not determined as representing the community as a whole but is probably close to the actual constitution. Table 2.3 shows the results of this survey among this population.

119 cats in Palmerston North were sampled during the same period as the dogs. 8 of these, or 7 ± 0.2 %, were excreting *Giardia*. Once again refuge shelter animals had the highest occurence of *Giardia*. (table 2.4)

Cats and dogs in Hamilton were sampled the last week before spring officially began (August) 1987. 81 dogs and 72 cats were sampled with the occurence of *Giardia* being 25 ± 0.5 % and 3 ± 0.2 % respectively. The separate results are shown in tables 2.5 and 2.6.

Table 2.3. Distribution of *Giardia* within dogs in Palmerston North according to age, sex and subpopulation.

		Boarding Pets			Vet Clinic Pets			Breeding			Refuge Shelter		
Age	Sex	*Giardia* +ve	-ve	Total	*Giardia* +ve	-ve	Total	*Giardia* +ve	-ve	Total	*Giardia* +ve	-ve	Total
> 3y	M	1	7	8	-	7	7	-	5	5	1	1	2
	F	-	20	20	-	8	8	-	5	5	1	5	6
1-3y	M	-	4	4	-	-	-	1	1	2	1	1	2
	F	-	9	9	-	2	2	-	2	2	2	3	5
< 1y	M	-	7	7	-	7	7	-	-	-	3	13	16
	F	-	3	3	1	1	2	-	5	5	-	10	10
un-known	M	-	-	-	-	3	3	-	-	-	-	-	-
	F	-	-	-	-	2	2	-	-	-	-	-	-
TOTAL		1	50	51	1	30	31	1	18	19	8	33	41
PERCENTAGE		1/51 = 2%			1/31 = 3%			1/19 = 5%			8/41 = 20%		
STANDARD DEVIATION		0.05			0.019			0.03			0.068		
STANDARD ERROR		0.005			0.003			0.011			0.011		
OVERALL TOTAL		11 / 142 = 8%			S.D. = 0.02			S.E. = 0.002					

Table 2.4. Distribution of *Giardia* within cats in Palmerston North according to age, sex and subpopulation.

		Boarding Pets			Breeding			Refuge Shelter		
Age	Sex	*Giardia* +ve	-ve	Total	*Giardia* +ve	-ve	Total	*Giardia* +ve	-ve	Total
> 3y	M	-	10	10	-	3	3	-	3	3
	F	-	8	8	-	13	13	-	-	-
1-3y	M	1	6	7	-	4	4	1	4	5
	F	-	11	11	-	1	1	1	8	9
< 1y	M	-	3	3	-	-	-	2	7	9
	F	-	2	2	1	2	3	1	9	10
un-known	M	-	4	4	-	-	-	-	5	5
	F	-	3	3	-	-	-	1	5	6
TOTAL		1	48	49	1	23	24	6	41	47
PERCENTAGE		1/48 = 2%			1/24 = 4%			6/47 = 13%		
STANDARD DEVIATION		0.02			0.04			0.05		
STANDARD ERROR		0.003			0.008			0.007		
OVERALL TOTAL		8 / 119 = 7% S.D. = 0.02 S.E. = 0.002								

Table 2.5. Distribution of *Giardia* within dogs in Hamilton according to age, sex and subpopulation.

		Boarding Pets			Breeding			Refuge Shelter		
Age	Sex	*Giardia* +ve	−ve	Total	*Giardia* +ve	−ve	Total	*Giardia* +ve	−ve	Total
> 3y	M	1	6	7	1	3	4	−	1	1
	F	1	−	1	−	12	12	−	2	2
1−3y	M	1	3	4	−	4	4	5	−	5
	F	−	4	4	1	3	4	−	2	2
< 1y	M	−	3	3	−	4	4	4	3	7
	F	1	2	3	1	5	6	4	4	8
TOTAL		4	18	22	3	31	34	13	12	25
PERCENTAGE		4/22 = 18%			3/31 = 9%			13/25 = 52%		
STANDARD DEVIATION		0.08			0.055			0.01		
STANDARD ERROR		0.02			0.01			0.02		
OVERALL TOTAL		20 / 81 = 25% S.D. = 0.045 S.E. = 0.005								

Table 2.6. Distribution of *Giardia* within cats in Hamilton according to age, sex and subpopulation.

		Boarding Pets			Breeding			Refuge Shelter		
Age	Sex	*Giardia* +ve	−ve	Total	*Giardia* +ve	−ve	Total	*Giardia* +ve	−ve	Total
> 3y	M	−	12	12	−	1	1	−	2	2
	F	−	11	11	−	2	2	−	1	1
1−3y	M	−	2	2	−	5	5	−	3	3
	F	−	4	4	−	5	5	−	8	8
< 1y	M	−	1	1	−	1	1	−	1	1
	F	−	2	2	−	2	2	−	5	5
TOTAL		−	32	32	2	18	20	−	20	20
PERCENTAGE		0/32 = 0%			2/20 = 10%			0/20 = 0%		
STANDARD DEVIATION		−			0.07			−		
STANDARD ERROR		−			0.015			−		
OVERALL TOTAL		2 / 72 = 3%			S.D. = 0.02			S.E. = 0.002		

A comparison of the results shows that between Hamilton and Palmerston North, occurence of *Giardia* among dogs are significantly different ($p < 0.01$) although there is no difference among cats. Cats and dogs on the other hand are not significantly different from each other in Palmerston North but are in Hamilton ($p < 0.01$).

Tables 2.7 - 2.10 show the occurence of *Giardia* amongst these populations as related to the age and sex of the animals. No significant difference was found between male and female animals although animals less than 3 years old are more likely to be infected with *Giardia* ($p < 0.01$). In nearly all cases, animals in refuge shelters are more likely to be infected with *Giardia* than those in stable homes ($p < 0.01$)

Also obtained was information on the breed of dogs in relation to the infection of *Giardia*. Table 2.11 shows this relationship along with the relative risk. No significant difference was found among the breeds ($p < 0.05$).

Table 2.7. Occurence of *Giardia* in dogs according to Sex, Area and Sample population

	Palmerston North			Hamilton		
Population	Male	Female	Total	Male	Female	Total
Pets	3%	2%	2%	14%	25%	18%
Breeders	14%	0%	5%	8%	9%	9%
Refuge Shelters	25%	14%	20%	69%	33%	52%
TOTAL	11%	5%	8%	31%	19%	25%

Table 2.8. Occurence of *Giardia* in cats according to Sex, Area and Sample population

	Palmerston North			Hamilton		
Population	Male	Female	Total	Male	Female	Total
Pets	4%	-	2%	-	-	0%
Breeders	-	6%	4%	-	15%	10%
Refuge Shelters	14%	12%	13%	-	-	0%
TOTAL	8%	6%	7%	0%	5%	3%

Table 2.9. Occurence of *Giardia* in dogs according to Age, Area and Sample population

	Palmerston North			Hamilton		
Population	⟨ 1 y	1-3 y	⟩ 3 y	⟨ 1 y	1-3 y	⟩ 3 y
Pets	1	-	1	2	1	1
Breeders	-	1	-	1	1	1
Refuge Shelters	3	3	2	8	5	-
Total *Giardia*	4	4	3	11	7	2
Total Animals	50	24	66	31	23	27
No infected	8%	16%	4%	35%	30%	7%
Relative Risk	1.0	2.8	0.4	2.0	1.4	0.2

The relative risk mentioned in this table and the one following is an indication to the possibility of an individual, within a particular group of animals, being infected with *Giardia*.

Table 2.10. Occurence of *Giardia* in cats according to Age, Area and Sample population

	Palmerston North				Hamilton		
Population	< 1 y	1-3 y	> 3 y	unknown	< 1 y	1-3 y	> 3 y
Pets	-	1	-	-	-	-	-
Breeders	1	-	-	-	-	-	-
Refuge Shelters	3	2	-	1	2	-	-
Total *Giardia*	4	3	-	1	2	-	-
Total Animals	26	37	37	18	16	27	29
No infected	14%	8%	-	-	13%	-	-
Relative Risk	3.7	1.3	0	-	high*	0	0

* exact number unobtainable due to division by zero.

Table 2.11. Distribution of *Giardia* within dog Breeds.

Breed	*Giardia* +ve	Total	% inf.	R.R
G.Shepherd	6	16	38 %	2.64
G.Shepherd X	2	11	19 %	1.14
G.Shepherd Doberman X	3	6	50 %	3.32
Doberman	2	6	33 %	2.10
Bord. Collie	1	8	13 %	0.77
C.Spaniel	1	2	50 %	3.17
Dacshound	1	1	100 %	6.40
Cavalier King Charles	1	7	14 %	0.86
Labrador	3	19	16 %	0.96
Beardie	3	4	75 %	5.04
Rhodesian Ridge back	3	7	43 %	2.84
Mongrel	3	16	18 %	1.10
Husky	1	1	100 %	6.40
Toy Poodle	1	8	13 %	0.77
Other Breeds	-	80	-	-
TOTAL	31	192	16 %	-

Information collected on the relationship between infection and diarrhoea (table 2.12) showed no significance between faeces form and *Giardia* infection.

Table 2.12. Relationship of faecal form and *Giardia* infection.

	Dogs			Cats		
	Formed	Semi Formed	No Form	Formed	Semi Formed	No Form
Giardia +ve	16	12	3	6	3	1
Giardia -ve	110	59	24	139	30	12
Total	126	71	27	145	33	13
Attack rate	13 %	17 %	11 %	4 %	9 %	8 %
Relative risk	2.55	1.37	0.77	0.46	2.00	1.58

Other pathogens were detected and found in high numbers within the animals sampled. Table 2.13 shows the occurence of these pathogens within all animals sampled and the relationship of these pathogens to the finding of *Giardia*. No statistical relationship was found between *Giardia* and other pathogens. 1 cat and 3 dogs were infected with more than two pathogens.

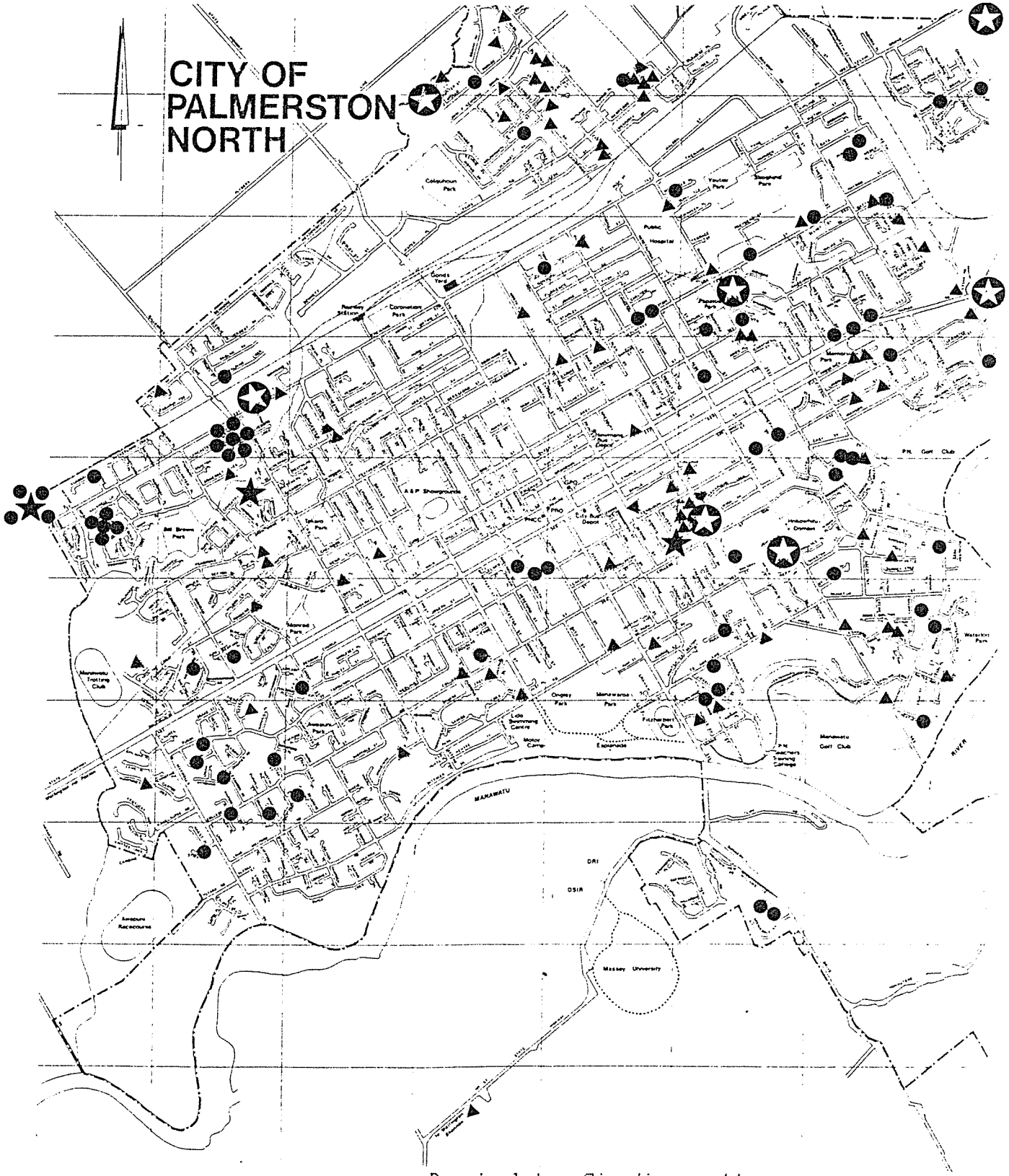
Table 2.13. Other pathogens found and their relationship to infection with *Giardia*.

	Dogs				Cats			
	Giardia +ve	*Giardia* -ve	Total	% age	*Giardia* +ve	*Giardia* -ve	Total	% age
Sarcocystis sp.	5	13	18	8%	1	6	7	4%
Worm eggs*	4	13	17	8%	2	8	10	5%
Cysto-isospora	2	3	5	2%	-	9	9	5%
Total Pathogens	11	29	40	18%	3	23	26	14%
No other Pathogens	23	163	186	-	8	157	165	-

* Nematodes of the species Toxocara and Toxoascaris.

A map of the available locations of all samples taken in Palmerston North was made to distinguish if the distribution pattern of the samples was random and to determine if any clustering of the positive isolates had occured (figure 2.2). The results show no clustering of *Giardia* although a large area of Palmerston North was unsurveyed, perhaps due to the use of a boarding kennels on the other side of the city.

Figure 2.2 Map of Palmerston North showing location of dogs and cats sampled including those which are positive for *Giardia*



● = Dog isolates *Giardia* negative.

★ = Dog isolates *Giardia* positive.

▲ = Cat isolates *Giardia* negative.

✪ = Cat isolates *Giardia* positive.

2.4 Discussion.

2.41 Sensitivity and Specificity.

The determination of the sensitivity and specificity of our test provided us with an indication to the usefullness of our test. Although only a maximum of 1 x 10^4 cysts per gram were determined, it was possible to see that in the higher range of cyst excretion most, if not all, samples are detected. In the lower range of excretion at least 40% of samples are detectable.

A possibile explanation of this work can be found in relating it to the pattern of cyst excretion given by Kirkpatrick (1986). In this work, *Giardia* excretion of a cat is detailed for a period of 60 days. 37 of these days are undetectable or less than 1 x10^4 cysts per gram of faeces. If this is combined with our reults, 40% of these 37 days are able to be detected, so that in reality 22 days or 36 % of the infections, maybe missed. Such an assumption however would require more study, utilizing a comparison of the available tests with a complete evaluation of the number of cysts excreted during infection to as low as one or two cysts.

The biggest problem with this test, and is in fact a problem with any floatation test, is the appearance of high concentrations of fat in the faecal specimens. The fat floats on the sucrose solution and generally makes concentration of the cysts much more difficult. One of the samples used in this sensitivity test contained alot of fat. This lead to not many samples being determined from that batch of faeces and may have given rise to the conservatively low test result. Obviously the ideal test is one that is exempt from this and other anomalies of faecal specimens and is capable of determining infections even in the event of low numbers of cysts. Various antigenic analysis such as described by Visvesvara *et al* 1980; Smith *et al* 1981; Ungar *et al* 1984; Winieka *et al* 1984; Green *et al* 1985; Vinyak *et al* 1985 are all future possibilities.

2.42 Survey of Cats and Dogs.

The survey shows that *Giardia* is a common parasite of cats and dogs within New Zealand. The predominant source of *Giardia* among these animals are those less cared for such as stray and stressed animals.

Significant results in this survey included the difference in occurence of *Giardia* in dogs between Hamilton and Palmerston North. This could have been due, either to the difference in geographical locality, or due to the differences in time that the occurence was surveyed i.e. between autumn and spring. The differences in season are known to affect man and the likelihood of them affecting animals is also very high. In general, it is found that during summer, when people drink more untreated water, giardiasis is more prevalent (Craun 1984). Whatever the reason, the occurence of *Giardia* among the dogs of Hamilton was high.

In comparison, cats were expected to follow the pattern shown by the dogs. However the occurence of *Giardia* in cats from Palmerston North was significantly higher than that of Hamilton. The reason for this is due to the refuge shelter cats in Hamilton being surprisingly free of *Giardia* perhaps indicating a subtle difference in the source of animals coming into the institution.

In comparison to the rest of the world, we find the occurence of *Giardia* in dogs to vary in the literature from between 1 and 60% (Zimmer and Burrington 1985) and in cats from 1 - 14% (Swan and Thompson 1986). Both the cats from Palmerston North and the dogs from Hamilton are near the higher levels of occurence mentioned in the literature. However, as mentioned previously, comparisons can only be made when consideration of season is taken into account and this is not possible.

As with other workers (Swan and Thompson 1986) we found no relationship between *Giardia* infection and sex or breed of the animal. *Giardia* was, however, more likely to appear in animals less than 3 years old.

No correlation was found between symptomatic disease (evaluated as the form of the faeces) and *Giardia* excretion. However it is likely that, as with humans, animals have intermittent periods of diarrhoea and that some were in fact suffering. This was observed during the course of these studies when a dog excreting cysts, and which seemingly had normal faeces, was later observed under the full blown constraints of a case of diarrhoea. Whether this was due to the *Giardia* or some other cause is unknown.

As well as *Giardia* we detected *Sarcocystis* species, and *Cystoisospora* species of Coccidia as well as species of *Toxocara* and *Toxoascaris* nematode worms in both cats and dogs. The appearance of these pathogen was unrelated to *Giardia* infection but was of significant proportions, i.e. 4 and 8 %, 5 and 2 %, and 5 and 8 % respectively for cats and dogs.

2.5 Conclusion.

The method of sucrose floatation was found to be suitable for a survey. It was used to find values of 8 % and 7 % in dog and cats within Palmerston North and obtained values in Hamilton of 25 % for dogs and 3 % for cats. In light of other work, the occurence of *Giardia* among the Hamilton dogs and the cats of Palmerston North were higher than average. Comparing the values obtained in this work, only that of the Hamilton dogs is significantly different from the others ($p < 0.01$). No relationship was found between breed or sex of the animal to *Giardia* infection, but, animals less than 3 years old were more likely to be infected ($p < 0.01$). Cats and dogs were also found to be infected with a number of other pathogens of significantly high percentage yet unrelated to dual infections with *Giardia*.

Chapter 3.

ISOLATION AND CULTIVATION OF *GIARDIA INTESTINALIS*.

3.1 Introduction.

Giardia intestinalis is not an easy organism to culture (Meyer 1979; Kasprzak and Majewska 1983,1985) and many attempts at cultivation do fail. This chapter sets out to describe the techniques used within this laboratory to isolate and routinely culture *G.intestinalis*.

3.2 Materials and Methods.

3.21 Materials.

3.21.1. TY1-S-33 Growth Medium.

Standard medium was usually made up to one litre although, with adjustment to the constituent weights, other volumes were also made. In making the medium, the following ingredients were added in order to 500 ml of distilled water.

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 N°C-7880)	1.5 g
Ferric Ammonium Citrate (Brown Pearls)	0.023g
L-Ascorbic acid	0.2 g
NCTC 135 (Gibco N°440-1100) or NCTC 109 (Difco No.5927-23)	100 ml
Bile Bacteriological (Sigma N° B 8381)	0.8 g
Benzyl Penicillin	0.06g
Gentamycin sulphate	0.05g
Vancomycin	0.02g
Bovine serum	100 ml
Distilled water	up to 1 litre

Once all the ingredients were added, the volume was made up to one litre with distilled water and the medium mixed using a magnetic stirrer. After mixing, the medium was adjusted to pH 7.0-7.2 with 1 M NaOH. Following this it was sterilized by positive pressure membrane filtration (De Jong Gorredijk B.V. / Sartorius G m.b.h). Clarification of the medium was, however, first necessary and was achieved by the use of non sterile 5.0µm and 0.45µm pore membranes. This removed large particles that clogged the 0.2µm membranes. Sterilization was achieved through 0.2 µm pore membranes that had been sterilized by autoclaving at 121°C for 15 minutes within the filter unit. Sterile medium was stored at 4°C until used or for a maximum of 10 days.

Bovine serum was initially acquired through GIBCO (NZ). but was later obtained through Waitaki freezing works and processed by ourselves.

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, at a final concentration of 10 µg/ml, was used.

3.21.2. Hanks Balnced 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	800ml
2	$CaCl_2$	2.8g
	Distilled water	100ml

Mix solutions 1 and 2 and make up to 1000 ml with distilled water. Add 2 ml chloroform and store at 4°C.

Solution B	$Na_2HPO_4.12H_2O$	3.04g
	KH_2PO_4	1.2 g
	Glucose	20.0 g
	Distilled water	800ml

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 and store at 4°C.

To make working strength HBSS.

Add 100 ml of solution A to 100 ml solution B and make up to 1 litre with distilled water. Sterilise either by membrane filtration or autoclaving at 121 °C for 15 minutes.

3.22 Isolation of *Giardia*.

Attempts at *Giardia* isolation were made from human and animal cysts from 11 distinct populations within New Zealand. Human source cysts were kindly provided by the pathology laboratories of Hamilton, Palmerston North, Hastings, New Plymouth, Whangarei, Blenheim and Tauranga. Animal source cysts came from *Giardia* positive cats and dogs mentioned in Chapter 2. All cysts were purified as described previous in Chapter 2. The excystation method required that the cysts were free of as much of the contaminating debris as possible and hence were washed many times. The purified cysts were then used in one or both of the following two methods of excystation.

3.22.1 *In vivo* excystation using suckling mice.

Wallis and Wallis (1986) report the use of gerbils as an *in vivo* method of *Giardia* excystation. These workers orally innoculated gerbils with cysts from human and animal sources. They used the trophozoites of the resultant infection, when applicable, to initiate an *in vitro* culture. The method used by this laboratory was similar except, instead of gerbils, suckling mice were used (Hill *et al* 1983; Vinayak *et al* 1979).

Initially New Zealand White mice were used until gross contamination by *Tritrichomonas muris, Hexamita muris* and ***Giardia muris*** rendered the use of these mice impossible. Cf1 Ib, an outbred specific pathogen free strain of mice were then employed until the end of the experiment.

To begin with, suckling mice were tested for susceptibility to *Giardia* cysts and axenically grown trophozoites. Once this was established, the cyst excretion pattern was determined as was the optimum age for inoculation of the mice. Mice from 4 days to 4 weeks old were intubed with 0.05 - 0.1 ml of cultured *Giardia* trophozoites or purified cyst suspension via 24 gauge tubing (Tygon microbore tubing, Cole and Porter - J 6417-21) connected to a 1 ml insulin syringe as described by Hill *et al* (1983). They were then left to incubate. *Giardia* infection

was determined by the collection of faeces every second day. Once retrieved the faeces were weighed and a known volume of water added. The sample was then mixed thoroughly and the number of cysts enumerated via an haemocytometer. Collection of faeces continued until no more cysts were found.

For *in vivo* excystation, mice were intubed as described above and, 6 days after being entubed, were monitored for *Giardia* infection by faecal examination. Examination of faeces continued every 2 days whether cysts were found or not, until the mouse was killed and the intestines examined about day 10 - 16 after being entubed.

Mice were killed by anaethetizing them with di-ethyl ether followed by cervical dislocation. Their intestines were then examined by excising the duodenum and jejunum and using one of the following methods. **1.** Washing the intestine by forcing cold HBSS supplemented with 0.2% l-cysteine HCl (for the purpose of an aid to attachment) through the lumen, or **2.** scraping the excised intestine in a glass petri dish filled with cold supplemented HBSS, or **3.** vigorously shaking the intestine in a universal bottle with 8 mls of cold supplemented HBSS for 5 minutes. Of the three methods, the latter was found to be the most effective. Once in solution, the intestinal contents were transfered to Kimax tubes and incubated horizontally for 20 minutes. During this time, most of the trophozoites, if present, attached to the surface of the tube. This incubation step determined whether or not the mouse had been infected with *Giardia*. If trophozoites were present the medium was discarded and the *Giardia* washed with warm fresh TY1-S-33 growth medium. Warm medium was used so as to not disrupt the attached trophozoites. The tube was then filled and incubated on an incline to the horizontal at 37°C.

3.22.2 *In vitro* excystation.

Two methods of *in vitro* excystation were used.

Method of Rice and Schaeffer 1981.

Into a 10 ml centrifuge tube a low pH induction medium was prepared. This comprised of 5 ml aqueous hydrochloric acid at pH 2.0, 2.5 ml of HBSS supplemented with 29 mM l-cysteine HCl and 67 mM glutathione, and 2.5 ml freshly made 0.1 M sodium bicarbonate. To this, 0.5 ml of cyst suspension was added and the tube incubated capped for 30 minutes in a 37°C waterbath. After incubation the suspension was centrifuged at 1000xg for 2 minutes and the supernatant discarded. The cysts were then washed, resuspended in excystation medium, and incubated inclined to the horizontal at 37°C in a 9 ml screw capped Kimax test tube completely filled with medium. This last step differed from the above workers who do not report attempts to culture *Giardia* using this method.

Method of Feely 1986.

The method of Feely also utilized a two step process. Firstly fresh HBSS was made and adjusted to pH 7.2 with sodium bicarbonate. Following this it was adjusted to pH 2.0 with 1M HCl. The cysts were then added to this solution in a centrifuge tube and incubated uncapped at 37°C for 25 minutes. Washing and incubation occured as described above although incubation was usually in both tubes and cavity slides (for determination of excystation percentage, see later).

The medium used as excystation medium varied during the course of these studies. Initially it was complete TY1-S-33 as described by Keister (1983) but this was soon modified by the addition of an extra 0.2% l-cysteine HCl (Kasprzak and Majewska 1983, 1985). Later due to an observable difference between excystation medium made with bovine serum and that made with foetal calf serum, another modification was made, that of using a 50/50 bovine and foetal calf serum mix in the excystation medium.

3.23 Development of a new excystment medium.

The aim of this experiment was to confirm the observations seen during some early excystations. In the presence of bovine serum newly excysted trophozoites were seen to attach but were unable to grow. On the other hand, those in the presence of foetal calf serum were not able to attach although multiplication of the trophozoites was occuring. In both cases, the trophozoites only survived a short while.

The method in this experiment employed making three batches of medium with the only difference being in the constitution of the serum component. Media was supplemented with either foetal calf serum (Sigma N° 200-6140), bovine serum or a 50/50 mix. Cysts were then induced, as described previously, and incubated in the different media both in slides (see later) and tubes. A strain of *Giardia*, already in culture, was used as a control to determine the quality of the medium for *Giardia* growth.

3.24 Determination of excystation percentage.

A modification of Bingham and Meyer (1979) was used in which a few drops of cysts in the excystation medium were transfered to cavity slides which were then sealed by piping a ring of vaseline around the cavity and covering with a cover slip. After an hour of incubation the slides were examined for total numbers of cysts and the number of empty cysts at 400 x magnification on a normal phase contrast microscope.

This method of using slides was also employed in the development of the new excystation medium and to photograph excystation taking place. Photography utilized a Leitz Ortholux 2 microscope with a Wild photoautomat MPS45 camera attachment. Tech pan black and white film was then used.

3.25 Culture of Isolates.

Kasprzak and Majewska (1985) describes TY1-S-33 (Diamond 1978) supplemented with bile (Keister 1983; Farthing *et al* 1983) as the medium of choice for the culture of *Giardia intestinalis*. This medium was used for the routine maintenance of the cultures in this laboratory.

Giardia isolates were maintained in culture vessels completely filled with medium. This helped to provide the anaerobic environment which *Giardia* require for growth. For normal maintenance, 9 ml Kimax screw capped test tubes were used. 50 ml plastic flasks (Nunc 1,63371) and 40 ml flasks (Falcon N° 3012) were also used for this purpose but primarily they were used, along with 15 and 20 ml Kimax tubes, for harvesting large numbers of trophozoites.

Tubes of *Giardia* were grown horizontally within a water jacket incubator at 37 °C. After three days, the inside surface of the tube was normally covered in a monolayer of attached trophozoites and was ready to be subcultured. Subculturing involved the method of Day (personal communication) in which the growing medium was discarded and replaced with 2 ml of cold sterile HBSS and the tube vigourously rolled between the hands. The combined physical stresses brought about detachment of the trophozoites from the glass. With motile trophozoites now in suspension, three to four drops, which equaled to about 1 - 5 x 10^5 cells, were transfered aseptically to two sterile tubes which were subsequently filled with different batches of TY1-S-33 medium and incubated at 37°C.

Subculturing was carried out every day so that, at any one time, we had cultures at various stages of growth.

Where the normal level of antibiotics did not control bacteria, the medium was discarded and the tube washed several times with warm medium containing extra antibiotics as described in the materials section. After washing, the tube was filled with this medium and incubated at 37°C. The process was repeated the next day if necessary.

If tubes did not form a monolayer by day three, the old medium was discarded and the tube refilled with fresh medium. This usually ensured healthy trophozoites ready to be subcultured the next day.

Cryopreservation was used to provide a backup store of cultures in case any were lost.

3.26 Cryopreservation of cultures.

The method of Phillips *et al* (1982) was used to preserve the cultures in liquid nitrogen. This involved harvesting trophozoites at the late log phase of growth by putting tubes of three day old cultures in ice water for 10 minutes. This detached the trophozoites which were then concentrated at 700 x g for 5 minutes. The pellet was resuspended in TY1-S-33 and the concentration of trophozoites estimated by use of a haemocytometer. The cell suspension was then adjusted to 1.2-1.5 x 10^6 trophozoites per ml with medium. 0.25 ml of this suspension was placed in 1 ml Nunc cryopreservat tubes (No.366656) and 0.25 ml of a 15% solution of Dimethyl Sulfate (DMSO) in TY1-S-33 was added slowly. The tubes were then mixed and wrapped in paper and tissue and placed within a polystyrene container which was then placed within the -80°C freezer. The wrapping up of the tubes was reduced the rate of freezing, protecting the trophozoites from the violent effects of rapid cooling. When frozen some of the tubes were transferred to liquid nitrogen and the rest were stored in the -80° freezer.

A modified method of Phillips *et al* (1982) and Wallis and Wallis (1986) was used to retrieve the cultures. This was accomplished by quickly thawing the cultures in a 37°C water bath, and transfering them to a Kimax tube which was imediately filled with medium and incubated horizontally at 37° for 15 - 25 minutes. During this time, healthy trophozoites attached to the tube. After they had attached, the medium was discarded and the tube refilled and reincubated. This latter medium was discarded 4 hours later to ensure that all the DMSO was out of the medium and cell membranes. The tubes were then refilled with fresh

medium and left for three days before either subculturing, or refilling them.

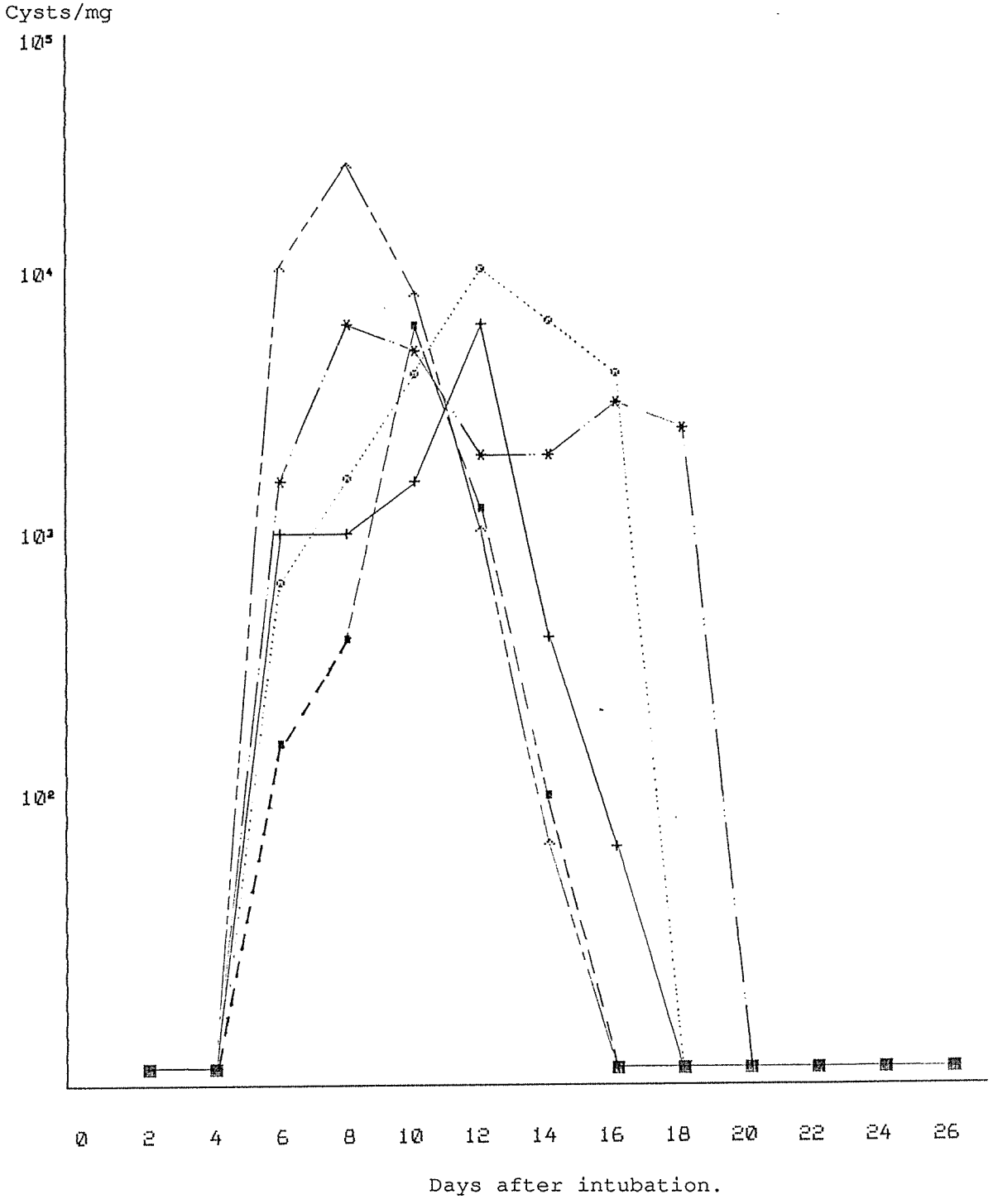
Retrieval was carried out a week after freezing to check that the process had worked.

3.3 Results.

3.31 *In vivo* excystment.

Both cysts and trophozoites were infective for suckling mice and in both a pattern of excretion could be seen lasting from day 6 to day 18 after intubation. On average, the highest excretion rate, 7 x 10^3 cysts per milligram of faeces, was produced on day 10 (Figures 3.1 and 3.2). All mice, no matter when intubed, relinquished the infection when they were about 25 - 30 days old. The optimum age was found to be between 8 to 14 days old. Any younger than this and the ability to intube the mice was manifoldly more dificult and the retrieval of faeces impossible. Older than this and the length of infection was reduced, as mentioned above, making isolation of *Giardia* more difficult.

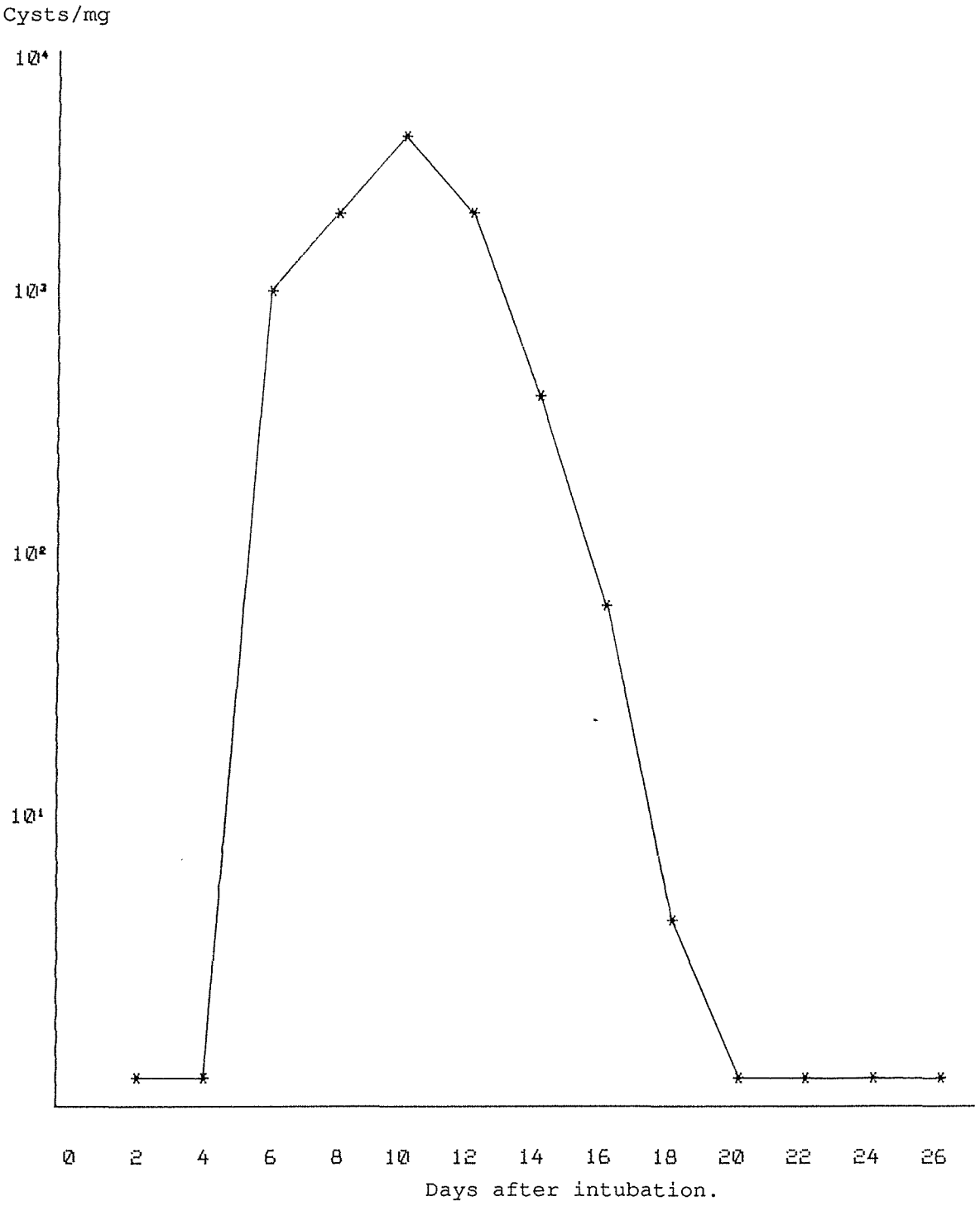
Figure 3.1. Graph of cyst excretion per milligram of faeces versus time in days after a single dose of 10^5 trophozoites of axenically grown *Giardia intestinalis*, strain P.Nth/86/MUGU/3 in five mice of the same litter.



■ = indicates all samples, unless otherwise shown, at undetectable levels

▪ / * / ° / ^ / + = individual mouse excretions.

Figure 3.1. Graph of average cyst excretion per milligram of faeces versus time in days after a single dose of 10^5 trophozoites of axenically grown *Giardia intestinalis*, strain P.Nth/86/MUGU/3 in five mice of the same litter.



Samples from 6 of the 11 populations were exposed to mice for the purpose of *in vivo* excystation. 2 out of 30 or 7% of these samples became cultures (table 3.1).

None of the samples used in mice were able to be cultured using the *in vitro* methods.

Table 3.1 *In vivo* excystment of *Giardia intestinalis*. using suckling mice

Area	No. Samples	No. +ve Infections	Culture +ve
Palmerston Nth	ND*	-	-
Hamilton	8	-	-
Hastings	4	3	2
Whangarei	ND	-	-
New Plymouth	ND	-	-
Tauranga	ND	-	-
Blenheim	ND	-	-
Dogs P.Nth.	9	-	-
Dogs Ham.	5	-	-
Cats P.Nth.	3	-	-
Cats Ham.	1	-	-
Total	30	3	2

* Not Done.

3.32 *In vitro* excystment.

Table 3.2 shows the results of *in vitro* excystation. In it, it can be seen that not all samples of cysts were able to be excysted in vitro and that the excystation percentage varied widely. This was mainly due to the variety of ages of the cysts. In vitro excystation to culure also yielded a total of 7%, that being 6 cultures from 88 samples.

Table 3.2 *In vitro* excystment of *Giardia intestinalis*.

Area	No. Samples used	No. +ve Excystments	Excystment Percentage	Culture +ve
Palmerston Nth	3	3	ND*	1
Hamilton	42	30	0.5% - 40%	2
Hastings	13	11	2% - 97%	1
Whangarei	2	2	30% - 70%	1
New Plymouth	2	1	ND	1
Tauranga	1	1	ND	-
Blenheim	1	1	10% - 40%	-
Dogs P.Nth.	11	4	10% - 30%	-
Dogs Ham.	4	1	0 - 10%	-
Cats P.Nth.	8	3	10% - 40%	-
Cats Ham.	1	1	1%	-
Total	88	58	0 - 97%	6

* ND = Not determined.

Photographs of *in vitro* excystment of *Giarida* .

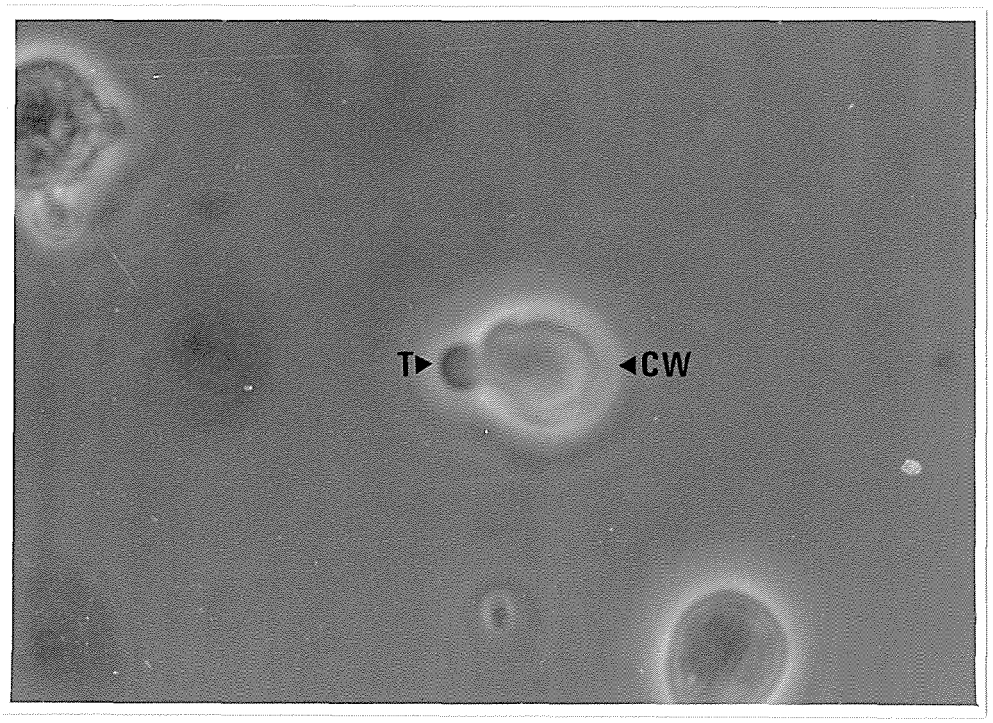
Plate 3.1. 15 minutes after resuspension in excystment medium. Cysts beginning to show signs of excystation. A small bud exudes from the cyst.

Key:

T. = Trophozoite

CW. = Cyst Wall.

Plate 3.2. 20 minutes after resuspension in excystment medium. The bud begins to take form. Flagella movement begins.


T
CW

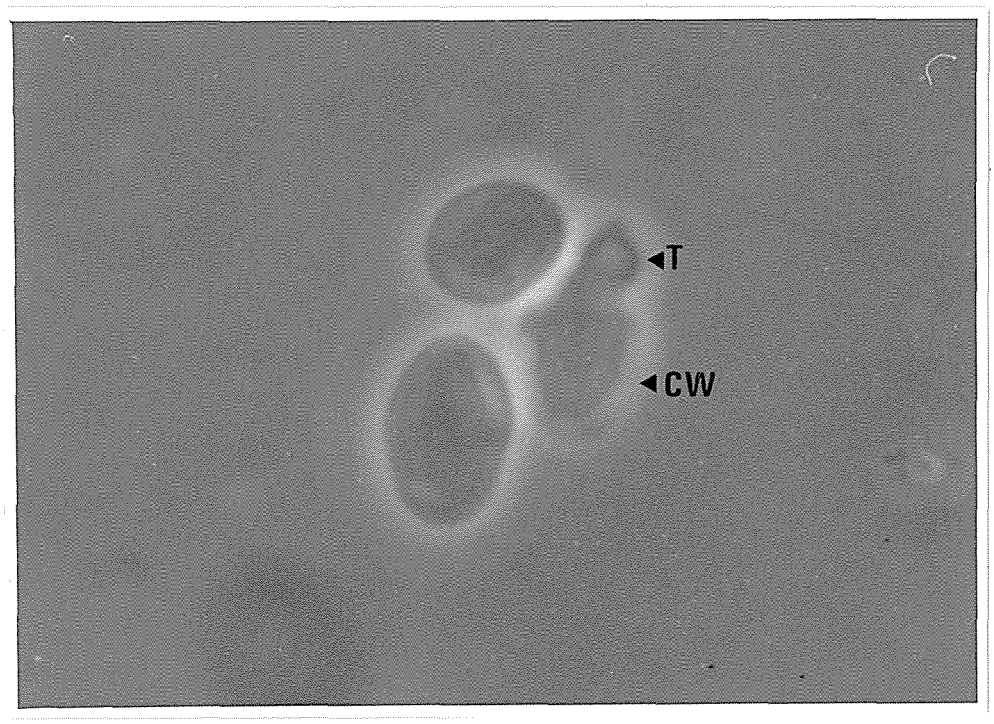
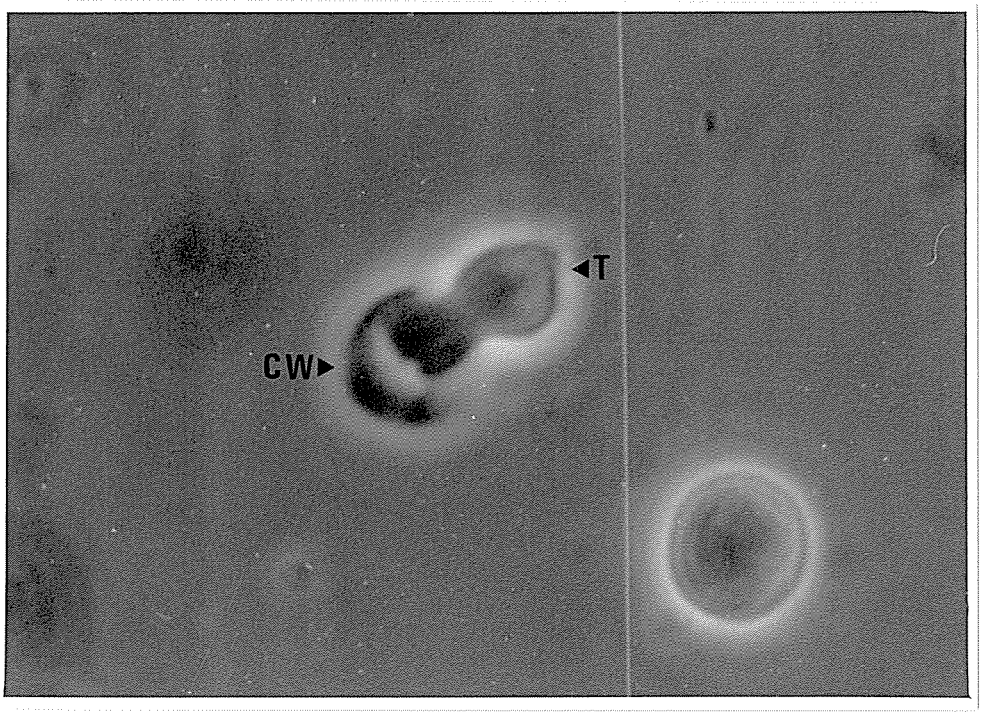

T
CW

Plate 3.3. 25 minutes after resuspension in excystment medium. Flagella movement is strong. The trophozoite is almost fully excysted.

Plate 3.4. 30 minutes after resuspension in excystment medium. One of two things may happen. Either the trophozoite disconects from the cyst wall and swims around or as in this photograph it stays connected until it divides in two. Either way, division eventually takes place.


◀T
CW▶

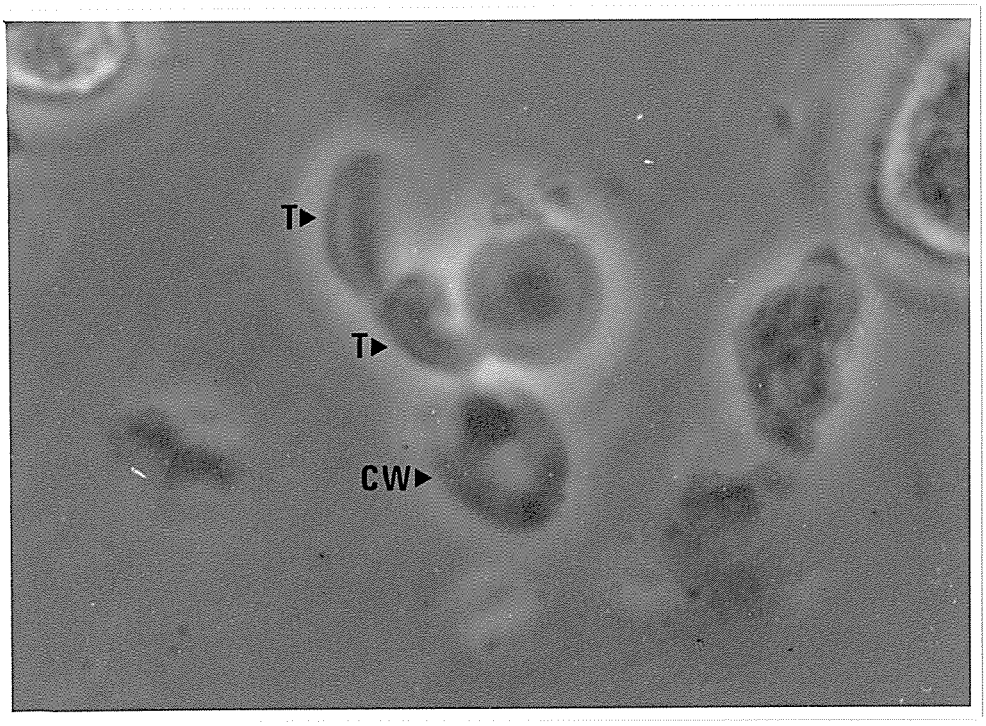
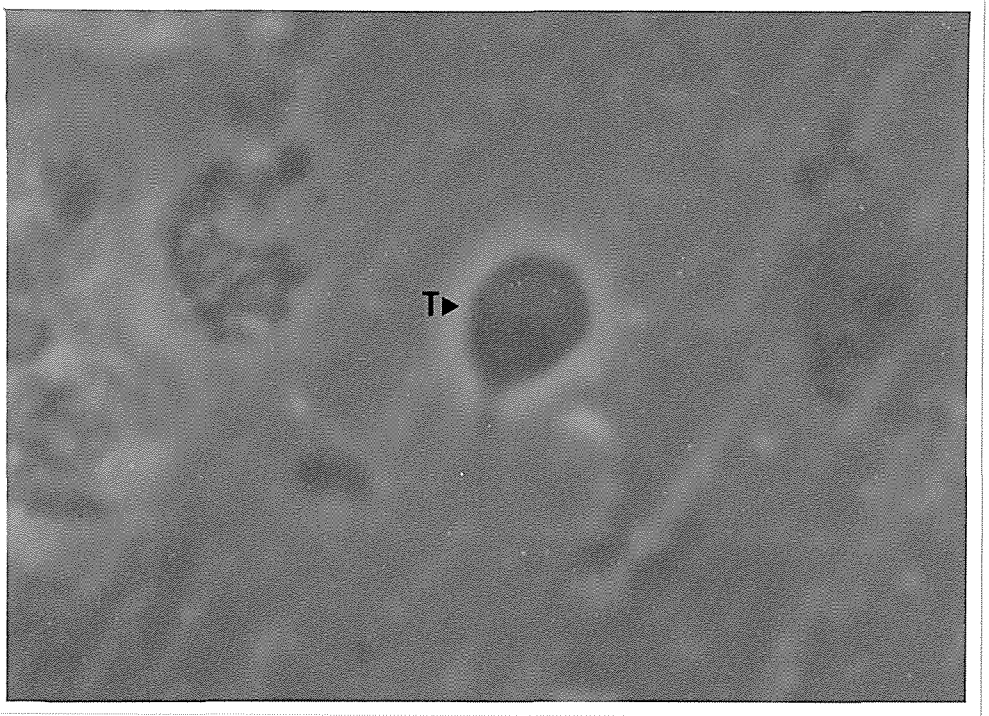

T▶
T▶
CW▶

Plate 3.5. 35 minutes after resuspension in excystment medium. Once divided the trophozoites attach to the substrate and grow.

Plate 3.6. In the case of many excystments, particularly with dogs, some, if not all, the trophozoites died upon excystment.


T▶

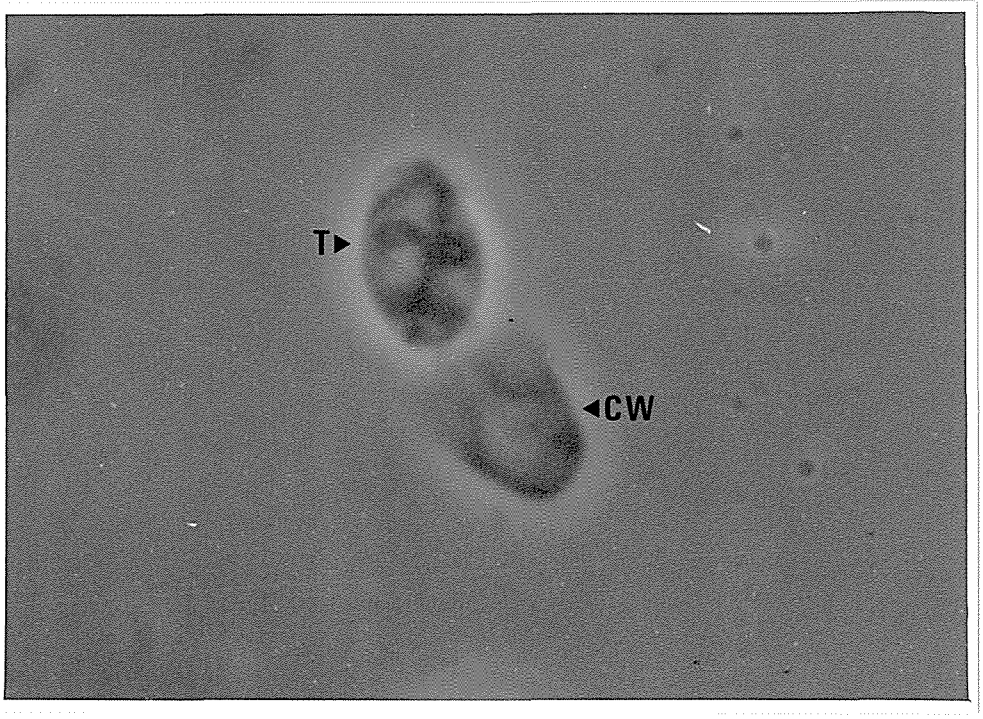

T▶
◀CW

Table 3.3 shows the result of the effect of different sera on excystment. The means of assesment was by determining the actual number of attachments and deaths within a cavity slide, and by determining the relative level of growth and attachment found in the culture tubes. Only excysted trophozoites were assessed using the cavity slide.

The results were evaluated over three days and suggest that the mixing of the sera protected the trophozoites from death, as well as increasing attachment and growth. Foetal calf and bovine sera, on their own, were unable to bring about culture of the excysted culture wherby the mixed sera did. The control culture died in the medium with foetal calf serum, yet was unaffected by the serum mixture. Overall it was found that the use of foetal calf serum by itself was hazardous to *Giardia* cultivation yet, when mixed with bovine serum, it provided a growing medium well suited to the isolation of *Giardia*.

Table 3.3 Effect of Bovine and Foetal calf serum upon excystation.

	Bovine serum		Foetal calf serum		Bovine/Foetal mix	
	Control culture	Excyst culture	Control culture	Excyst culutre	Control culture	Excyst culture
Day 1 slide	ND	30% att 30% dead	ND	10% att 10% dead	ND	30% att 10% dead
tube	Gro ++ Att ++	Gro + Att ++	Gro ++ Att ++	Gro ++ Att +	Gro + Att +++	Gro + Att ++
Day 2 slide	ND	30% att 30% dead	ND	6% att 60% dead	ND	15% att 15% dead
tube	Gro ++ Att ++	Gro + Att ++	Gro ++ Att +	Gro ++ Att -	Gro ++ Att +++	Gro + Att ++
Day 3 slide	ND	- all dead	ND	- all dead	ND	10% att 25% dead
tube	Gro +++ Att ++	Gro + Att ++	Gro ++ Att +	Gro + Att -	Gro +++ Att +++	Gro ++ Att +++
Outcome	OK	Died	Died	Died	OK	Eventual culture

Key:

Gro = growth

Att = Attachment.

Dead = Non viable.

ND = Not determined.

Pluses indicate relative levels of attachment and growth, +++ is better than ++ which is better than +.

3.33 Culture of Isolates.

In summary, 8 cultures were obtained from 91 samples on which 171 attempts had been made. Samples came from 11 distinct populations within 7 areas of New Zealand. A total sample to culture percentage of 9% was obtained. An outline of the samples processed can be seen in table 3.4.

Table 3.4 Summary of excystment of *Giardia intestinalis*.

Area	No. Samples	No. of attempts	Culture +ve
Palmerston Nth	3	10	1
Hamilton	42	55	2
Hastings	13	37	3
Whangarei	2	12	1
New Plymouth	2	6	1
Tauranga	1	1	-
Blenheim	1	8	-
Dogs P.Nth.	11	19	-
Dogs Ham.	7	9	-
Cats P.Nth.	8	12	-
Cats Ham.	1	2	-
Total	91	171	8

Table 3.5 outlines the eight strains isolated within this laboratory and includes the methods of isolation, the time to the first monolayer to appear and the strain designation. As can be seen by the strain designation number (see key) most of our isolations occured during the later part of this work. The culture supplied to us from Boreham in Brisbane Australia is included for completeness.

All strains were from symptomatic human cases and were successfully cultured except P.Nth/86/MUGU/3 and Ham/87/MUGU/30 which were lost due to bacterial contamination.

3.34 Cryopreservation.

Trophozoites were examined one week after storage at -80°C and liquid nitrogen temperatures. Upon thawing, trophozoite movement averaged 70% within the strains with a range of 50 to 85%. Reculturing from both temperatures was possible as long as the cells were well washed, by the method described, of DMSO which exerts a killing effect if left in the tube. In this relation, the wash step 4 hours after setting up the cultures, was found to be vital. Attachment for 25 minutes yielded the highest number of viable trophozoites.

Legend:

1/ Methods

A. Rice and Schaeffer (1981). Standard TY1-S-33 used as excystment medium.

B. Feely (1986). Standard TY1-S-33 used as excystment medium.

C. Feely (1986). TY1-S-33 modified with the addition of an extra 0.2% L-cysteine HCl and with a 50/50 mix of bovine and foetal calf sera used as excystation medium.

D. Duodenal isolation from an infected suckling mouse. TY1-S-33 modified with the addition of an extra 0.2% l-cysteine HCl and with a 50/50 mix of bovine and foetal calf sera was used as isolation medium.

E. Duodenal isolation from an infected symptomatic child

2/ Designated according to Boreham (1984). MUGU stands for Massey University Giardia Unit, 87 is the year of recovery and 3 stands for the sample number processed in this laboratory

3/ Includes incubation time within the mouse. Between 10 -14 days.

4/ Imported culture from Boreham Brisbane Australia.

Table 3.5 Result of excystment and culture of *Giardia intestinalis*.

Area	Sample Label	Method of Excystment[1]	Time to first sub-culture	Strain designation[2]
Palmerston Nth	2	A	2 weeks	P.Nth/86/MUGU/3
Hamilton	18	B	2 weeks	Ham/87/MUGU/30
	37	C	2 weeks	Ham/87/MUGU/84
Hastings	6	D	4 weeks[3]	Hast/87/MUGU/68
	10	D	7 weeks[3]	Hast/87/MUGU/76
	11	C	3 weeks	Hast/87/MUGU/86
Whangarei	1	C	5 weeks	Whan/87/MUGU/48
New Plymouth	2	C	3½weeks	N.Pl/87/MUGU/77
Brisbane[4]	1	E	-	Bris/83/HEPU/106

3.4 Discussion.

3.41 Excystation.

The excretion pattern of the mice was found to be similar to that of Vinayak (1979) who used cysts.

Mice provided a useful and effective means of *in vivo* isolation of *Giardia*. The use of live animals, however, presented many problems. Our research showed that suckling mice 8 - 14 days old were the best for use, and also that young cysts were preferable to old ones. This meant a coordination between cyst supply and mouse supply was required. Due to the irregularity of supply, many times old cysts were given to young mice or vice versa decreasing the chance of isolating a culture. Another problem was the contamination of the mice with other parasites. *Hexamita muris, Tritrichomonas muris* and *Giardia muris* were all found in this regard. These parasites made establishment of infections of *G. intestinalis* difficult. Wallis and Wallis (1986) report experiencing the same problems when working with gerbils.

In vitro excystment, as mentioned, provided the same ratio of success as mice with many more samples proceessed. The adjustment to the medium by the mixing of sera was effective, although, the result seen may have been purely an artefact of the particular batch of foetal calf serum we obtained. An important part of the isolation of *Giardia* was found to be in persistent refilling of the culture tube every three days. This is not a new discovery as Kasprzak and Majewska (1985) also report the necessity of perserverance, indicating that most cultures appear only after several weeks.

Overall the excystation percentages, and the ability to form a culture showed no direct corelation. Cultures were made from cyst supplies that produced both very high and very low excystation percentages (see also Kasprzak and Majewska 1985). In comparing the methods of Feely (1986) and Rice and Schaeffer (1981) in terms of excystation percentage, the variation in cysts supplied to us was so variable, no comparison could be made.

Isolation and culture of *Giardia* from animals was unsuccessful. Reports of *Giardia* cultivation from cats (Meyer 1970; Nash *et al* 1985; Meloni - personal communication) and dogs are available. Cultivation from the dogs, however, was only possible using an *in vivo* method of isolation (Wallis and Wallis 1986). *In vitro* excystation and cultivation using trophozoites from duodenal aspirates from dogs has been unsuccessful (Meloni - personal communication). The isolates we processed, revealed similar results. All dog trophozoites that excysted did not last longer than a couple of hours, for what reason we are not sure, perhaps the *Giardia* of dogs resembles that of mice, which also cannot be cultured. With the cats there was a chance of culture and, although no culture eventuated, some trophozoite lasted seven days.

3.42 Culture of Isolates.

During the course of these studies the subculturing routine was modified as seen fit with a need for exceptional aseptic technique. Two types of contaminant were occasionally encountered, fast growing contaminants and slow growing contaminants. Fast growing contaminants appeared overnight and were generally responsible for the complete overgrowth and death of that particular tube. In two severe cases, all tubes of three of the strains P.Nth/86/MUGU/3, Bris/83/HEPU/106, and in a separate occasion, Ham/87/MUGU/30, were overcome by fast growing bacteria, which although undergoing strenuous recovery operations still ended with the death of these cultures. Slow growing contaminants of yeast, actinomycetes and *Pseudomonas* were also encountered and although they still posed a threat to the health of the culture, they were able to be controlled.

Problems with different batches of media constituents were encountered but their effects were negligable.

Cryopreservation, as developed was found a useful tool in the storage of *Giardia* both in liquid nitrogen and in the - 80°C freezer. We found it particularly important to leave at least 25 minutes for the *Giardia* to attach before discarding the medium and to wash the trophozoites four hours later.

3.5 Conclusion

8 human source strains of *Giardia* were established. Isolates of cats and dogs were unable to be made. The use of *in vivo* and *in vitro* methods of excystment provided effective means of isolation as long as the medium was suitable for growth and persistant refiling of the excystment tubes was maintained.

Giardia intestinalis is a difficult organism to culture from the environment the biggest problem being bacterial contamination. Aseptic technique and antibiotics are therefore of vital importance in control of bacteria. Cryopreservation early in the isolation of strains can ensure the preservation of the cultures if overwhelming bacterial contamination occurs.

Chapter 4.

COMPARISON OF *GIARDIA INTESTINALIS* ISOLATES BY GROWTH RATE AND TOTAL PROTEIN ANALYSIS BY SDS-PAGE.

4.1 Introduction.

Comparing and determining strains of *Giardia* is important in the determination of host specificity and the zoonotic potential of giardiasis (Woo 1984). Strain differentiation has traditionally been by morphometric and morphological means (Grant and Woo 1978; Bertram *et al* 1984[b]) but methods of antigenic, genetic and metabolic differentiation have lately been experimented with (Smith *et al* 1982; Moore *et al* 1982; Bertram *et al* 1984[a]; Nash and Keister 1985; Nash *et al* 1985). This study used two methods of differentiation utilizing two sections of the organism. Firstly the growth rate and secondly a comparison of total proteins of the organism. It was intended in this Chapter to compare isolates from humans, cats and dogs but, as described in Chapter 3, only human source *Giardia* were isolated.

4.2 Materials and Methods.

4.21 Materials.

4.21.1 Phosphate Buffered Saline (PBS).

	NaCl	8.5 g
	$Na_2HPO_4.12H_2O$	13.5 g
or	Na_2HPO_4 anhydrous	5.35g
	$NaH_2PO_4.12H_2O$	0.32g
	Distilled water	up to 1 litre

PBS was made by mixing the above ingredients and sterilizing at 121°C for 15 minutes.

4.21.2 Preparation of Coomasie Brilliant Blue Protein Reagent.

Coomasie Brilliant Blue G-250	100 mg
95% ethanol	50 ml
85% (w/v) phosphoric acid	100 ml
Distilled water	up to 1 litre

The dye was dissolved in a mixture of the ethanol and phosphoric acid and made up to 1 litre with distilled water. The solution was then filtered through two layers of Whatman N° 1 filter paper and stored in the dark at room temperature.

4.21.3 Preparation of SDS-PAGE Solutions.

Running Gel Acrylamide

Acrylamide	30.0 g
Methylene bis acrylamide	0.5 g
Distilled water	up to 100 ml

Stacking Gel Acylamide

Acrylamide	30.0 g
Methylene bis acrylamide	1.6 g
Distilled water	up to 100 ml

Acrylamide solutions were prepared by dissolving the acrylamide in 70 ml of distilled water. The methylene bis acrylamide was then dissolved and the solution made up to 100 ml.

Lower Tris Buffer

Trizma base	18.17 g
10% SDS in distilled water	4.0 ml
12M HCl	to pH 8.8
Distilled water	up to 100 ml

Upper Tris Buffer

Trizma base	6.06 g
10% SDS in distilled water	4.0 ml
12M HCl	to pH 6.8
Distilled water	up to 100 ml

Trizma base was added to 70 ml of distilled water and the pH was adjusted to the appropriate valus with HCl. SDS was added and the solution made up to 100 ml with distilled water. The pH was then checked and adjusted.

Ammonium Persulphate

Ammonium persulphate	0.1 g
distilled water	up to 1.0 ml

A fresh solution was prepared immediately prior to use.

Tris-Glycine Resevoir Buffer (pH 8.3)

Trizma base	6.07g
Glycine	28.8 g
SDS	2.0 g
Distilled water	up to 2 litres

SDS Sample Buffer

2-mercaptoethanol	10.0 ml
SDS	6.0 g
Upper tris buffer	25.0 ml
Distilled water	up to 100 ml

Bromophenol Blue Tracking Dye

Bromophenol Blue	0.05 g
Glycerol	40.0 ml
Distilled water	up to 50 ml

Isopropanol Stain

Isopropanol	250 ml
Glacial acetic acid	100 ml
Coomasie Brilliant Blue R-250	0.4 g
Distilled water	up to 1 litre

Storage.

The above solutions were stored at 4°C except for the tracking dye and the isopropanol stain which were stored at room temperature.

4.22 Growth Rates.

7 human isolates, 6 from this laboratory and 1 from Brisbane, Australia, were used for this and the folllowing experiments. The *Giardia muris* used in the total protein analysis by SDS-PAGE were isolated from naturally infected mice by the method described in Chapter 2.

For this experiment, 14 tubes of TY1-S-33 growth medium per isolate were prepared. To this, 1 $x10^5$ trophozoites were added. Trophozoites were harvested in late log phase of growth by immersing a culture tube in ice water for 10 minutes and when detached, enumerating the number of *Giardia* by an haemocytometer. Tubes were then incubated horizontally at 37°C.

At the same time each day, duplicate tubes of each isolate were retrieved and placed in ice water for 10 minutes to detach the trophozoites. When detached, the tube was thoroughly mixed and the number of trophozoites per ml was evaluated with the use of an haemocytometer. With the information gained, a graph of growth was plotted and the growth rate was determined over the area of logrithmic growth by the equation:

$$\text{Growth Rate} = \frac{1}{G} = \frac{\log n_t - \log n_o}{\log 2.(t_t - t_o)} = \text{generations / hour}$$

where : t_o = time at origin.

t_t = time at second reading.

n_o = number of cells at time o.

n_t = number of cells at time t.

G = Generation time in hours.

4.23 SDS-PAGE Comparison of Total Proteins.

Moore *et al* (1982) and Smith *et al* (1982) used SDS-PAGE to determine the number of protein bands in each isolate and therefore the difference between isolates. Moore *et al* (1982) used an acrylamide gel percentage of 9%. When this experiment was repeated with a concentration of 10%, it was found that it did not adequately express the banding pattern of *Giardia*. Determination of the best percentage of acrylamide that would give reproducable results was therefore first assayed. Once this was established, the isolates were run parrallel to determine any differences between the strains.

The method of preparation and running of SDS-PAGE gels is as described by Ionas (1983).

A. Estimation of Protein Content.

Preparation of Standard Curve.

To prepare the standard curve a range of bovine serum albumen solutions from 0-100µg per 0.1 ml were made in 0.2 M NaOH. These samples were then sealed with tape and placed in a boiling water bath for 3 minutes to simulate the treatment the *Giardia* proteins would recieve. When cooled, 5 ml of Coomasie Brilliant Blue protein reagent was added and mixed by inversion. The absorbance of the samples was then read at 595 nm using a Cecil 272 spectrophotometer. Each new batch of Coomasie Brilliant Blue protein reagent required a new standard curve to be prepared.

Estimation of Protein Content of Samples.

An alliquot of cell suspension was diluted 1/3 and 1/10 in 0.2M NaOH. 100µl of each dilution was placed in a boiling water water bath for 3 minutes to solubilise the proteins. Once the samples had cooled 5 ml of Coomasie Brilliant Blue protein reagent was added and the absorbance read at 595 nm. The protein content of the diluted sample was then read off the standard curve.

B. Preparation and Pouring of Acrylamide Gel.

Preparation of Glass plates.

The apparatus and technique used for SDS-PAGE was that of Ionas (1983), where the vertical gel slab was polymerised between two glass plates. One of the glass plates was a rectangle measuring 13 cm by 17 cm and 0.55 cm thick. The second was the same size but had a notch 2 cm deep and 10 cm long cut 1.5 cm from the corner on one of the shorter sides. Cleanliness of the plates is essential so the plates were washed well before use with detergent, plenty of hot water and followed by rubbing with alcohol soaked tissue. A thread of petroleum jelly was then piped from a syringe along the three straight sides of the notched plate, approximately 0.5 cm from the edge. Three 0.15 cm thick perspex spacers were then pressed into position over the threads of petroleum jelly. Another thread of petroleum jelly was then piped upon these spacers and the other plate was pressed into position. To facilitate gel pouring the plates were clamped on to a vertical perspex stand using bulldog clips. The notched plate was placed outermost and upright.

Preparation and Pouring of Running Gel Acrylamide.

Acylamide gel percentages were tried at 10%; 15% with glycerol; 15% without glycerol; and 12.5% without glycerol. The latter concentration gave the best results and was used for the rest of the gels. Running gel acrylamide for the appropriate concentrations was prepared by adding the following solutions in the order given and then adequately mixing.

	Acrylamide concentrations			
	10%	15%	15%	12.5%
Lower tris buffer	5.0 ml	5.0 ml	5.0 ml	5.0 ml
Running gel acrylamide	6.7 ml	10.0 ml	10.0 ml	8.3 ml
Distilled water	8.3 ml	-	5.0 ml	6.7 ml
40 % Glycerol	-	5.0 ml	-	-
Ammonium persulphate	0.1 ml	0.05ml	0.05ml	0.1 ml
N N N'N'-tetramethylethylenediamine	0.01ml	0.05ml	0.05ml	0.01ml

The resulting solution was poured, avoiding air bubbles, between the plates to a depth of 11 cm. Immediately, the solution was gently overlayed with distilled water. This provided conditions sufficiently anaerobic to enable complete polymerisation of the acrylamide solution. The gels were left 45-60 minutes at room temperature for polymerisation to occur.

Preparation and Pouring of Stacking Gel Acrylamide.

A stacking gel solution was prepared by adding the following solutions in the order given, ensuring adequate mixing after after each addition.

Upper tris buffer	2.5 ml
Stacking gel acrylamide	1.5 ml
Distilled water	6.0 ml
Ammonium persulphate	0.5 ml
N N N'N'-tetramethylethylenediamine	0.01ml

Water and any unpolymerised acrylamide were poured off the running gel. The exposed surface was washed twice with aliquots of freshly prepared stacking gel acrylamide and the space above the gel was filled with the remaining gel solution. A perspex comb (with 10, 7.5 mm wide teeth, 3mm apart and 17mm long) was inserted between the glass plates into the stacking gel, leaving a gap of 8 mm between the running gel and the teeth of the comb. It is essential to avoid getting air bubbles on the teeth of the comb as this creates air bubbles in the stacking gel. Polymerisation of the stacking gel required 5-10 minutes at room temperature. Once polymerisation was complete the comb was carefully removed and the wells washed with tris-glycine resevoir buffer.

The plate/gel apparatus was then removed from the vertical stand and the bottom spacer was withdrawn.

Next, a thick thread of petroleum jelly was applied around the buffer portal to the upper reservoir on the electrophoresis apparatus. Another thread was applied midway between the upper and lower reservoirs. This

was to provide a liquid-tight seal between the chambers, thus ensuring that the current would flow through the gel. The plate/gel apparatus was then pressed onto the electrophoresis stand with the notch in the plates open to the buffer portal of the upper reservoir. The plates were then secured to the stand by bulldog clips. Once in place, the resevoirs were filled with Tris-glycine reservoir buffer and the bubbles of air eliminated from the lower gel surface by a syringe with a bent needle full of Tris-glycine reservoir buffer.

C. Preparation of Protein Samples.

Growth of samples for SDS-PAGE.

Giardia cultures were grown in large Kimax screw capped test tubes in TY1-S-33 for three days. When the cultures were at late log phase the medium was poured off and the tubes refilled with cold Phosphate Buffered Saline (PBS). This facilitated the detachment of the trophozoites. Once detached, the tubes were centrifuged at 700 x g for 10 minutes. The supernatant was discarded and the pellets of individual tubes of each isolate were pooled and washed in PBS. Finally the cells were resuspended in a small volume of PBS and stored at -80°C.

Sample Preparation for SDS-PAGE.

To prepare the sample for SDS-PAGE the cells were lysed and the proteins solubilised by boiling for 3 minutes in the following:

Cell suspension	100 µl
SDS sample buffer	25 µl
Bromophenol blue tracking dye	12.5 µl

When cooled, samples containg either 5, 10, 15, 20, 30, 40, 50, 60, 70, 80 or 90 µg of protein, as determined by the previous method, were applied to each track to ascertain the optimum loading. 50µg was eventually established as the optimum for characterization and was used in the comparison of the cultures.

D. Running of the Gel.

Electrophoresis of the Protein Sample.

After all the samples were added a current of 15 mA was applied to the gel until the tracking dye reached 1 cm from the bottom of the running gel. It took a total of 4.5 - 6 hours for this to take place.

Staining Protein Bands in Gels.

On completion of electrophoresis the gel was removed from the apparatus. The two perspex spacers were withdrawn and the glass plates levered apart. A segment of the lower left hand corner of the gel was then removed to enable correct orientation of the gel once stained. The gel was then carefully allowed to fall off the plate into a container of isopropanol stain. This was left rocking over night at room temperature.

Destaining the Gel.

Stain was decanted off the gel and the gel was covered in 10 % aqueous acetic acid and left to rock. Every 2 - 3 hours the destaining solution was replaced with fresh 10 % acetic acid until the background of the gel was clear. The gel was then washed with distilled water until no acetic acid smell remained. A permanent record of the gel was made by photographing the gel with 5" x 4" techpan film.

As well as the comparison of the 7 isolates, an isolate of *G. muris* and a human strain (Hast/87/MUGU/76) isolated from an experimentally infected mouse were compared with axenic Hast/87/MUGU/76. The method of obtaining these isolates was as it is described in Chapter 2. The preparation was the same as for the axenic *Giardia.*

This method was also used to compare cyst samples that had been purified. They were treated and run with a comparison trophozoite sample. The concentration of cysts, and thus protein, was low so the results shown are only an indication of the differences that exist.

4.3 Results.

4.31 Growth Rates.

Table 4.1 and figure 4.1 show the differences observed between the seven isolates of *Giardia intestinalis*. Statistically with a mean of 0.09 and a standard deviation of 0.01 there is no difference between the isolates ($p < 0.05$). The difference between N.P1/87/MUGU/77 and Whan/87/MUGU/48 is significant although in relation to the other isolates, the results do not lend themselves to strain differentiation.

Table 4.1 Growth rate and Generation time among 7 isolates of *Giardia intestinalis*.

Strain	Growth rate gen. / hour	Generation time in hours
Ham/87/MUGU/84	0.096	10.39
Hast/87/MUGU/68	0.093	10.78
Hast/87/MUGU/76	0.093	10.77
Hast/87/MUGU/86	0.080	12.47
Whan/87/MUGU/48	0.079	12.61
N.P1/87/MUGU/77	0.109	9.2
Bris/83/HEPU/106	0.080	12.46

Legend

■ = indicates all samples, unless otherwise shown.

#——# = Bris/83/HEPU/106

^—··—^ = N.Pl/87/MUGU/77

°——° = Whan/87/MUGU/48

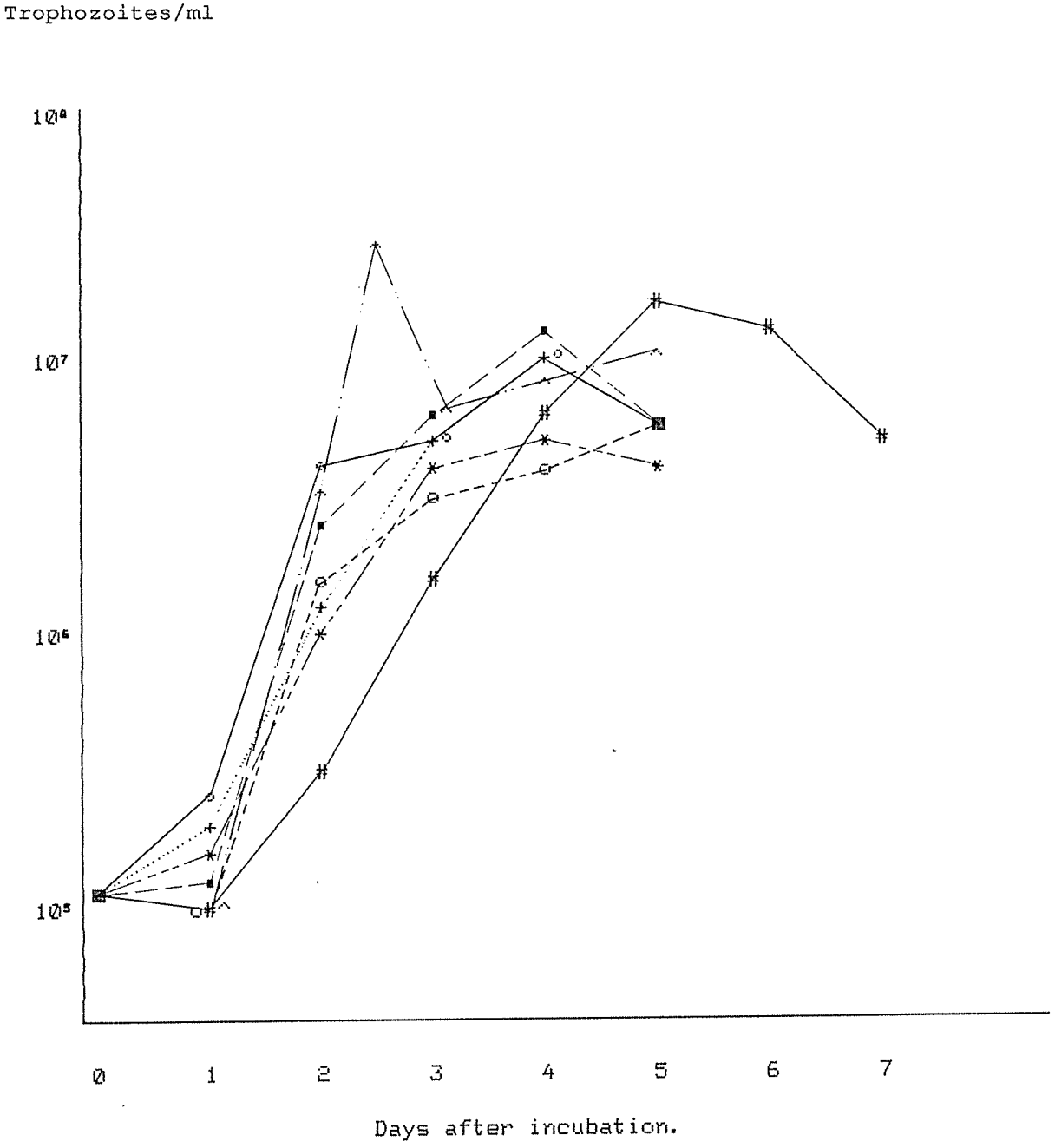
■---■ = Ham/87/MUGU/84

---- = Hast/87/MUGU/68

o----o = Hast/87/MUGU/76

+·····+ = Hast/87/MUGU/86

Figure 4.1. Graph of growth of 7 isolates of *Giardia intestinalis* over 7 days in TY1-S-33 medium.



4.32 SDS-PAGE Comparison of Total Proteins.

Four attempts were made to find the optimum acrylamide percentage. Eventually, 12.5 % without glycerol and 50 µg of protein was decided upon as the best combination for determination between distinct banding patterns. (Plates 4.1 - 4.5.)

Comparison of the isolates can be seen in plate 4.6. A total of 42 distinct Coomasie Blue staining bands could be determined. The differences between the isolates is small. An obvious single band in isolates Whan/87/MUGU/48, Hast/87/MUGU/76, Hast/87/MUGU/86 and a few faint bands within the other isolates show the only differences. No characterization of the strains can be made from these results.

The *Giardia muris* isolate shows that there is a significant difference between it and the cultured *Giardia intestinalis* strains. The Hast/87/MUGU/76 isolate retrieved from the mouse, although faint and not particularly well represented by the photograph, shows the difference between the cultured form and the *in vivo* form is significant. Comparison of the *G. muris* isolate and the human isolate from the mouse reveals that the differences between these two "species" of *Giardia* may be proteinaceious.

Although only representative, the results from the comparison of the cysts of the three species and the cultured trophozoites, show that all have different banding patterns. The comparison of patterns shows that greatest similarity exists between the human cyst and the human cultured trophozoite, although this is not by much. The differences between the cysts could be strain or host variations.

Plates 4.1 - 4.5. SDS-PAGE Analysis: Determination of optimum gel and protein concentration using protein prepared from trophozoites of Bris/83/HEPU/106.

Plate 4.1 10 % acrylamide gel concentration.
Protein concentrations from left to right
5, 10, 20, 30, 40, 50, 60 µg of protein per track.

Note that the gel front is darkly stained indicating that it contains many protein bands. Low molecular weight proteins are therefore unable to be distinguished at this concentration.

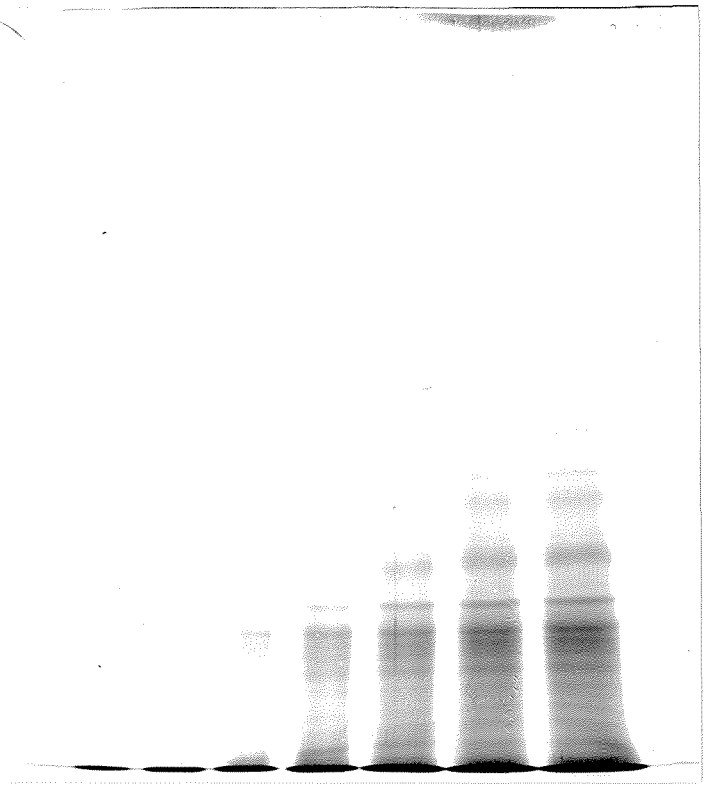


Plate 4.2. 15 % acrylamide gel concentration with glycerol.
Protein concentrations from left to right
20, 30, 40, 50, 60, 70, 80 µg of protein per track.

Note that this concentration does not give good
differentiation of the high molecular weight proteins.

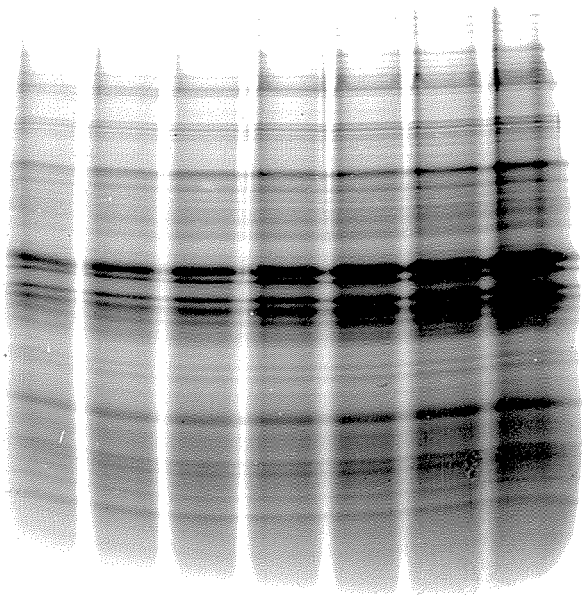


Plate 4.3. 15 % acrylamide gel concentration without glycerol.
Protein concentrations from left to right
30, 40, 50, 60, 70, 80, 90 µg of protein per track.

Even without glycerol, the bands still do not run the length of the gel.

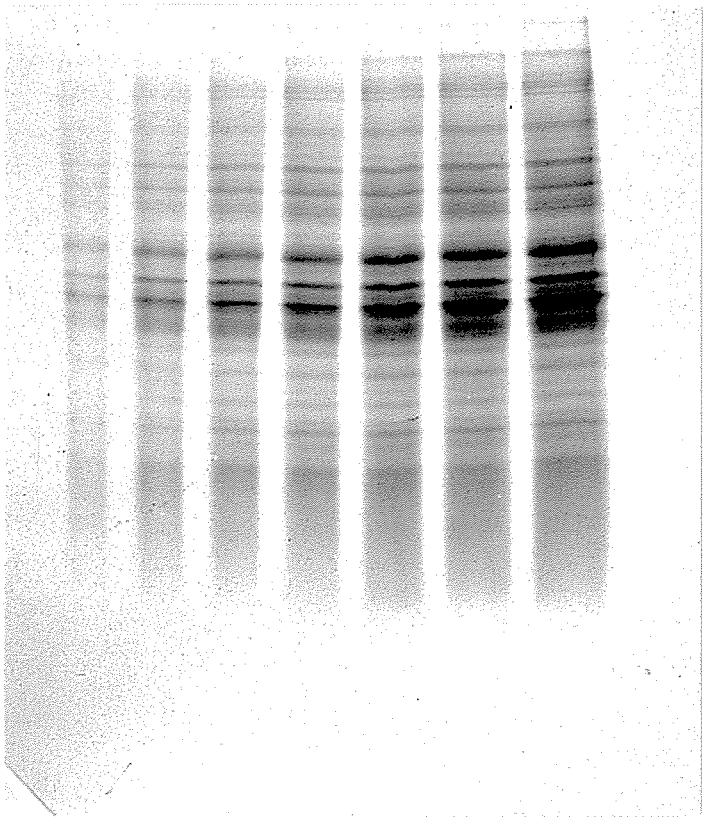


Plate 4.4. 12.5 % acrylamide gel concentration without glycerol.
Protein concentrations from left to right
30, 40, 50, 60, 70, 80 µg of protein per track.

Note how the pattern of banding at this concentration, although not as clear as those at 15 %, does show the maximum differentiation of the bands, from low molecular weight proteins to high.

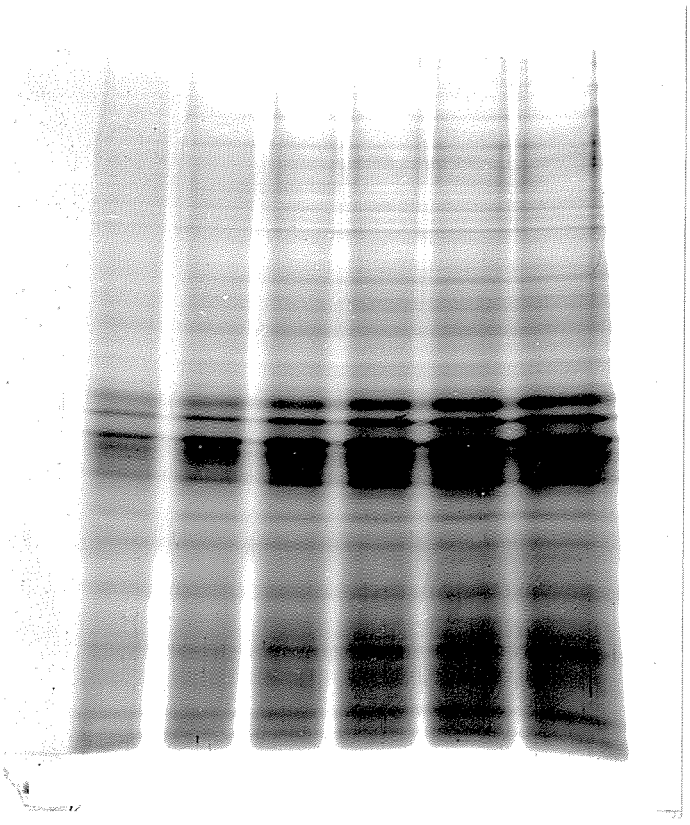


Plate 4.5. 12.5 % acrylamide gel concentration without glycerol. This shows a comparison between strain Bris/83/HEPU/106 and Whan/87/MUGU/48. This was done to ensure that the concentration of 12.5 % and 50 µg was optimum concentrations for determination of patterns.

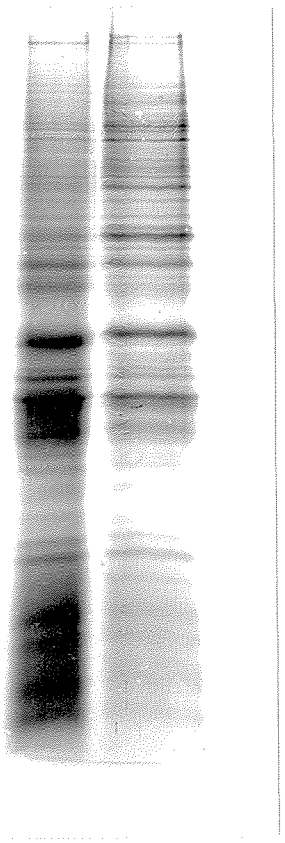


Plate 4.6. 12.5 % acrylamide gel concentration without glycerol. Comparison of the seven isolates of *Giardia intestinalis* culured in this laboratory.
From left to right.
Bris/83/HEPU/106 : Whan/87/MUGU/48 : N.Pl/87/MUGU/77 : Ham/87/MUGU/84 : Hast/87/MUGU/68 : Hast/87/MUGU/86 : Hast/87/MUGU/76.

Note that distinct single band differences appear in lanes 2, 6 and 7.

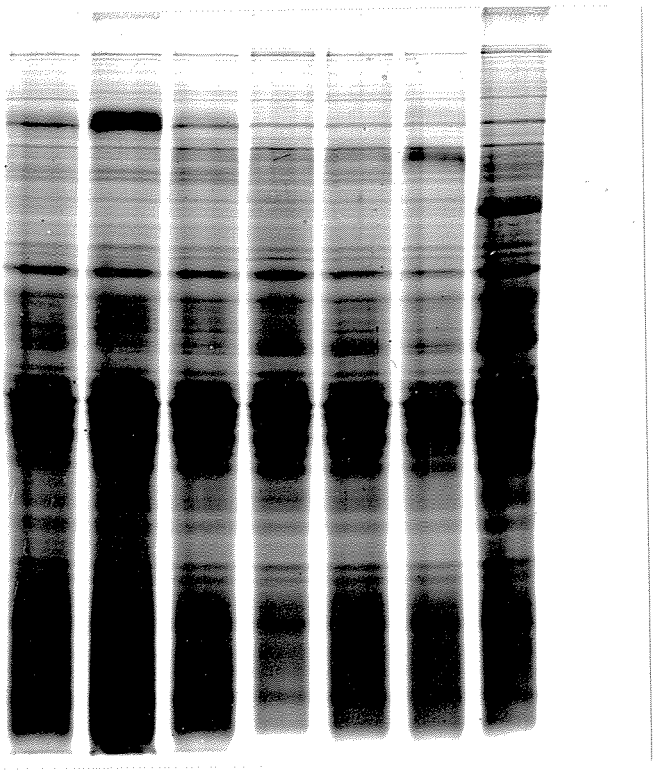


Plate 4.7. 12.5 % acrylamide gel concentration without glycerol. Comparison of cultured Hast/87/MUGU/76 (right) and trophozoites of the same strain taken from a mouse with trophozoites (*Giardia muris*) from a naturally infected mouse (far left).

Note that the patterns contain many differences.

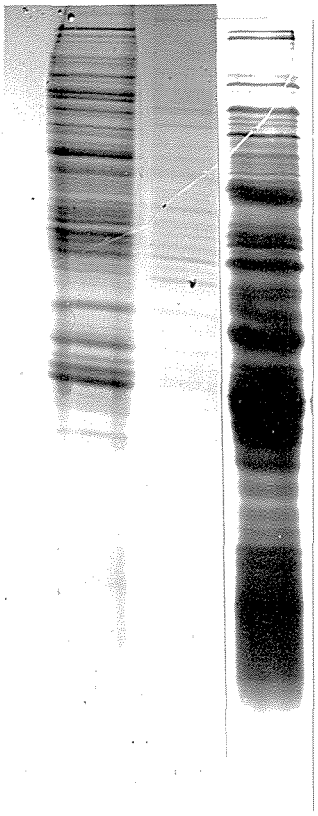
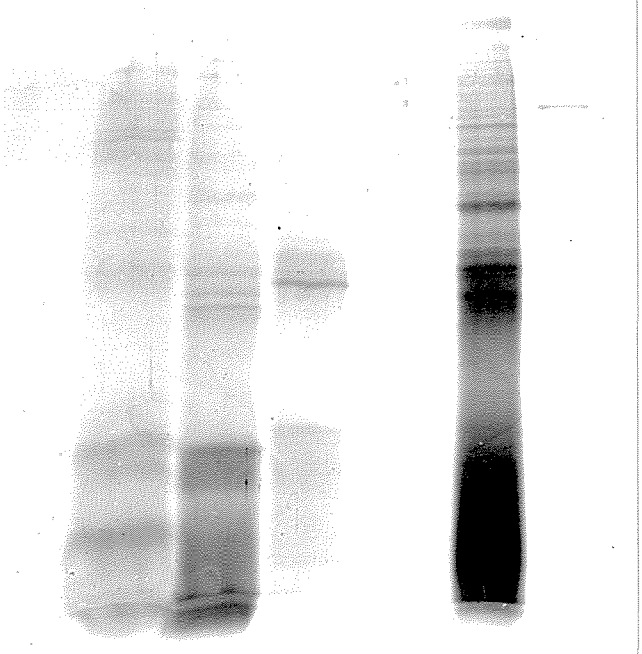


Plate 4.8. 12.5 % acrylamide gel concentration without glycerol. Comparison of Hast/87/MUGU/68 trophozoites (right), and 3 cyst samples. From the left, Dog cysts; Cat cysts; and Human cysts from Hastings.

Note that all have distinct banding patterns.



4.4 Discussion.

The isolates, 6 from New Zealand and 1 from Australia, were compared by two methods for indications of strain variations. The methods employed were growth rate and total protein banding patterns by SDS-PAGE.

Growth rates of our 7 isolates averaged 0.09 ± 0.01 generations / hour with a range of 0.079 to 0.109. Although the upper and lower values differ significantly, as a whole, the isolates show no variation.

Other workers have measured growth rates from various strains (Keister 1983; Kasprzak and Majewska 1985). Kasprzak and Majewska 1985 record from 23 isolates of *Giardia*, generation times which range from 8.1 - 16.6 hours. Discounting their isolate which had a generation time of 16.6 hours, the rest were very similar to each other. The reason for the 16.6 hour generation time was not given, although the sample had been frozen for 48 hours.

In comparison to Moore *et al* (1982) and Smith *et al* (1982), we found that to compare isolates on SDS-PAGE gels we first had to find an optimum gel concentration. Moore *et al* (1982) had used 9% gels which we found unable to distinguish low molecular weight proteins. Smith *et al* (1982) used a gradient 5 - 15% gel which although giving a nice spread of low and high molecular weight proteins, is difficult to reproduce and may give distortions (Ionas 1983).

12.5% acrylamide was used as the optimum gel concentration in the comparison of isolates. At this concentration, 42 distinct Coomasie Blue staining bands were determined. This compares to Moore *et al* (1982) who found 20 and Smith *et al* (1982) who reported 26. The ability to distinguish a higher number of staining bands meant that the sensitivity of attempting to ascertain differences was increased.

Of the 7 strains, Whan/87/MUGU/48, Hast/87/MUGU/76 and Hast/87/MUGU/86 were the only ones to show any distinct band differences although fainter differences were also able to be detected. All these differences were found in the high molecular weight bands.

In general we see from the results that the axenically cultured *Giardia intestinalis* isolates have few differences. This observation could mean one of two things. Firstly, all the isolates are of the same strain which may have originated from Australia (the source of the control isolate). Secondly a further possibility is indicated by the intersting result obtained with the comparison of the *in vitro* and *in vivo* human strain, Hast/87/MUGU/76. These isolates differed in many bands indicating a protein synthesis dictated by the environment and the availability of amino acids. In work done on the metabolism of trophozoites, Lindmark and Jarrol (1984) showed that the main method of incorporation of lipids, purines and pyrimidines into *Giardia* is not by synthesis but by salvage pathways. If this is also true in relationship to proteins, then the differences seen here are those of differing environments. Whatever the cause, the effect is that using these methods, strain variation is difficult to determine on cultured trophozoites.

From the previous experiment we can also that the differences between *G. muris* and *G. intestinalis* from within similar animals show that the subdivision of the two species is reasonable.

The result of the cysts versus trophozoites showed definite differences in the banding patterns. Variations between the dog, cat and human cysts could easily be seen although the resolution of plate 4.8 is poor. The correlation of the banding patterns between the cyst samples is low and may represent strain or host differences of the *Giardia*. The difference between the human cyst sample and the cultured trophozoite showed some similarities although the cyst sample looked to have more in common with the *in vivo* human isolate from the previous gel.

4.5 Conclusion.

7 isolates of *Giardia intestinalis* were compared and found to lack any distinct differences. Among the growth rates an average of 0.09 ± 0.01 generations / hour was obtained and none of the isolates varied significantly from this mean. 42 distinct bands were seen by SDS-PAGE total protein analysis with only single band differences appearing among the isolates. *Giardia intesinalis* was found to differ greatly between *in vivo* and *in vitro* cultivation and the differences between *G. muris* and *G. intestinalis* isolates taken from the mouse were significant. *Giardia* cysts from cat, dog and human sources were compared using SDS-PAGE alongside a cultured trophozoite sample. Differences were found to occur between all four samples.

Chapter 5.

GENERAL DISCUSSION.

The main aim and purpose of this study was to make comparisons of *Giardia* from humans, cats and dogs in an attempt to determine if any relationship between the *Giardia* found within these animals exists.

To carry this out, the occurence of *Giardia* within cats and dogs was surveyed. *Giardia* was found in 7% and 3% of cats and 8% and 25% of dogs in Palmerston North and Hamilton respectively. These figures represent a significant infection with *Giardia* in both these communities . Overseas studies have shown the occurrence of *Giardia* to range from 1 - 60% in dogs and 1 - 14% in cats with an average of approximately 8% and 3% respectively (Barlough 1979; Kirkpatrick 1986; Swan and Thomson 1986).

Most of the surveys done by previous workers do not take into account the season in which the survey was carried out. Craun (1984) clearly reports that a seasonal variation is seen when *Giardia* outbreaks are analysed. His research shows that *Giardia* outbreaks tend to follow a consistent pattern of occurrence with autumn, early winter and spring showing peaks for *Giardia* cases within a community. Summer outbreaks appear to coincide with tourists visiting other communities. From this information it is reasonable to conclude that the level of *Giardia* within the cat and dog populations will also vary depending on the season. One would expect that, like humans, autumn and spring will coincide with the highest level of *Giardia* infection. Therefore, comparisons of survey data must take account of seasonal variation.

The suvey showed that *Giardia* was more commonly found in stray and stressed animals. This result is similar to that obtained by Swan and Thomson (1986) who suggest the reason for this is that the animals are poorly fed. Comparable to the findings made by these same workers is the discovery that animals less than three years of age are more likely to be infected. This is not a surprising result as most surveys have shown that young animals are more likely to be infected. Bemrick (1961) discovered this when, in dogs, he found that those younger than 1 year were more likely to be infected with *Giardia*. Similarly Swan and Thomson found in their survey that *Giardia* was more prevalent in animals less than 3 years old. These findings correlate with early reports of giardiasis being only a disease of children (Miller 1922; Burke 1977; Wolfe 1984).

Other information obtained showed that the sex, breed and area had no relationship to *Giardia* infection. The appearance of clinical symptoms of giardiasis, determined by the appearance of non formed faeces, were also not found to correlate to cyst excretion.

Further in the determination of the occurrence of *Giardia*, we found a significant number of other parasites rarely associated in dual infections with *Giardia*. Swan and Thomson (1986) looked for a correlation between the occurence of *Giardia* and other parasites and, were also unable to establish one. The coccidian parasites *Sarcocystis* and species of *Cystoisospora* were found in 4% and 8%, and 8% and 5% respectively for cats and dogs. Nematode worm eggs of the species *Toxoascaris* and *Toxocara* were present in 2% of cats and 5% of dogs. None of these parasites are regarded as highly pathogenic within these animals. However, in occasions of overwhelming infection, particularly of Nematodes, symptoms may be evident. As a result of infection with *Toxoascaris* and *Toxocara*, animals and humans may present with pneumonitis, myelitis, abdominal and chest pains and other symptoms (Schantz and Glickman 1981).

The veterinary significance of this level of infection is difficult to ascertain. *Giardia* may be a significant pathogen of cats and dogs but because the symptoms such as nausea, cramps and other subjective complaints, are difficult to detect, the proof of *Giardia's* pathogenicity in these animals is difficult to determine. Predominately it is found that both cat and dog *Giardia* infections are asymptomatic (Barlough 1979; Kirkpatrick 1986; Swan and Thomson 1986; this study) Occasionally a low number of animals present either acute or chronic symptoms. Symptomatic giardiasis in animals is debilitating, not usually associated with mortality, and can be treated with metronidazole, quinacrine and other drugs (Zimmer and Burrington 1986[b]). In relating animal to human giardiasis, it seems that humans are more susceptible to clinical illness than animals (Wolfe 1984). Asymptomatic cases of human giardiasis are not often completly devoid of symptoms and bursts of diarrhoea and other symptoms are often intermittently experienced (Wolfe 1984). Discomfort in animals, if similar to asymptomatic disease in humans, should thus, in most cases, be intermittent and minimal.

Apart from the veterinary aspect, concern relating to the level of infection among cats and dogs is founded upon the possibility that infection in these animals may directly relate to that within the human population (Schantz 1983; Swan and Thomson 1986). Giardiasis occurs as epidemic "outbreaks" of disease resulting from contamination of water supplies (Craun 1984), and as endemic disease within the community (Owen 1984). Both in epidemic and endemic giardiasis, animals may be providing the source of infective cysts. Certainly in one major outbreak, beavers were the implicated source of the infection (Dykes *et al* 1978). If cats and dogs and other animals are found to be reservoir hosts of *Giardia* that infect humans, then maybe the number of epidemics and the high level of endemic disease could be reduced.

To pursue our aim and investigate the relationship between the *Giardia* of animals and humans we attempted to culture *Giardia* from these sources. Two techniques in the isolation of *Giardia* were used. Firstly an *in vivo* technique, similar to that described by Wallis and Wallis (1986) but using suckling mice as described by Vinayak *et al* (1979) and

Hill *et al* (1981). And secondly, an *in vitro* method employing an excystation procedure developed by Feely (1986). In work done on the excystment and viability of *Giardia* cysts, Kasprzak and Majewska (1983[b]) showed that the infectivity of cysts was not always related to the cysts ability to excyst *in vitro*. Using both *in vivo* and *in vitro* methods in the isolation and culture of *Giardia* may therefore increase the yeild of excysted trophozoites and hence, cultures.

Wallis and Wallis (1986) successfully infected mongolian gerbils with cysts from a wide range of sources including dogs, humans, beaver and meadow voles. After establishing an infection, they sacrificed the gerbils and retrieved large numbers of viable trophozoites from the duodenum of the animals. With these trophozoites, *in vitro* cultures were set up. Axenic cultivation was thus established following the initial foundation period. Our method used suckling mice between 8 and 14 days old to establish *in vivo* infections. From these infections axenic cultures were set up via a similar method to that of Wallis and Wallis (1986).

In vitro excystation which was first reported in 1926 by Hegner was not much used until the development of suitable *in vitro* culture media. Since the appearance of such media a variety of *in vitro* excystation methods have been developed. Initially we used the method of Rice and Schaffer (1981) for *in vitro* excystation but with the introduction of Feely's method (1986) which was simpler, and no less effective, the latter method was used.

Using both these methods 8 strains from 91 samples were successfully cultured. 2 of these strains, however, were lost later through bacterial contamination. Both methods gave a success rate of 7% although overall a success rate of 9% was achieved. No dog or cat strains were isolated. Cat source trophozoites were seen to survive for over a week in culture medium. Dog source trophozoites were never viable longer than 24 hours. *Giardia* from the cat has been isolated, even as early as 1970 (Meyer 1970) with subsequent isolations occurring (Meloni - personal communication). Culturing from dogs has not had such success. Wallis and

Wallis (1986) provide one of the few reports of such an isolation. The method used by these workers used mongolian gerbils to excyst and produce high numbers of viable trophozoites that were then transfered into axenic culture. Meloni (personal communication) used high numbers of trophozoites taken straight from the duodenum of infected dogs and was unable to axenically culture *Giardia*. The success of Wallis and Wallis (1986) may thus be associated to an adaption of the *Giardia* within another host that made it more readily adaptable to *in vitro* cultivation.

In this study the number of trophozoites that grew successfully was small compared to the size of the inoculation (see also Kasprzak and Majewska 1983, 1985; Wallis and Wallis 1986). Both duodenal trophozoites and excysted trophozoites showed a high death rate before logarithmic growth took place. The reason for this is perhaps the heterogeneity of the population found in an infection. Only some of the trophozoites may have the right genetic and/or physiological features which enable them to adapt to the culture medium. Rendtorff, (1978) in discussing the difference in symptomology and infectivity of *Giardia* within his 1954 study on transmission within humans, suggested that *Giardia* might have varying degrees of virulence and infectivity. These factors might be related, either positively or negatively, to the ability of the parasite to culture. If this is the case and the medium is "selective", then culturing itself may determine strain differences between *Giardia*. Cultures then, would show very little variation.

Since successful culturing of *Giardia* was developed, many attempts to determine strain variations have been reported. Originally, strains of protozoa were determined by morpholgy and morphometric variations. Filice (1952) in his studies compared strains of both *G. muris* and *G. intestinalis* by such means. Differences within different hosts of the same species were found but were disregarded with an explanation from the work of Tsuchiya (1930, 1931). Tsuchiya had noted that cyst sizes in both humans and dogs varied on a day to day basis. Filice thus suggested that trophozoites could similarly vary, hence the differences he had obtained were not significant. Grant and Woo (1978) disagree with

Filice's conclusions suggesting that with enough samples, the error would be reduced and significant variations would be found. These workers carried out studies on morphology and morphometric variations of *Giardia* within different hosts and found that variations within trophozoites from the same host did occur. These variations occured particularly in relation to the presence or absence of the median bodies. Accounting for these differences by a large sample size, they went on to find significant variations between the "species" of different hosts. The significance of their results however do not include the possibility of the host actually inducing the variation in the trophozoites.

The morphometric approach has also been applied to axenically cultured *Giardia intestinalis*. Bertram *et al* (1984[b]) used this method to conclude that variations were small between trophozoites of widely diverse origins. These workers also used a method of isoenzyme analysis to compare these strains. This latter method distinguished three definite groupings of the *Giardia*. Two groups contained *Giardia* that were very closely analogous to each other while the other group was composed of *Giardia* more diversley related (Bertram *et al* 1984[a]). Korman *et al* (1986) also used this method to determine strains of *Giardia*. His results found that the groups identified by isoenzyme analysis could also be identified antigenically by agglutination with rabbit anti-*Giardia* sera. In other tests for antigenic differentiation, variations have been very subtle. Such comparisons have used SDS-PAGE, immunoelectrophoresis, crossed immunoelectrophoresis and enzyme linked immunosorbent assay (Moore *et al* 1982; Smith *et al* 1982). Apart from some minor variations between isolates, distinguished from the latter two methods, the results showed the general similarity. That is all except one isolate which had been in culture the longest, which was found to lack a significant antigen which all the other isolates had (Smith *et al* 1982). This shows that *Giardia* are perhaps similar to bacteria and other parasites that when they are cultured, they lose their virulence after a few passages.

Other methods of strain determination have been developed by Nash and associates. One of their methods determined differences between 19 isolates by comparing firstly the reactivity of anti-*Giardia* sera to excretory-secretory products and surface antigens and secondary the electrophoretic mobility patterns of these products (Nash and Keister 1983; Nash *et al* 1985[a]). The other method involved techniques of molecular biology to compare the genetic material of the *Giardia* (Nash *et al* 1985[b]). This latter method distinguished two groups of *Giardia* on the basis of restriction endonuclease patterns which could be distinguished further after southern blotting the DNA and hybridizing them to a DNA probe. The results from this method were found to correlate to the results of the previous method such that three groups were able to be distinguished. Group 1 contained 5 isolates, all closely affiliated; Group 2 contained 11 isolates rather more diverse; and Group 3 were more diverse still and found only to cross react amongst themselves.

What relationship the reults of these experiments have to the actual differentiation of the cultures has yet to be determined. The variations seen may define an altered virulence relating to a potentially different host response, or they may have no physiological significance to *in vitro Giardia*.

This study compared 7 human strains and found that variation between the isolates was small when compared by growth rates and SDS-PAGE protein analysis. These results correlate with similar work done by Moore *et al* (1982) Smith *et al* (1982) and Kasprzak and Majewska (1985) a full discussion of which is found in Chapter 4. Further work done in this study used SDS-PAGE protein analysis to compare cultured trophozoites to trophozoites from an *in vivo* source and to trophozoites of *G. muris*.

The comparison of *G. muris* to *G. intestinalis*, both *in vivo* and *in vitro*, showed a large number of bands differing between isolates. More bands were different between the *in vivo* isolate and the *G. muris* isolate than the *in vivo* isolate and the *in vitro* one. Hence we conclude that retaining *G. muris* as a separate species is justified on the grounds of total protein composition.

Total protein analysis was able to show differences between the human isolate of *Giardia* taken from the mouse and its cultured counterpart. This result gives evidence to the suggestion that *Giardia* either adapts, conforms to, or is selected for growth in the culture medium. No similar experimentation has been done to confirm this although Lindmark and Jarroll (1984) in work done on metabolism of trophozoites have shown that *Giardia* scavenges for both lipids and nucleic acid precursors. Such results allow us to ask the question, do *Giardia* scavenge within other pathways? If such a situation exists, then the composition of the *Giardia* cell must be determined by its environment and hence cultured isolates will vary very little.

Experiments using trophozoites from both *in vivo* and *in vitro* sources must then take account of the various media and host interactions. Such things as antigenicity; enzymatic activity; conformation, ie. morphometric and morphological differences, may be affected. Any molecular biological approach must also account for the possiblity of a heterogeneously composed infection or, culture imposed homogeneity.

The first problem is to establish if a heterogeneous infection exists. Infections may be established with very low numbers of cysts (Rendtorff 1954, 1978; Kasprzak and Majewska 1983[b]) but *in vivo* infections might be produced by many hundreds of cysts all from different sources. Patients suffering from giardiasis may be infected with many "strains" of *Giardia* of which only one is responsible for the symptoms, and another one is the only one able to adapt to culture.

With the unknown diversity of *Giardia*, the potential for zoonotic transmission of giardiasis is great. Our knowledge from *in vitro* experimentation may take a long time before the surety of any zoonoses that exists is known. Until then epidemiological investigations must provide us with the greatest leads. Such investigations should evaluate the source of the infection for every case of human giardiasis not disregarding pets as a possible source of *Giardia* infection. With such evidence a picture of giardial ubiquity could be established that will give greater results and proof than any *in vitro* test, at present, could give.

It is evident that little is known about *Giardia* and giardiasis even though much work has been done. Just recently, *Giardia* was included in the World Health Diarrhoeal Disease Control Program (Wilson 1984).This indicates the need for the acurate identification of strains of *Giardia* among different hosts so that the reservoirs of human giardiasis are known and the potential for infection reduced.

Conclusion.

In this research a high number of *Giardia* infections in both cats and dogs from Palmerston North and Hamilton were found. These infections were largely associated with animals less than 3 years old. A significantly higher number of infections were found in Hamilton although the survey was carried out within different seasons and this may account for this observation. Overall more dogs than cats were infected and no relationship to clinical symptoms in either animal could be seen. In attempting to relate *Giardia* of cats and dogs to *Giardia* from humans, *in vitro* cultures were established. However, out of 91 samples, only 8 human isolates were retrieved. 6 of these isolates were then compared by growth rate and total protein banding patterns for strain variations. Although some differences were observed, the results indicated an overwhelming similarity between the strains. This study showed that *in vivo* and *in vitro* trophozoites of the same "strain" were different and that *G. muris* and *G. intestinalis* were distinguishable by total protein analysis.

BIBLIOGRAPHY.

Al-Issa, T., Jawad, A.H. and Asal, A. 1986.
Detection of *Enterobius vermicularis* eggs using two different methods in Iraq. Bull. End. Dis., **27**. 25-30.

Ament, M.E. 1972.
Diagnosis and treatment of giardiasis. J. Pediatr., **80**. 633-637.

Ament, M.E. and Rubin, C.E. 1972.
Relation of giardiasis to abnormal intestine strucure and function in gastrointestinal immunodeficiency syndromes. Gastroenterology, **62**. 216-226.

Anand, B.S., Kuay, M., Chakravonti, R.N., Sehgal, A.K. and Chuttoni, P.N. 1980.
Pathogenesis of malabsorption in *Giardia lamblia* infection : an experimental study in rats. Trans. Roy.Soc. Trop. Med. Hyg., **74**. 565-570.

Anand, B.S., Mahmoud, A., Ganguly, N.K., Rehani, M.M., Dilawari, J.B., and Mahajan, R.C. 1982.
Transport studies and enzyme assays in mice infected with human *Giardia lamblia*. Trans. Roy. Soc. Med. Hyg., **76**. 616-619.

Anderson, T., Forsell, J. and Sterrer, G. 1972.
Outbreak of giardiasis - effect of a new antiflagellate drug, Tinidazole. Br. Med. J., 2. 449-451.

Barbieri, D., DeBrito, T., Hoshino, O., Nascimento, O.B., Martins-Campos, J.V., Quarentei, G. and Marcondes, E. 1970.
Giardiasis in childhood. Arch. Dis. Child., 45. 466-472.

Barlough, J.E. 1979.
Canine giardiasis: a reveiw. J. Small. Anim. Pract., 20. 613-23.

Barton, J.J., Burke, J.D. and Casey, E.P. 1986.
Reactive arthritis - *Giardia lamblia*, another new pathogen? Irish Med. J., 79. 223.

Bassily, S., Farid, A., Mikhail, J.W., Kent, D.C. and Lehman, J.S. 1970.
The treatment of *Giardia lamblia* infection with mepacrine, metronidazole and furazolidone. J. Trop. Med. Hyg., 73. 15-18.

Bateson, M.C. and Boucher, I.A.D. 1981.
Clinical investigation of gastrointestinal function. Publ. by Blackwell Scientific Publishers. 2nd Edition 1981.

Baughn, R.E. and Morales, A.R. 1971.
Am. J. Clin. Path., 55. 221.

Belosevic, M., Faubert, G.M., MacLean, J.D., Law, C. and Croll, N.A. 1983.
Giardia lamblia infections in Mongolian gerbils: An animal model. J. Infect. Dis., 147. 222-226.

Bemrick, W.J. 1961.
A note on the incidence of three species of *Giardia* in Minesota. J. Parasitol., 47. 87-89.

Bertram, M.A., Muller, P. and Meyer, E.A. 1984.
Giardia isoenzymes. In Giardia and Giardiasis. Biology, Pathogenesis and Epidemiology. Ed. Erlandsen, S.L. and Meyer, E.A. Publ by Plenum Press New York. p. 81-86.

Bertram, M.A., Meyer, E.A., Anderson, D.L. and Jones, C.T. 1984.
A morphometric comparison of five axenic *Giardia* isolates. J. Parasitol., 70. 530-535.

Bingham, A.K., Jarroll, E.L.Jr., Meyer, E.A. and Radulescu, S. 1979.
Giardia sp.: physical factors of excystation *in vitro*, and excystation vs eosin exclusion as determinants of viability. Exper. Parasitol., 47. 284-291.

Bingham, A.K. and Meyer, E.A. 1979.
Giardia excystation can be induced *in vitro* in acidic solutions. Nature, 277. 301-302.

Black, R.E., Dykes, A,C., Sinclair, M.S. and Wells, J.G. 1977.
Giardiasis in day care centres: Evidence of person to person transmission. Pediatrics., 60. 486-491.

Blackmore, D.K. and Harris, R.E. 1979.
Introductory veterinary epidemiology. A study guide printed by Massey Univesity.

Blanchard, R. 1888.
Remarques sur le *Megastome intestinal*. Bull. Soc. Zool. Fr., 30. 18-19.

Boeck, W.C. 1927.
Giardiasis in man. Its prevalence and relation to diarrhea and to gallbladder disease. Arch. Intern. Med., 39. 134.

Boreham, P.F.L., Phillips, R.E. and Shepherd, R.W. 1984.
The sensitivity of *Giardia intestinalis* to drugs *in vitro*. J. Antimicrob. Chemother., 14. 449-461.

Box, E.D. 1981.

Observations on *Giardia* of Budgerigars. J. Protool., **28**. 491-94.

Brandborg, L.L., Tankersley, C.B., Gottlieb, S., Barancik, M. and Sartor, V.E. 1967.

Histological demonstration of mucosal invasion by *Giardia lamblia* in man. Gastroenterology, **52**. 151.

Brightman, A.H. and Slouka, G.F. 1976.

A reveiw of five clinical cases of giardiasis in cats. J. Am. Anim. Hosp. Assoc., **12**. 492-497.

Brodsky, R.E., Spencer, H,C., and Schultz. M.G. 1974.

Giardia in american tourists to the Soviet Union. J. Infect. Dis., **130**. 319.

Brown, E.H. 1948.

Giardia lamblia. The incidence and results of infestations of children in residential nurseries. Arch. Dis. Child., **23**. 119.

Brumpt, L. 1935.

Traitement experimental de la lambliase. C. R. Seances Soc. Biol. Paris, **124**. 1040.

Buckley, A. 1986.

Severe megaloblastic anaemia due to giardiasis. Br. Med. J., **292**. (6526) 992.

Burke, J.A. 1977.

The clinical and laboratory diagnosis of giardiasis. CRC Crit. Rev. Clin. Lab. Sci., 373-391.

Center for Disease Control 1979.

Giardiasis in apes and zoo attendants in Kansas City Missouri. CDC Vet. Publ. Health Notes., **Jan. 1979**. 7-8.

Chatterjee, G.C. 1927.

On cultivation of *Giardia intestinalis*. Ind. Med. Res., **47**. 33.

Chester, A.C., MacMurray, F.G., Restifo, M.D. and Man, O. 1985.

Giardiasis as a chronic disease. Dig. Dis. Sci., **30**. 215-218.

Chochillon, C., Gobert, J.G., Buchel, L.A. and Savel, J. 1986.

Giardia intestinalis; étude comparée des débits fécaux lipidiques et azoté chez des adultes et des enfants parasités. [Giardia lamblia: comparitive study of faecal lipid and nitrogen levels in infected adults and children.] Pathologie biologie., **34**. 830-834.

Conroy, D.A. 1960.

A note on the occurence of *Giardia* sp. in a Christmas pudding. Rev. Iber. Parasitol., **20**. 567-571.

Craft, J.C. 1982.

Experimental infection with *Giardia lamblia* in rats. J. Infect. Dis., **145**. 495-498.

Craft, J.C. and Nelson, J.D. 1982.

Diagnosis of giardiasis by counter immunoelectrophoresis of faeces. J. Inf. Dis.. **145**. 499-504.

Craft, J.C., Murphy,T. and Nelson, J.D. 1981.

Furazolidone and quinacrine - Comparitive study of therapy for giardiasis in children. Am. J. Dis. Child., **135**. 164-166.

Craige, J.E. 1948.

Differential diagnosis and specific therapy of dysentries in dogs. J. Am. Vet. Med. Assoc., **113**. 343.

Craun, G.F. 1979.

Waterborne outbreaks of giardiasis. In Waterborne Transmission of Giardiasis, Ed. Jabukowski, W. and Hoff, J.C. 127-149.

Craun, G.F. 1984.

Waterborne outbreaks of giardiasis: current status. In Giardia and Giardiasis. Biology, Pathogenesis and Epidemiology. Ed. Erlandsen, S.E. and Meyer, E.A. 243-263.

Danciger, M. and Lopez, M. 1975.

Number of *Giardia* in the faeces of infected children. Am. J. Trop. Med. Hyg., **24**. 237-242.

Davies, R.B. and Hibler, C.P. 1979.

Animal resevoirs and cross-species transmission of *Giardia*. In Waterborne transmission of Giardiasis. Ed. Jabukowski, W. and Hoff, J.C. 104-126.

DeCarneri, I. and Trane, F. 1978.

Murine giardiasis can be transmitted by food soiled with a few trophozoites of *Giardia muris*. Trans. Roy. Soc. Trop. Med. Hyg., **72**. 533.

Develaux, M., Mouchet, F. and Labo, R. 1986.

Parasitoses digestives de l'enfant scolarisé en République du Niger. [Gastrointestinal parasites in school children in Niger.] Bull. Soc. Pathol. Exot. Fil., **79**. 571-575.

Diamond, L.S. 1968.

Techniques of axenic cultivation of *Entamoeba histolytica* schaudin and *E. histolytica* the amoebae. J. Parasitol., **54**. 1047-1056.

Diamond, L.S., Harlow, D.K. and Currick, C.C. 1978.

A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans. Roy. Soc. Trop. Med. Hyg., **72**. 431-432.

Dobell, C. 1920.

The discovery of the intestinal protozoa of man. Proc. R. Soc. Med., **13**. 1-15.

Dobell, C. 1932.

"Anton van Leeuwenhoek and His 'Little' Animals." Harcourt, Brace, New York.

Dobell, C. and Laidlaw, P.P. 1926.

On the cultivation of *Entamoeba histolytica* and some other entozoic amoebae. Parasitology, **18**. 283-318.

Dykes, A.C., Juranek, D.D., Lorenz, R.A., Sinclair, S., Jakubowski, W. and Davies, R. 1980.

Municipal waterborne giardiasis. Epidemiological investigation - beavers implicated as possible resevoir. Ann. Int. Med., **92**. 165-170.

Erlandsen, S.L. and Feely, D.E. 1984.

Trophozoite motility and the mechanism of attachment. In Giardia and Giardiasis. Biology, Pathogenesis and Epidemiology. Ed. Erlandsen, S.E. and Meyer, E.A. 33-61.

Farthing, M.J.G., Vanon, S.R. and Keusch, G.T. 1983.

Mammalian bile promotes growth of *Giardia lamblia* in axenic culture. Trans. Roy. Soc. Trop. Med. Hyg., **77**. 467-469.

Feely, E. 1986.

A simplified method for *in vitro* excystation of *Giardia muris*. J. Paraistol., **72**. 474-475.

Filice, F.P. 1952.

Studies on the cytology and life history of a *Giardia* from the laboratory rat. Univ. Calif. Publ. Zool., 57. 53-146.

Finegold, S.M. 1980.

Metronidazole. Ann. Intern. Med., **93**. 585-587.

Frost, F. and Harter, L. 1980.

Giardiasis in Washington State, Office of Environmental Health Programs, Washington State Department of Social and Health Services (June 1980).

Galli-Valerio, B. 1937.

La lambliase et son traitment par l'atebrine. Schweitz. Med. Wochenschr., 67. 1181.

Gillin, F.D. Reiner, D.S. 1982.

Attachment of the flagellate *Giardia lamblia*: role of reducing agents, serum, temperature and ionic composition. Molec. Cell. Biol., 2. 369-377

Gillin, F.D. 1984.

The role of reducing agents and the physiology of trophozoite attachment. In Giardia and Giardiasis. Biology, Pathogenesis and Epidemiology. Ed. Erlandsen, S.E. and Meyer, E.A. 111-131.

Gillin, F.D. and Diamond, L.S. 1979.

Axenically cultivated *Giardia lamblia*: Growth attachment and the role of L-cysteine. In. Waterborne transmission of Giardiasis. Ed. Jakubowski, W. and Hoff, J.C. 270-272.

Gillon, J. and Ferguson, A. 1984.

Changes in small intestinal mucosa in giardiasis. In Giardia and Giardiasis. Biology, Pathogenesis and Epidemiology. Ed. Erlandsen, S.E. and Meyer, E.A. 163-183.

Giugliano, L.G., Bernardi, M.G.P., Vasconelos, J.C., Costa, C.A. and Giugliano, R. 1986.

Longtintudinal study of diarrhoeal disease in a peri-urban community in Manaus (Amazon, Brazil.). Ann. Trop. Med. Parasit., 80. 443-450.

Gordts, B., Hemelhof, W., Tilborgh, K., Van Beliore, P., Cadranel, S. and Butzler, S.P. 1986.

Evaluation of a new method for routine *in vitro* cultivation of *Giardia lamblia* from human duodenal fluid. J. Clin. Micro. **22**. 702-704.

Gordts, B., Retoré, P., Cadranel, S. Hemelhof, W., Rahmon, M. and Butzler, S.P. 1984.

Routine culture of *Giardia lamblia* trophozoites from human duodenal aspirates. Lancet, **ii** : 137.

Grant, D.R. and Woo, P.T.K. 1978.

Comparitive studies of *Giardia* species in small mammals in Southern Ontario. I. Prevalence and identity of the parasites with a taxanomic discussion of the genus. Can. J. Zool., **56**. 307-313.

Grassi, B. 1881.

Di un nuovo parassita dell'uomo, *Megastoma entericum*, Gaz. Degli Ospidali, **2**. 577.

Grassi, B. and Schewiakoff, W. 1888.

Beitrag zur Kenntnis des *Megastoma entericum*, Z.Wiss.Zool., **46**. 143.

Green, E.L., Miles, M.A. and Warhurst, D,C. 1985.

Immunodiagnostic detection of *Giardia* antigen in faeces by a rapid visual enzyme-linked immunosorbent assay. Lancet, **ii** : 691-693.

Hartman, H.R. and Kyser, F.H. 1941.

Giardiasis and its treatment. J. Amer. Med. Assoc., **116**. 2835-2839.

Hegner, R.W. 1922.

A comparitive study of the *Giardia*'s living in man, rabbits and dog. Amer. J. Hyg., 2. 442-454.

Hegner, R.W. 1926.

Giardia beckeri n. sp. from the ground squirrel and *Endamoeba dipodomysi* n. sp. from the kangaroo rat. J. Parasit., **12**. 203-6.

The biology of host-parasite relationships among protozoa living in man. Quart. Rev. Biol., 1. 393-418.

Hegner, R.W. 1927.

Excystation *in vitro* of human intestinal protozoa. Science, **65**. 577-578.

Hermans, P.E., Diaz-Buxo, J.A. and Stobo, J.D. 1976.

Idiopathic late-onset immunoglobulin deficiency: Clinical observations in 50 patients. Am. J. Med., **74**. 903-910.

Hewlett, E.L., Andrews. J.S., Ruffier, J. and Schaefer, F.W. 1982.

Experimental infection of mongrel dogs with *Giardia lamblia* cysts and cultured trophozoites. Infect. Dis., **145**. 89-93.

Heyworth, M.F. 1986.

Antibody response to *Giardia muris* trophozoites in mouse intestine. Inf. Immun., **52**. 568-571.

Heyworth, M.F., King, J.E. and Eriksson, E.C. 1986.

Clearance of *Giardia muris* infection in mice deficient in natural killer cells. Inf. Immun., **54**. 903-904.

Hill, D.R., Guerrant, R.L., Pearson, R.D. and Hewlett, E.L. 1983.

Giardia lamblia infection in suckling mice. J. Infect Dis., **147**. 217-221.

Hiral, K., Sawa. H., Yamashita, T., Shimakura, S. and Hashimoto, A. 1980.

Giardia infection in Budgerigars. Jap. J. Vet. Sci., **42**. 615-17.

Ionas, G. 1983.

Studies of *Mycoplasma Ovipneumoniae* in New Zealand sheep: Epidemiology and comparison of isolates. Thesis, M.Sc. Massey University.

Isaac-Renton, J., Proctor, E.M., Prameya, R. and Wong, Q. 1986.

A method of excystation and culture of *Giardia lamblia*. Trans. Roy. Soc. Trop. Med. Hyg., 80. 989-991.

Isaacs, P.E.T. and Warhurst, D.C. 1975.

Psychiatric manifestations of giardiasis. Br. Med. J., **1** (5948). 38.

Johnson, D.D. 1972.

Enteritis secondary to *Giardia lamblia* in students traveling on tour in Russia. J. Am. Col. Health Assoc., **20**. 207.

Jokipii, L. 1979.

Giardiasis leningradista. Duodecim, **88**. 522.

Kamath, K.R. and Murugasa, R.A. 1974.

A comparitive study of four methods of detecting *Giardia* in children with diarrheal disease and malabsorption. Gastroenterology, **66**. 16.

Karapetyan, A.E. 1960.

Methods of *Lamblia* cultivation. Tsitologiia, **2**. 379.

Karapetyan, A.E. 1962.

In vitro cultivation of *Giardia duodenalis*. J. Parasitol., **48**. 337-340.

Kasprzak, W. and Majewska, A.C. 1981.

Przenoszenie cyst *Giardia* I. Rola owadow synantropowych. [Transmission of *Giardia* cysts I. Role of Synanthropic insects.] Wiadomosci Parazytologiczne, **27**. 555-563.

Kasprzak, W. and Majewska, A.C. 1983.

Isolation and axenic growth of fresh *Giardia intestinalis* strains in TPS-1 medium. Trans. Roy. Soc. Trop. Med. Hyg., **77**. 223-224.

Infectivity of *Giardia* sp. cysts in relation to eosin exclusion and excystation *in vitro*. Tropenmed. Parasit., **34**. 70-72.

Kasprzak, W. and Majewska, A.C. 1985.

Improvement in isolation and axenic growth of *Giardia intestinalis* strains. Trans. Roy. Soc. Trop. Med. Hyg., **79**. 551-557.

Kasprzak, W., Rauhut. W. and Mazur, T. 1981.

Przenoszenie cyst *Giardia* II. Owocoe i jazyny jako zrodlo inwazji. [Transmission of *Giardia* cysts II. Fruit and vegetables as the source of the infection.] Wiadomosci Parazytologiczne, **27**. 565-571.

Kavousi, S. 1979.

Giardiasis in infancy and childhood : a prospective study of 160 cases with comparison of quinacrine (Atarbrine) and Metronidazole (Flagyl). Am. J. Trop. Med. Hyg., **28**. 19-23.

Keister, D.B. 1983.

Axenic culture of *Giardia lamblia* in TY1-S-33 medium supplemented with bile. Trans. Roy. Soc. Trop. Med. Hyg., **77**. 487-488.

Kent, W.S. 1880.

A manual of infusoria. Publ. by Bogue, London.

Khan, A.A. 1974.

Psychiatric manifestations. Br. Med. J., **4**. (5941) 407.

Kirkpatrick, C.E. 1986.

Feline giardiasis: a reveiw. J. Small. Anim. Pract., **27**. 69-80.

Kofoid, C.A. and Christiansen, E.B. 1915.

On *Giardia microti* sp. nov., from the meadow mouse. Univ. Calif. Publ. Zool., **16**. 23-29.

On binary and multiple fission in *Giardia muris* (Grassi). *Ibid.*, 30-54.

Korman, S.H., Blanq, S.M.Le., Spira, D.T., ElOn, J., Reifen, R.M. and Deckelbaun, R.J. 1986.

Giardia lamblia identification of different strains from man. Zeit. Parasiten., **72**. 173-180.

Kuhn, O. and Owades, J.C. 1985.

Beer and giardiasis. Brewers Digest, **1985**. 18.

Kulda, J. and Nohynkova, E. 1978.

Flagellates of the human intestine and of intestines of other species. In Protozoa of Veterinary and Medical interest. Ed. Krier, J.P. **2**. 1-138. Academic Press, New York.

Künstler, J. 1882.

Sur cinq protozoaires nouveaux. C. R. Acad. Sci. Paris, 95. 347-349.

Lall, R. 1985.

Intestinal parasitic infections in a section of population of Port Blair, Andaman and Nicobar Islands. J. Commun. Dis., **17**. 249-250.

Lambert, R.A. 1970.

Parasitology: Identifiacation of protozoa. Publ. by Butterworth, London.

Lambl, W. 1859.

Mikroskopische Untersuchongen der Darm-Excrete. Vierscr. Prakt. Heil. Praug., 61. 1-58.

Lavier, G. 1924.

Deux espèces de *Giardia* du rat d'égout parisien (*Epimys norvegicus*). Ann. Parsit. Hum. Comp., 2. 161-168.

Lindmark, D.G. and Jarroll, E.L. 1984.

Metabolism of trophozoites. In Giardia and Giardiasis. Biology, Pathogenesis and Epidemiology. Ed. Erlandsen, S.E. and Meyer, E.A. 65-80.

Lopez, C.E., Dykes, A.C., Juranek, D.D., Sinclair, S.P., Conn, J.M., Christies, R.W., Lippy, E.C., Schultz, M.G. and Mires, M.H. 1980.

Waterborne giardiasis: a community wide outbreak of disease and a high rate of asymptomatic infection. Am. J. Epidemiol., **112**. 495-507.

Love, J. and Taylor, C.B. 1940.

Atebrine in the eradication of *Giardia lamblia*. U.S. Nav. Med. Bull., **38**. 239.

Macfie, J.W.S. 1922.

Observations on the role of cockroaches in disease. Ann. Trop. Med. Parasit., **16**. 441-448.

McNeish, A.S. and Anderson, C.M. 1974.

Clin. Gast., **3**. 127.

Malstrandrea, G. and Micarelli, A. 1968.

Search for parasites in vegetables from the local markets in the city of Rome. Arch. Ital. Sci. Med. Trop. Parassitol., **49**. 201-208.

Meyer, E.A. 1970.

Isolation and axenic cultivation of *Giardia* trophozoites from the rabbit, chinchilla and cat. Exp. Parasitol., **27**. 179.

Meyer, E.A. 1976.

Giardia lamblia: Isolation and axenic cultivation. Exp. Parasitol., **39**. 101-105.

Meyer, E.A. 1979.

The propagation of *Giardia* trophozoites *in vitro*. In. Waterborne transmission of Giardiasis. Ed. Jakubowski, W. and Hoff, J.C. 211-216.

Meyer, E.A. and Radulescu, S. 1984.

In vitro cultivation of *Giardia* trophozoites. In Giardia and Giardiasis. Biology, Pathogenesis and Epidemiology. Ed. Erlandsen, S.E. and Meyer, E.A. p 99-110.

Miller, R. 1926.

Coeliac disease: its definition and diagnosis. Lancet, **i** : 330.

Lambliasis as a cause of chronic enterises in children. Arch. Dis. Child., **1**. 93.

Miotti, P.G., Gilman, R.H., Santosham, M., Ryder, R.W. and Yolken, R.H. 1986.

Age related rate of seropositivity of antibody to *Giardia lamblia* in four diverse populations. J. Clin. Micro., **24**. 972-5.

Monzingo, D.L. Jr. 1985.

The prevalence of *Giardia* in a beaver colony and the resulting environmental contamination. Dissertatation, Ph.D., Colorado State University, 1985.

Moore, G.T., Cross, W.M. and McGuire, C.D. 1969.

Epidemic giardiasis at a ski resort. N. Eng. J. Med., **281**. 402-407.

Moore, G.W., Soganares-Bernal, F. and Dennis, M.V. 1982.
Characterization of *Giardia lamblia* trophozoite antigens using polyacrylamide gel electrophoresis, high performance liquid chromotography and enzyme linked immunosorbent assay. Vet. Parasitol., **10**. 229-237.

Morecki, R. and Parker, J.G. 1967.
Ultrastructural studies of the human *Giardia lamblia* and subjacent mucosa in a subject with steatorrhea. Gastroenterology, **52**. 151.

Moritz, F. and Holzl, H. 1892.
über Hâufigkeit und Bedeutung des Vorkommens von *Megastoma entericum* im Darmkanal des Menschen. Muench. Med. Wocenschar., **39**. 831.

Morrison, L.M. and Swalm, W.A. 1939.
A new effective parasiticide in giardiasis. Am. J. Dig. Dis., **38**. 239.

Musgrave, W.E. 1922.
Flagellate infestations and infections. J. Am. Med. Assoc., **79**. 2219-20.

Nash, T.E. and Keister, D.B. 1985.
Differences in secretory and surface antigens among 19 *Giardia* isolates. J. Infect. Dis., **152**. 1166-1171.

Nash, T.E., Gillin, F.D. and Smith, P.D. 1983.
Excretory - secretory products of *G. lamblia*. J. Immun., **131**. 2004-10.

Nash, T.E., McCutchan. T., Keister, D., Dame, J.B., Conrad, J.D. and Gillin, F.D. 1985.
Restriction-endonuclease analysis of DNA from 15 *Giardia* isolates obtained from humans and animals. J. Infect. Dis., **152**. 64-73.

Nasliowska, M. 1983.

Pasozyty/jelitawe - rok. [Intestinal parasites - 1983] Przeglad Epidemiologiczny, **39**. 181-187.

Nasliowska, M. 1984.

Pasozyty/jelitawe - 1984 rok. [Intestinal parasites - 1984] Przeglad Epidemiologiczny, **40**. 181-187.

Ormiston, G., Taylor, J. and Wilson, G.S. 1942.

Enteritis in a nursery home associated with *Giardia lamblia*. Br. Med. J., **2**. 151.

Osterholm, M.T., Forfang, J.C. and Ristinen, T.L. 1981.

An outbreak of foodborne giardiasis. N. Eng. J. Med., **304**. 24-28.

Owen, R.L., Dritz, S.K. and Wibbelsman, C.J. 1979.

Veneral aspects of gastroenterology. West. J. Med., **130**. 236-46.

Owen, R.L., Allen, C.L. and Stevens, D.P. 1981.

Phagocytosis of *Giardia muris* by macrophages in Peyer's patch epithelium in mice. Inf. Immun., **33**. 591-601.

Padchenko, I.K. and Stolyarchuk, N.C. 1969.

On the possible circulation of *Lamblia* in nature. Prog. Protozool., **3**. 311-312.

Perroncito, E. 1988.

Note sur l'enkystement du *Megastoma intestinale*. Bull. Soc. Zool. Fr., **13**. 16.

Poindexter, H.A. 1931.

Studies on the cultivation of parasitic intestinal protozoa. Puerto Rico J. Publ. Health, **7**. 417-434.

Phillips, R.E., Boreham, P.F.L. and Shepherd, R.W. 1982.
Cryopreservation of viable *Giardia intestinalis* trophozoites. Trans. Roy. Soc. Trop. Med. Hyg., **78**. 604-606.

Radulescu, S. and Meyer, E.A. 1981.
Opsonization *in vitro* of *Giardia lamblia* trophozoites. Inf. Immun., **32**. 852-856.

Rendtorff, R.C. 1954.
The experimental transmission of human intestinal protozoan parasites. II *Giardia lamblia* cysts given in capsules. Am. J. Hyg., **59**. 209.

Rendtorff, R.C. 1979.
The experimental transmission of *Giardia lamblia* among volunteer subjects. In Waterborne Transmission of Giardiasis, Ed. Jabukowski, W. and Hoff, J.C. 64-81.

Rendtorff, R.C. and Holt, C.J. 1954.
The experimental transmission of human intestinal protozoan parasites. III. Attempts to transmit *Endamoeba coli* and *Giardia lamblia* cysts by flies. Am. J. Hyg., **60**. 320-326.

The experimental transmission of human intestinal protozoan parasites. IV. Attempts to transmit *Endamoeba coli* and *Giardia lamblia* cysts by water. Am. J. Hyg., **60**. 327-328.

Rice, E.W. and Schaeffer, F.W. 1981.
Improved *in vitro* excystation procedure for *Giardia lamblia* cysts. J. Clin. Micro., **14**. 709-710.

Roberts-Thomson, I.C., Stevens, D.P., Mahmoud, A.A.F. and Warren, K.S. 1976.

Giardiasis in the mouse; an animal model. Gastroenterology, **71.** 57-61.

Aquired resistance to infection in an animal model of giardiasis. J. Immunol., **117.** 2036-2037.

Roberts-Thomson, I.C. and Mitchell, G.F. 1978.

Giardiasis in mice. I. Prolonged infections in certain mouse strains and hypothymic (nude) mice. Gastroenterology, **117.** 2036-2037.

Saha, T.K. and Ghosh, T.K. 1977.

Giardiasis: Association with homosexuality. Ann. Intern. Med. **88.** 801-804.

Schantz, P.M. 1983.

Compend. Contin. Educ. Prac. Vet., **3.** 773.

Schleinitz, P., Justus, P., Stenzal, P. Owen, R. and Meyer, E. 1983.

Successful introduction of culture adapted *Giardia* into a rabbit model: Ultrastructural features. Gastroenterology, **84.** 1301.

Schneider, J. 1961.

Traitment de la giardiase (lambliase) par le metronidazole. Bull. Soc. Pathol. Exot., **54.** 84.

Schuman, S.H., Arnold, A.T. and Rowe, J.R. 1982.

Giardiasis by inhalation? Lancet, **i** (8262). 53.

Sehgal, A.K., Grewal, M.S., Chakravati, R.N., Broor, S.L., Deka, N.C. and Chuttan, P.N. 1976.

Experimental giardiasis in albino rats. Ind. J. Med. Res., **64.** 1015-18

Shaw, P.K., Brodsky, R.E., Lyman, D.O., Wood, B.T., Hibler, C.P., Healy, G.R., MacLeod, K.I.E., Stahl, W. and Schultz, M.G. 1977.
A community wide outbreak of giardiasis with evidence of transmission by a water supply. Ann. Int. Med., **87**. 426-432.

Simon, C.E. 1922.
Giardia enterica: a parasitic flagellate of man. Amer. J. Hyg., **1**. 440-491.

Smith, P.D. 1984.
Human Immune responses to *Giardia lamblia*. In Giardia and Giardiasis. Biology, Pathogenesis and Epidemiology. Ed. Erlandsen, S.E. and Meyer, E.A. 201-218.

Smith, P.D., Gillin, F.D., Brown, W.R. and Nash, T.E. 1981.
IgG antibody to *Giardia lamblia* detected by enzyme linked immunosorbent assay. Gastroenterology, **80**. 1476-1480.

Smith, P.D., Hornsburgh, C.M. and Brown, W.R. 1981.
In vitro studies on bile acid deconjugation and lypolysis inhibition by *Giardia lamblia*. Dig. Dis.Sci., **26**. 700-704.

Smith, P.D., Gillin, F.D., Kausal, N.A. and Nash, T.E. 1982.
Antigenic analysis of *Giardia lamblia* from Afghanistan, Puerto rico, Equador and Oregon. Inf. Immun., **36**. 714-719.

Smith, P.D., Gillin, F.D., Spira, W.M. and Nash, T.E. 1982.
Chronic giardiasis: Studies on drug sensitivity, toxin production and host immune response. Gastroenterology, **83**. 797-803.

Smith, P.D., Keister, D.B. and Elson, C.O. 1982.
Human host response to *Giardia lamblia*. II. Antibody-dependent killing *in vitro*. Cell. Immunol. **82**. 308-315.

Snider, D.P. Underdown, B.J. 1986.

Quantitative and temporal analysis of murine antibody response in serum and gut secretions to infection with *Giardia muris*. Inf. Immun., **52**. 271-278.

Solomons, N.W. 1982.

Giardiasis: Nutritional implications. Rev. Infect. Dis., **4**. 859-869.

Speghelyi, P.V. 1940.

Giardiasis. Am. J. Dis. Child., **59**. 793.

Steele, T.W. and McDermott, S. 1977.

Diagnosis of giardiasis. Med. J. Austr., **Dec. 24/31**. 876-877.

Sutherland, R.J. 1982.

Giardiasis in Saanen goats. Surveillance, **9**. 25.

Sutherland, R.J. and Clarkson, A.R. 1984.

Giardiasis in intensively reared Saanen kids. NZ Vet. J., **32**. 34-35.

Suteu, E. and Coman, S. 1974.

Observations sur la giardose du chein, fréquence et association avec l'dautres parasitoses. Recl. Med. Vet. Ec. Alford, **150**. 593.

Swan, J.M. and Thompson, R.C.A. 1986.

The prevalence of *Giardia* in dogs and cats in Perth, Western Australia. Austr. Vet. J., **63**. 110-112.

Thornton, S.A, DuPont, H.L., West, A.H. and Pickering, L.K. 1983.

Comparison of methods for identification of *Giardia lamblia*. Am. J. Clin. Pathol., **80**. 858-860.

Tsuchiya, H. 1930.

A comparitiave study of two diverse strains of *Giardia lamblia*. Am. J. Hyg., **12**. 467-491.

Tsuchiya, H. 1931.

Changes in morphology of *Giardia canis* as affected by diet. Proc. Soc. Exp. Biol. Med., **28**. 708-709.

Ungar, B.L.P., Yolken, R.H., Nash, T.E. and Quinn, T.C. 1984.

Enzyme linked immunosorbent assay for the detection of *Giardia lamblia* in faecal specimens. J. Inf. Dis., **149**. 90-97.

Veazie, L. 1969.

Epidemic giardiasis. N. Eng. J. Med., **281**. 853.

Veghelyi, P. 1939.

Absorption studies in children with *Giardia lamblia* infection, Arch. Dis. Child., **14**. 155.

Veghelyi, P.V. 1940.

Giardiasis. Am. J. Dis. Child., **59**. 793.

Vinayak, V.K., Sharma, G.L. and Naik, S.R. 1979

Experimental *Giardia lamblia* infection in Swiss mice - a preliminary report. Ind. J. Med. Res., 70. 195-198.

Vinayak, V.K., Kum, K., Chandra, R. Venkatesuonlu, K. and Mehta, S. 1985.

Detection of *Giardia lamblia* antigen in faeces by counterimmunoelectrophoresis. Ped. Inf. Dis., **4**. 383-386.

Visvesvara, G.S. 1980.

Axenic growth of *Giardia lamblia* in Diamonds TPS-1 medium. Trans. Roy. Soc. Trop. Med. Hyg., **74**. 213-215.

Visvesvara, G.S. Healy, G.R. and Meyer, E.A. 1977.
Comparative antigenic analysis of *Giardia lamblia* trophozoites grown in medium supplemented with human, bovine, or rabbit serum. Fifth Int. Congr. Protozool.

Visvesvara, G.S. Smith, P.D. Healy, G.R. and Brown, W.R. 1980.
An immunofluorescent test to detect serum antibodies to *Giardia lamblia*. Ann. Intern. Med., **93**. 802-805.

Voltrina, E.N. 1941.
Sur la question du traitment de la lambliose par l'acrichine (Atebrine). Trop. Dis. Bull., **38**. 275.

Walia, B.N.S., Ganguly, N.K., Mahajan, R.C., Kumar, D., Madan, I.J., Gambhir, S.K. and Kanwar, S.S. 1986.
Morbidity in preschool *Giardia* cyst excretors. Trop. Geog. Med., **38**. 367-370.

Wallis, P.M. and Wallis, H.M. 1986.
Excystation and culture of human and animal *Giardia* sp. by using Gerbils and TY1-S-33 medium. App. Env. Micro., **51**. 647-651.

Walzer, P.D., Wolfe, M.S., and Schultz, M.G. 1971.
Giardiasis in travellers. J. Infect. Dis., **124**. 235.

Weiden, S.C., Keister, D.B. and Kenen, D.S. 1983.
Mass cultivation of *Giardia lamblia* in serum free medium. J. Parasit., 69. 1181-1182.

Wenyon, C.M. 1915.
Observations on the common intestinal protozoa of man: their diagnosis and pathogenicity, Lancet, **ii** : 1173.

Wenyon, C.M. 1926.
Protozoology. 2 volumes New York, publ. William Wood and Co.

Wenyon, C.M and O'Connor, F.W. 1917.

Human intestinal protozoa in the Near East. Publ. by Wellcome Bureau of Scientific Research. 1st ed., London. John Bale, Sons and Danielson, ltd.

Wilson, A. 1984.

World Health, 3. 22.

Winiecka, J., Kasprzak, W., Kocecka, W., Plotkowiak, J. and Myjak, P. 1984.

Serum antibodies to *Giardia intestinalis* detected by immunofluoresence using trophozoites as antigen. Tropenmed. Parasit., 35. 20-22.

Wolfe, M.S. 1975.

Giardiasis. JAMA, 233. 1362.

Wolfe, M.S. 1979.

Managing the patient with giardiasis: Clinical, diagnostic and therapeutic aspects. In Waterborne Transmission of Giardiasis, Ed. Jabukowski, W. and Hoff, J.C. 39-53.

Wolfe, M.S. 1984.

Symptomolgy, diagnosis and treatment. In Giardia and Giardiasis. Biology, Pathogenesis and Epidemiology., Ed. Erlandsen, S.L. and Meyer, E.A. Publ. by Plenum press, New York, NY. 147-161.

Woo, P.T.K., 1984.

Evidence for animal resevoirs and transmission of *Giardia* infection between animal species. In Giardia and Giardiasis. Biology, Pathogenesis and Epidemiology., Ed. Erlandsen, S.L. and Meyer, E.A. Publ. by Plenum press, New York, NY. 341-364.

Woo, P.T.K. and Patterson, W.P. 1986.

Giardia lamblia in children in day care centres in Southern Ontario, Canada, and susceptibility of animals to *G. lamblia*. Trans. Roy. Soc. Trop. Med. Hyg., 80. 56-59.

World Health Organization 1980.

Parasite-related diarrhoeas. Bull. WHO, **58**. 819-830.

Wright, S.G, Tomkins, A.M. and Ridley, D.S. 1977.

Giardiasis - clinical and therapeutic aspects. Gut, **18**. 343-350.

Yoeli, M., Most, H. Hammond, J. and Scheinsson, G.P. 1972.

Parasitic infections in a closed community; Results of a 10 year survey in Willowbrook State School. Trans. Roy. Soc. Trop. Med. Hyg., **66**. 754-776.

Zimmer, J.F. and Burington, D.B. 1986.

Comparison of four techniques of faecal examination for detecting canine giardiasis. J. Am. Anim. Hosp. Assoc., **22**. 161-167.

Zimmer, J.F. and Burington, D.B. 1986.

Comparison of four protocols for the treatment of canine giardiasis. J. Am. Anim. Hosp. Assoc., **22**. 168-172.