Differentiation of human and calf isolates of *Giardia intestinalis*

A thesis presented in partial fulfilment of the
requirements for the degree of
Master of Science
in
Microbiology
at Massey University, Palmerston North,
New Zealand.

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1999

ABSTRACT

Traditionally farm runoff has been blamed for the contamination of aquatic waterways with *Giardia* cysts especially during the natural calving seasons. But despite *Giardia intestinalis* being one of the most commonly acquired waterborne gastrointestinal parasites in humans little is known about the extent of *G.intestinalis* transmission between humans and animals throughout a defined geographical region. This study examines the characterisation of human, calf and laboratory adapted isolates of *G.intestinalis*.

Specific amplification primers were developed to target a section of the ribosomal DNA (rDNA) unit. This locus is considered to be rapidly evolving and therefore suitable for use in the elucidation of phylogenetic relationships between *G.intestinalis* isolates. The isolates characterised were collected in the Waikato district from naturally infected humans and calves throughout 1998 but especially during the spring calving season of August and September. *Giardia* from calves from a second province as well as laboratory adapted isolates cultured from a variety of hosts were also surveyed. Sequence analysis of human, calf and laboratory adapted *G.intestinalis* isolates showed the presence of three distinct groups. All calf *G.intestinalis* isolates clustered together despite differences in the collection time and site. The human *G.intestinalis* isolates split into two clusters, corresponding to recognised 'Polish' and 'Belgian' subtypes. Surprisingly the laboratory adapted isolates grouped with the human 'Polish' subtype despite striking differences in isolate origin. The current data strongly suggests that host specific *G.intestinalis* strains are present in the environment. Cross-transmission has so far not been detected.

The occurrence of isolate specific rDNA sequences enabled the development of diagnostic polymerase chain reaction (PCR) amplification primers. In conjunction with the existing primers these allow the identification of human specific *Giardia* as well as differentiating between the 'Belgian' and 'Polish' subtypes. These primers offer the ability to quickly and economically identify potential sources of human giardiasis in the environment. Using such molecular tools may lead to an overall decrease in human giardiasis resulting from environmental contamination sources.

ACKNOWLEDGMENTS

As with any research there are a number of individuals who stuck with me through the many ups and downs experienced during this particular research. They all deserve much more than a mention in this acknowledgment but at least it's a start.

My supervisors Professor Tim Brown and Dr George Ionas supported me thoroughly throughout my time in the Protozoa Research Unit with an occasional hint of compassion, or muffin, when needed. This project reflected ideas they had been kicking around for awhile and felt I was the right person to be up to my elbows in faecal matter for two years! Thanks to the Protozoa Research Team; Jim Learmonth, Liz Keys, Trish McLenachan, and Michiko Kakubayashi who shared the smells with me, even when the rest of the building would rather have not.

Thanks to the many staff in the Department of Microbiology and Genetics and now the Institute of Molecular BioSciences who assisted me with many aspects of the research. There are far too many to name.

Thanks to all my flatmates who over the years put up with, although not quietly, all of my many calf faeces collection trips. After completing this work I don't think our flat fridge has ever been so empty with no one complaining!

Most importantly thanks goes out to my ever-loving and supportive family. I am grateful for having a "1990's" family of many members who at different times gave me bursts of support. Fortunately the one upcoming addition to my family means I will always have someone to help me build trees, order primers and collect specimens should I have the need! Thanks Richard.

Thank you all.

Cindy Lee Lou Dunny Hunt dedicates her thesis to her brothers,
Clynton and Vaughan,
and looks forward to playing basketball again with them both one day.

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CHAPTER ONE: INTRODUCTION

1.1 *GIARDIA* – THE ORGANISM

1.1.1 Discovery

Giardia was first observed by van Leeuwenhock, a Dutch amateur lens maker, in 1681 while examining his own diarrhoeic faeces (Adam, 1991). Although he made no illustrations of the organisms his descriptions were later recognised to be *Giardia* trophozoites. In 1859 a Czech physician, Lambl, isolated *Giardia* from children suffering with dysenteric diarrhoea (Adam, 1991). Lambl, for whom the organism was later named after, described *Giardia* trophozoites in greater detail. The connection between the cyst and trophozoite life form of *Giardia* was recognised by Grassi, a French biologist, in 1879, nearly 200 years after the organism was first observed.

Until 25-30 years ago, *Giardia* was considered a commensal organism living in the mucosal lining of its hosts' small intestine. Due to an increased occurrence in waterborne outbreaks of diarrhoea, the observation of severe giardiasis in patients with hypogammaglobulinemia (Faubert, 1996) and the fulfilment of Koch's postulates in experimental human infections (Nash *et al.*, 1987) *Giardia* was identified as a human pathogen (Adam, 1991). *Giardia intestinalis* (synonyms *Giardia lamblia* and *Giardia duodenalis*) has been found to be one of the most commonly acquired waterborne parasites causing gastrointestinal infections in humans. In this thesis *Giardia* capable of infecting humans and other mammalian hosts will be referred to as *G.intestinalis*.

1.1.2 Taxonomy

Giardia is a flagellated, aerotolerant anaerobic protozoan parasite in the phylum Sarcomastigophora, subphylum Mastigophora, class Zoomastigophorea, order Diplomonadida (Cox, 1981). Phylogenetic reconstruction's based on rDNA sequences indicate that *Giardia* is one of the most ancient representatives of the eukaryotes (Vellai *et al.*, 1998). Ribosomal size comparisons and the presence of

both prokaryotic and eukaryotic like features also support the basal position of *Giardia* within eukaryotes. Prokaryotic-like features of *Giardia* include anaerobic metabolism, a plasmid, the bacterial enzyme pyruvate, toxin-like genes and the use of cysteine in maintaining redox balance. *Giardia* lacks several prominent eukaryotic features, such as mitochondria and peroxisomes, but does possess a complex, highly developed cytoskeleton, bounded nuclei and linear chromosomes capped with telomeric repeats which are found in eukaryotes (Upcroft and Upcroft, 1998).

Prior to the development of a morphological taxonomy (Filice, 1952) there were more than forty *Giardia* species recognised based primarily on the host from which the parasite was originally isolated. The taxonomy developed by Filice identified three taxa species, *Giardia agilis*, *Giardia muris*, and *Giardia intestinalis*. This division was based on fixed morphological differences in trophozoite size, median body shape, caudal flagella, and ventrolateral flange presence as well as differences in hosts (Thompson and Lymbery, 1996). *G.agilis,* with a teardrop-like appearance and measuring 20μm by 4μm, is a parasite of amphibians and has not been isolated from mammals. Measuring 10μm by 7μm with a smaller, rounded median body *G.muris* has been isolated from rodents and possums (Ortega and Adam, 1997). *G.intestinalis* has been isolated from a wide range of mammalian hosts and to date is the only species known to parasitise humans (McKenna, 1998; Koudela and Vítovec, 1998; Majewska *et al.,* 1998; Monis *et al.,* 1998). Measuring 13μm by 8μm *G.intestinalis* trophozoites have claw hammer shaped median bodies.

Recently three additional *Giardia* species have been identified due to differences in morphological and molecular characteristics. *Giardia ardeae* which infects wading birds shows variable median body morphology and is distinguishable by the presence of only a single caudal flagella (van Keulen *et al.*, 1990; Erlandsen *et al.*, 1990). A second form also infects an avian host. *Giardia psittaci* infects budgerigars and despite having claw hammer shaped median bodies *G.psittaci* is distinguishable from *G.intestinalis* by the absence of a ventrolateral flange (Erlandsen and Bemrick, 1987). A sixth taxon, *Giardia microti*, which is

morphologically distinguishable from *G.intestinalis* under light microscopy, has recently been isolated from voles and muskrats (van Keulen *et al.*, 1998).

Species-specific characteristics, such as median body shape, are important morphological features in the taxonomic characterisation of different *Giardia* species although considerable variation has been noted among different isolates (Thompson and Lymbery, 1996). Recently combinations of morphological and molecular characteristics have been used in the study of *Giardia* taxonomy. The diversity exhibited by *G.intestinalis* isolates has prompted extensive investigation into the taxonomic classification of these isolates.

1.1.3 Morphology and Life Cycle

Giardia has a relatively simple asexual life cycle consisting of two morphological stages, a cyst and a trophozoite phase. Both cysts and trophozoites are present at defined stages during the life cycle and, with few exceptions, only one morphological phase is found at any given time.

In the environment cysts are the infectious form of *G.intestinalis* and are introduced into the host body by oral ingestion. Host ingestion initiates cyst excystation, a process which degrades the thick cyst wall due to highly acidic stomach conditions, typically pH 1.3-4.0, and the presence of pancreatic fluid (Ortega and Adam, 1997). Excystation results in the release of two vegetative trophozoites from each ingested cyst into the small intestine. Trophozoites are the replicative form of *G.intestinalis*, multiplying by repeated asexual binary fission and are typically only found in the gastrointestinal tract of the infected host (Marshall *et al.*, 1997). The binucleate trophozoites are unilaterally symmetrical and are approximately 11-15μm long by 6-10μm wide giving them a pear-shape appearance. Both nuclei, one positioned either side of the centreline, are simultaneously transcriptionally active. Four pairs of flagella (anterior, caudal, posterior and ventral) provide the trophozoite with mobility while a large ovoid sucking disc on the anterior ventral surface facilitates adherence of the trophozoite to the lining of the small intestine. A ventrolateral flange, a flexible ring surrounding the suction disc, aids trophozoite adherence (Luján *et al.*, 1997).

G.intestinalis trophozoites are non-invasive and do not normally penetrate the intestinal epithelium microvilli of infected hosts. Mucosal penetration by *G.intestinalis* trophozoites has been observed but should be regarded as a rare event (Farthing, 1997).

The small intestine, with relatively little microbiological competition, is the usual environment for *G.intestinalis* trophozoites. However trophozoites will pass down the gastrointestinal tract due to overcrowding as the result of rapid multiplication or the continuous shedding of the gastrointestinal epithelial lining. As these trophozoites pass down the gastrointestinal tract, localised environmental changes induce the transformation to cysts. Increasing levels of bile in addition to cholesterol deprivation triggers the two-stage encystation process (Luján *et al.,* 1997). Consisting of an intracellular and an extracellular stage, encystation takes fourteen to sixteen hours to complete and results in mature infectious cysts (Erlandsen *et al.,* 1996). An encysting trophozoite undergoes nuclear division so that the mature cyst contains two binuclear trophozoites. However, mature *G.intestinalis* cysts do not undergo active replication. *G.intestinalis* cysts are oval in shape, approximately 5μm by 8μm, with a smooth 0.3μm thick cyst wall (Adam, 1991). The cyst wall protects the two enclosed trophozoites from adverse environmental conditions encountered during transfer between hosts (Erlandsen *et al.,* 1996). Mature infectious cysts are excreted from the host within faecal material. Typically only cysts are expelled from the host, except during severe infections when incompletely encysted trophozoites may be excreted. These trophozoites are however non-infectious and as they do not complete encystation quickly die (Gillin *et al.,* 1996).

The ingestion of mature *G.intestinalis* cysts from a faecally contaminated source by a second host initiates excystation of ingested cysts and the release of trophozoites, therefore beginning a new cycle of infection. Cysts survive well under favourable environmental conditions, so do not require an immediate host (Owen, 1984).

1.1.4 Transmission of *G.intestinalis*

The transmission of *G.intestinalis* can occur *via* one of many routes.

1.1.4.1 Person to person transmission

Person to person transfer of *G.intestinalis* occurs by direct faecal/oral transmission. Closed communities, such as daycare centres, institutions, and convalescent homes, generally show higher levels of giardiasis (Olsen *et al.*, 1996). Generally hygiene practices at such centers are substandard as the individuals are usually either young children, the elderly or those with lowered mental capabilities. The exploratory behaviour of young children and the mentally impaired, both anally and orally, increases the rate of giardiasis (Hall, 1994). Outside of the closed communities *G.intestinalis* can be transmitted by way of poor hygiene to parents, siblings and care givers (Adam, 1991).

People participating in anal intercourse, especially male homosexuals, also show an increased incidence of giardiasis (Hall, 1994). Due to the relatively low infectious dose of *G.intestinalis* direct anal contact appears to be an important mode of transmission.

1.1.4.2 Foodborne transmission

Viable *G.intestinalis* cysts can be introduced onto foods through washing, cooking or crop food propagation. Foodborne transmission of *G.intestinalis* usually occurs when asymptomatically infected food handlers contaminate prepared foods with viable cysts due to poor personal hygiene (Islam, 1990). Foodborne outbreaks of *G.intestinalis* can also result from uninfected food handlers who, prior to food preparation, were exposed to viable *G.intestinalis* cysts, usually due to interpersonal contact (Bemrick, 1984). Both commercially and privately prepared foods have been reported as sources for giardiasis (Barnard and Jackson, 1984). Crop plants fertilised with excrement or grown using water containing viable *G.intestinalis* cysts can also promote giardiasis (Pell, 1997; Bemrick, 1984). Owen (1984) reported that *G.intestinalis* cysts remain viable for at least three

months under favourable environmental conditions. Shellfish, due to filter feeding methods, can concentrate *G.intestinalis* cysts from marine waters and consumption of raw shellfish can result in giardiasis (Johnson *et al.,* 1995; Graczyk *et al.,* 1998). However, cyst viability is limited in seawater, Brown *et al.* (1999) noted a 30-50% drop in cyst viability after seven days in seawater at 15°C. Care should therefore be taken in food preparation preferably through thorough cleaning and cooking.

1.1.4.3 Waterborne transmission

Waterborne transmission of *Giardia* cysts occurs readily, currently accounting for most parasitic gastrointestinal infections in humans (Marshall *et al.,* 1997). Waterborne transmission of *G.intestinalis* can occur through two generalised scenarios.

Inadequately treated municipal water supplies are potential sources for large scale *G.intestinalis* infections. Contamination of water supplies generally occurs through the introduction of wastewater containing either animal or human faeces infected with *Giardia* cysts. During the late 1970's a number of waterborne *G.intestinalis* outbreaks occurred as the result of inadequately filtered municipal water supplies (Hibler and Hancock, 1990). Although there were few human cases of giardiasis reported, the poor treatment procedures used by many municipal water suppliers was highlighted. In New Zealand, 320 out of 1669 (19.2%) waterways tested throughout the country over a six year period were positive for *Giardia* cysts (Brown *et al.*, 1998b). Although no particular pattern was evident, extensively populated areas, both urban and rural, contained a high proportion of the positive locations. Seasonal occurrence of *Giardia* has been identified in dairy farming areas, such as the Manawatu and Waikato regions, although *Giardia* presence was not confined to these regions and appeared to be widespread throughout New Zealand (Brown *et al.*, 1998a).

Waterborne transmission can also occur through direct exposure of individuals to *G.intestinalis* cysts present in contaminated recreational aquatic environments. The introduction of recently excreted human or animal faecal matter containing

G.intestinalis cysts or inadequately sanitised wastewater into recreational aquatic environments has been reported to result in human giardiasis (Ortega and Adam, 1997).

1.1.4.4 Zoonotic transmission

Throughout the world both domestic and feral animals are infected with *G.intestinalis* and the potential risk these reservoirs pose to humans remains unclear. In New Zealand large numbers of animal reservoirs, in terms of animal population size and *G.intestinalis* infection frequency, have been identified (Chivlers *et al.*, 1998). Brown *et al* (1997) reported *G.intestinalis* occurrence rates of 28.4% in cattle, 30% in sheep, 57.8% in black rats, 22% in mice, 15.7% in possums, 34% in blackbirds and 61% in chaffinches. Companion animals, such as cats and dogs, also appear to be reservoirs of *G.intestinalis*. In two New Zealand cites, Palmerston North and Hamilton, 3 to 25% respectively of cats and dogs excreted faeces containing *G.intestinalis* cysts (Tonks *et al.*, 1991). Worldwide studies have been unable to conclusively eliminate animals as a source of *G.intestinalis* cysts capable of causing human giardiasis (Monis *et al.*, 1998; Baruch *et al.*, 1996; Meloni et al., 1995).

Hopkins *et al.* (1997) genetically characterised isolates from human and canine faeces in an Australian aboriginal community, the first reported genetic characterisation of *Giardia* from humans and dogs inhabiting the same local environment. The 5' end of the 16S rDNA gene was sequenced from PCR amplified fragments for all isolates. Four genetically distinct groups were reported. The first two groups consisted of human and canine representatives which corresponded to the 'Polish' and 'Belgian' (Homan *et al.*, 1992) groups respectively. The second two groups contained only dog isolates. Although most human and dog isolates fell into genetically separate groups, cross-transmission of *G.intestinalis* between the host species appears to occur at a low frequency, as indicated by the genetic similarity of some human and dog isolates to each other. Monis *et al.* (1998) confirmed the division of human and canine *G.intestinalis* isolates into four sections based on sequence analysis of the glutamate dehydrogenase locus. The four groups, termed Assemblages A, B (corresponding

to Andrews *et al.* 1989) and C, D (new assemblages) are identical to those identified by Hopkins *et al.* (1997).

Although the transmission frequency of *G.intestinalis* between human and companion animal hosts through direct faecal/oral contact remains unclear, waterborne transmission between animals and humans has been investigated. Although controversial, the link between aquatic environments contaminated from an animal source and subsequent human infections has been documented. Using isoenzyme electrophoresis and pulsed-field gel electrophoresis (PFGE) Isaac-Renton *et al.* (1993) characterised *G.intestinalis* isolates from water, animal and human sources during a waterborne outbreak of giardiasis. The parasite was genetically identical, as determined by these techniques, whether isolated from the original source, the contaminated water or infected human hosts. In this case the source of infection was traced to beavers inhabiting areas upstream of the municipal water supply intake. Although beavers were linked to the *G.intestinalis* outbreak in this example they are not the only mammals connected with the contamination of aquatic environments with *G.intestinalis* cysts.

1.2 THE DISEASE

1.2.1 Giardiasis

G.intestinalis causes a severe infection in humans called giardiasis. Giardiasis may result following oral ingestion of as few as ten to a hundred viable cysts (Ortega and Adam, 1997). Generally *G.intestinalis* infections are apparent after a period of seven to ten days following cyst ingestion, although latency can be as long as seventy-five days (Wolfe, 1984). During the latent period the host jejunum microvilli are densely colonised by *G.intestinalis* trophozoites and the host may suffer nausea, low grade fevers, chills and general malaise. Eventually colonisation results in abdominal cramps, malabsorption, foul smelling excess gas and profuse, watery, foul smelling diarrhoea. Typically weight loss occurs during giardiasis, although it is experienced most often during chronic giardiasis. Temporary post giardiasis lactose intolerance can occur during infections and may continue after all other symptoms, including cyst excretion, have ceased (Wolfe,

1990). If untreated, symptoms of a *G.intestinalis* infection may continue for two to four weeks although up to 25% of untreated *G.intestinalis* infections in travellers are symptomatic for up to seven weeks or longer (Farthing, 1994). Chronic infections, developing from untreated acute *G.intestinalis* infections, occur in an estimated 30-50% of cases.

The immune response to *G.intestinalis* infections involves both humoral and cellular immunity, in addition to other non-specific responses. In some patients *G.intestinalis* can be spontaneously eliminated while some patients like young children and patients with compromised immune responses tend to experience an increased severity and prevalence of infection indicating that humoral immunity is of particular importance (Rabbani and Islam, 1994). Also indicative of humoral immunity is the partial resistance to reinfection conferred by previous exposure to *G.intestinalis* (Rabbani and Islam, 1994). Expression levels of the secretory immunoglobulin, IgA, are elevated during giardiasis due to the non-invasive nature of *G.intestinalis* at the gastrointestinal mucosal level (Roberts-Thomson and Anders, 1984). Smith (1984) showed, *in vitro*, that spontaneous cell-mediated cytotoxicity (SCMC) and antibody dependent cellular cytotoxicity (ADCC) are important responses to *G.intestinalis* infections. Similar studies had shown earlier that, *in vivo*, *G.intestinalis* stimulated the SCMC and ADCC response (Smith, 1984).

1.2.2 Treatment of giardiasis

Once an infection has been detected the symptoms of giardiasis can be treated. Currently there are several drug regimes available for the treatment of giardiasis however each has limitations.

Quinacrine, the preferred antiprotozoal drug, has 95% efficiency against *G.intestinalis* when prescribed orally over a five to ten day period. However gastrointestinal side effects, such as nausea, cramps, and diarrhoea, are relatively common. Animal studies, mainly involving rats, indicate an increase in foetal death rates during exposure to this drug (Long and Rybacki, 1994). Although adequate human pregnancy studies are not available women currently pregnant or

breast-feeding are generally not prescribed Quinacrine. Typically Quinacrine is not well tolerated by children or the elderly.

The broad-spectrum antibiotic, Paromomycin is recommended for the treatment of giardiasis in women either currently pregnant or breast-feeding due to possible foetal side effects shown in animal models by other more effective frontline antiprotozoal agents (Ortega and Adam, 1997). Paromomycin is typically 55% effective in eliminating *Giardia* infections but shows none of the side effects on the foetus found for other more effective antiprotozoal agents.

Furazolidone (Furoxone™) is generally used for the treatment of children due to greater tolerance although it is less effective than Quinacrine. Although Furazolidone has shown carcinogenic properties in animal models it demonstrates no such effects in humans (Boreham, 1994).

Two other drugs, Metronidazole (Flagyl™) and Tinidazole, both nitromidazoles, are highly effective in the treatment of giardiasis. Tinidazole is well tolerated and highly effective when given as a single dose while Metronidazole is typically 90% effective when given over a five day course. Gastrointestinal side effects are common for both drugs. Metronidazole has in animal studies led to increased cancer rates although no such increases have been demonstrated in humans following treatment (Adam, 1991).

1.3 *G.INTESTINALIS* DIVERSITY

The biological diversity reported for *G.intestinalis*, in particular different phenotypic characteristics such as host specificity, growth rate, infectivity, virulence and drug sensitivity, has highlighted inadequacies in current intraspecific taxonomy. A number of recent studies investigating *G.intestinalis* classification have reported greater variation between isolates from the same host species than between isolates from different host species (Andrews *et al.*, 1998; Monis *et al.*, 1998; Meloni *et al.*, 1995; Weiss *et al.*, 1992). The molecular characterisation and comparison of *G.intestinalis* isolates from both human and animal hosts has revealed that considerable genotypic diversity exists within this

species. Using allozyme electrophoresis Andrews *et al.* (1989) characterised and compared axenically cloned *G.intestinalis* isolates from throughout Australiasia. The 58 isolates were found to cluster in four distinct groups, Group I-IV. Human isolates propagated by the infection of suckling mice, fell into two main assemblages which covered the variation observed in Groups I-IV as identified by Andrews *et al.* (1989). Later work by Ey *et al.* (1992, 1993) using restriction fragment length polymorphism's (RFLP) of genomic *G.intestinalis* DNA and sequence analysis of genes encoding cysteine-rich surface proteins supported the division of *G.intestinalis* into four genetic groups.

Homan *et al.* (1992) used isoenzyme analysis and RFLP analysis to compare *G.intestinalis* taken from hospital patients throughout the world. This study identified two major genetic groups termed 'Polish' and 'Belgian'. RLFP pattern differences in surface antigen expression and the presence/ absence of a group-specific gene identified three assemblages within the human isolates investigated (Nash and Mowatt, 1992). The Assemblage A of Andrews *et al.* (1989), Groups 1 and 2 of Nash and Mowatt (1992) and Homan *et al.* (1992) 'Polish' strain are all equivalent. Similarly Assemblage B of Andrews *et al.* (1989), Group 3 of Nash and Mowatt (1992) and Homan *et al.* (1992) 'Belgian' strain are all equivalent.

The classification of *G.intestinalis* isolated from human hosts has revealed distinct evolutionary lineages within this species and it has been suggested that a taxonomic complex would more appropriately reflect this variability (Upcroft and Upcroft, 1998). The significance of molecular characteristics and the possible inclusion of animal isolated *G.intestinalis* to these classification groups remains controversial. A number of researchers have investigated the possible zoonotic transmission of *G.intestinalis* between human and animal hosts although current data is contradictory.

Mayrhofer *et al.* (1995) compared sixty Australian isolates from human faeces representing Groups I–IV of Andrews *et al.* (1989) using enzyme electrophoresis analysis. Despite the high level of variability displayed by these isolates two genetic groups consistent with the earlier studies were observed. In addition to the human samples an isolate of *G.muris* and an isolate of *G.intestinalis* from a feline

host were included. Despite being *G.intestinalis* the feline isolate did not associate with the human *G.intestinalis* subgroups. The exclusion of the feline isolate from either assemblage supported an earlier study that had found cats to be highly resistant to infection by human *G.intestinalis* (Kirkpatrick and Green, 1985). As expected *G.muris* did not group within either *G.intestinalis* cluster.

The characterisation of *G.intestinalis* isolates by the above studies all required some form of *in vitro* or *in vivo* culturing. Axenisation is not only difficult and time consuming but introduces a selection bias for isolates which perform better *in vitro*. This bias is an important factor when comparing strains from host species that have proven difficult to culture axenically. Brown *et al.* (1992) reported difficulties when culturing New Zealand human *G.intestinalis* isolates establishing only eight culture lines from 129 attempts. Similarly Hopkins *et al.* (1997) were unable to establish *in vitro* cultures of *G.intestinalis* isolates from canine hosts over a ten year period. Mayrhofer *et al.* (1995) reported that *G.intestinalis* isolates not maintained by axenic culture revealed a greater degree of genetic heterogeneity than that found from axenic *G.intestinalis* isolates. This suggests that strong selective pressures are placed on the organism by this culture method. The isolates characterised by Mayrhofer *et al.* (1995) had however been inoculated into suckling mice, which introduces a selective bias for *G.intestinalis* strains that are better adapted to survival in mice. Clearly, techniques reliant on the growth of *Giardia* either *in vitro* or *in vivo* have limited applications when characterising and comparing genotypic attributes of isolates from different host species. Ideally molecular characterisation should be carried out on *Giardia* cysts directly isolated from naturally infected hosts. Recently random amplified polymorphic DNA (RAPD) analysis (Ionas et *al.*, 1997) and DNA sequencing (Hopkins et *al.*, 1997) has been used for the molecular characterisation of *G.intestinalis* as these do not rely on culturing the organism in growth medium.

1.4 OBJECTIVES AND APPROACHES

Worldwide studies have shown that farm animals, in particular cattle, sheep, horses, deer and goats, secrete large numbers of *G.intestinalis* cysts into the environment while infected (Majewska *et al.*, 1998; Ey *et al.*, 1997; Olson *et al.*, 1997; Quílez *et al.*, 1996). The direct contamination of aquatic environments with animal faecal matter containing *G.intestinalis* cysts *via* general farm runoff has been well documented although the implications for humans remains unclear (Crockett and Haas, 1997; States *et al.*, 1997). Most cattle are naturally infected with *G.intestinalis* within the first eight weeks of life and infections typically last a week (Quílez *et al.*, 1996). Despite excreting up to 1×10^9 cysts per day infected calves show few side effects and resolve infections without specific medical treatment (Pell, 1997). In large dairy farming regions during the spring and autumn calving seasons, the number of *G.intestinalis* cysts excreted daily increases dramatically in comparison to non-calving periods. An increase in the number of reported human giardiasis cases during the spring and autumn has also been documented however, the characterisation of calf and human *G.intestinalis* isolates during this period has yet to be investigated (Brown *et al.*, 1998a). This thesis investigates the relationship of *G.intestinalis* infections present in naturally infected calves and humans living in the same dairy farming region.

The *Giardia* genome is approximately 1.2×10^7 base pairs (bp) in size, but it is not clear whether this is the haploid or diploid size. The genome consists of five to eight major chromosomes and over forty minor chromosomes that appear to be duplications of most or all of the major chromosomes (Upcroft and Upcroft, 1998). Like the majority of eukaryotes, *G.intestinalis* carries a set of tandemly repeated units containing the ribosomal DNA operon. This operon consists of a small subunit rDNA (16S rDNA) gene, an internal transcribed spacer (ITS) region, a 5.8S rDNA gene, a second ITS region, a large subunit rDNA (23S rDNA) gene and an external non-transcribed spacer (van Keulen *et al.*, 1992). The ribosomal DNA operons for *G.intestinalis* and *G.muris* are approximately 5566bp and 7668bp in size respectively (van Keulen *et al.*, 1992; Healey *et al.*, 1990). Each trophozoite contains approximately sixty-three copies of the tandemly repeated rDNA operon (Weiss *et al.*, 1992). The rDNA operon of *Giardia* has

both prokaryotic features; a relatively small 16S rDNA gene and the small size of transcription unit overall, and eukaryotic features; a 5.8S rDNA gene separate from the 23S rDNA gene and spacer regions of variable size (van Keulen *et al.*, 1992). The nucleotide composition of the *Giardia* rDNA is biased. The GC content of *G.intestinalis* is approximately 75% while in *G.muris* this figure is 62% (van Keulen *et al.*, 1992). The 5.8S rDNA gene and the two flanking ITS regions are considered to be rapidly evolving regions making them a suitable target for the characterisation of phylogenetically closely related organisms, for example different species of the same genus (Felleisen, 1997). Primers derived from the conserved sequences at the 3' end of the 16S rDNA gene and the 5' end of the 23S rDNA gene were used in this thesis and the PCR amplified fragments were then directly sequenced in order to investigate the genetic relatedness of different *G.intestinalis* isolates originating from both human and calf hosts.

We characterise *G.intestinalis* cysts isolated directly from calf and human faecal matter obtained from the Waikato Region, North Island, New Zealand. No attempt was made to culture the isolates collected in this study either *in vitro* or *in vivo* which may, as previously noted, introduce a selection bias. A collection of laboratory adapted strains that had previously been cultured axenically were also characterised and compared to the environmental isolates collected.

CHAPTER TWO: MATERIALS AND METHODS

2.1 COLLECTION OF *GIARDIA* FOR NUCLEIC ACID EXTRACTION

2.1.1 Revival of cryopreserved *Giardia* trophozoites

Materials

- Cryopreserved *Giardia* trophozoites
- TY1-S-33 growth medium

TYI-S-33 Growth Medium (pH 7.5)

Trypticase soy broth (Becton Dickson)	20g
Yeast extract powder (DifcoLaboratories)	10g
Glucose (BDH)	10g
NaCl (Sigma)	2g
K_2HPO_4 (BDH)	1g
KH_2PO_4 (BDH)	0.6g
L-Cysteine monohydrochloride (Sigma)	1.5g
Ferric ammonium citrate (BDH)	0.023g
L-Ascorbic acid (BDH)	0.2g
NCTC-135 medium (Sigma)	0.94g
Bile bacteriological (Sigma)	0.8g
Benzyl penicillin (Biochemie)	0.06g
Bovine serum	100mL
Distilled H_2O to	1000mL

All dry ingredients were dissolved in 500mL distilled H_2O followed by the bovine serum. The mixture was made to 1L total volume with the addition of distilled H_2O. The medium was adjusted to pH 7.5 and mixed for thirty minutes using a magnetic stirrer prior to filtration. The medium was initially filtered by positive pressure filtration through five sheets of Whatman filter paper followed by non-sterile 0.45μm and 0.2μm pore membranes (Advantec MFS, Inc). The medium was filter sterilised using a Supor™ 0.2μm pore membrane (Gelman Sciences)

that had previously been autoclaved at 121°C and 103 kilopascals (kPa) for fifteen minutes. Aliquots of 500mL sterile medium were dispensed into sterile Schott bottles. The growth medium was stored at 4°C for up to fourteen days, after which time any unused medium was discarded.

Preparation of Bovine Serum:

Samples of bovine serum were prepared in the lab using bovine blood collected from the freezing works. Bovine blood samples were left to clot overnight in cheesecloth bags suspended over collection buckets at 4°C. One litre aliquots of whole blood were centrifuged at 1000 × g for fifteen minutes in a RC3B centrifuge. The upper layer containing the serum was collected and stored at –20°C until required and the pellet containing the red blood cells was discarded.

Method

Laboratory adapted cultures of *Giardia* previously cryopreserved and stored at -80°C were revived and grown *in vitro*. A cryopreservation tube containing *Giardia* trophozoites was thawed quickly at 37°C immediately following removal from liquid nitrogen storage with the lid loosened. Once thawed the cryopreservation tube was emptied into a 70ml culture flask (NUNC Brand Product, Inc) half-filled with pre-warmed (37°C) TY1-S-33 growth medium. The empty cryopreservation tube was then rinsed with pre-warmed TY1-S-33 growth medium and emptied into a second culture flask also half-filled with pre-warmed TY1-S-33 growth medium. Both culture flasks were overfilled with pre-warmed TY1-S-33 growth medium to eliminate all air bubbles and incubated at 37°C for thirty minutes. The medium from each of the culture flasks was replaced with fresh pre-warmed TY1-S-33 growth medium. Both culture flasks were incubated at 37°C and trophozoite growth was monitored daily using an inverted microscope at 80× magnification.

Trophozoite cultures were maintained by replacing the old TY1-S-33 growth medium every three to four days, or when confluent growth was observed, with fresh pre-warmed TY1-S-33 growth medium.

2.1.2 Maintenance of *Giardia* trophozoite cultures

Materials

- *Giardia* trophozoite cultures
- TYI-S-33 growth medium (see Section 2.1.1)

Method

Trophozoite cultures were subcultured weekly into fresh culture flasks. This was to ensure that healthy growing stocks of trophozoites were maintained. The growth medium was discarded from the culture flask once a complete monolayer of *Giardia* trophozoites had formed through attachment to the flask surface. The culture flask was then half-filled with fresh cold (4°C) TYI-S-33 growth medium and placed on ice for ten minutes, trophozoite side down. The culture flask was tapped sharply at one end to dislodge the trophozoites and then viewed under an inverted microscope at 80× magnification. This procedure ensured that the trophozoite monolayer had completely lifted from the culture flask surface. If detachment of the trophozoite monolayer was not complete after ten minutes the culture flask was left a further five minutes on ice. Once the trophozoite monolayer had completely lifted from the culture flask surface the contents of the culture flask was divided equally into two fresh sterile 70mL culture flasks and topped up with fresh pre-warmed (37°C) TYI-S-33 medium and incubated at 37°C.

If large numbers of *Giardia* trophozoites were required 750mL culture flasks (NUNC Brand Products, Inc.) were inoculated instead of 70mL culture flasks.

2.1.3 Cryopreservation of *Giardia* trophozoites

Materials

- *Giardia* trophozoite cultures
- 15% Dimethyl sulphoxide (DMSO) in TY1-S-33 growth medium
- TY1-S-33 growth medium (see Section 2.1.1)

Method

Giardia trophozoites in the log phase of growth, typically three to four days old were prepared for cryopreservation. The 70ml culture flask containing trophozoites was half filled with cold TY1-S-33 growth medium stored at 4°C. The culture flask was left to stand for ten minutes on ice until the majority of the trophozoites had detached from the flask surface, as viewed using an inverted microscope at 80× magnification. The half-filled culture flask was decanted into a sterile 50ml centrifuge tube (NUNC Brand Products, Inc) and centrifuged at 1000 × g for ten minutes. The supernatant was discarded, the pelleted trophozoites were resuspended in 5mL TY1-S-33 growth medium and maintained on ice. An equal volume of 15% DMSO was added dropwise to the constantly shaking centrifuge tubes to give a final concentration of 7.5% DMSO. The cell suspension was dispensed in aliquots of 1mL volume to cryopreservation tubes (NUNC Brand Products, Inc) and frozen slowly overnight at -80°C. The cryopreservation tubes were transferred for storage into liquid nitrogen for up to six months.

2.1.4 Harvesting of large scale *Giardia* trophozoite cultures

Materials

- *G.intestinalis* trophozoite culture
- TYI-S-33 growth medium (see Section 2.1.1)
- Ethylene diamine tetra-acetic acid disodium salt (EDTA, BDH)
- Phosphate buffered saline (PBS)
- Tris (Gibco BRL)
- Tris-EDTA (TE) buffer

0.2M EDTA (pH 7.2)

EDTA	7.44g
Distilled H_2O to	1000mL

The solution was adjusted to pH 7.2 by the addition of 5M NaOH and stored at room temperature.

PBS

NaCl	8.5g
$Na_2HPO_4.12H_2O$	2.7g
$NaH_2PO_4.2H_2O$	0.39g
KCl	2g
Distilled H_2O to	1000mL

The solution was sterilised at 121°C and 103 kPa for fifteen minutes and stored at room temperature until required.

1M Tris-HCl (pH 7.5)

Tris	121.1g
Distilled H_2O to	1000mL

The solution was adjusted to pH 7.5 by the addition of 5M HCl and stored at room temperature.

TE Buffer

1M Tris-HCl (pH 7.5)	1mL
0.2M EDTA (pH 7.2)	5mL
Distilled H_2O to	10mL

The solution was sterilised at 121°C and 103 kPa for fifteen minutes and stored at room temperature until required.

Method

Large culture preparations of *Giardia* trophozoites were prepared as described in Section 2.1.2. When a complete monolayer of trophozoites had developed on the flask surface, usually after three to four days growth, the trophozoites were collected for DNA extraction.

To detach the trophozoites from the flask surface, the culture flask (750mL) was placed on ice for ten minutes, trophozoite side down. The flask was vigourously shaken to detach the trophozoites. The detachment of trophozoites from the culture flask surface was monitored visually using an inverted microscope at 80× magnification. Approximately two thirds of the supernatant containing the detached trophozoites was decanted into a centrifuge tube and centrifuged at 1080 × g for ten minutes. The supernatant was discarded and the pellet containing the trophozoites was resuspended in 10mL PBS. The trophozoites were centrifuged twice more. Finally the pellet was resuspended in 1mL of sterile TE buffer and either used immediately for DNA extraction or stored at –20°C until required.

2.1.5 Collection of calf faecal specimens

During a five week period, between August 16 and September 12, 1998, calf faecal specimens were collected from twelve dairy farms throughout the Waikato region, as indicated in Figure 2.1. Calf management and housing on each farm varied and has been outlined briefly. Reference numbers were given to each of the twelve farms for later analysis of isolates. An average of ten samples were collected weekly from the same group of calves on each of the twelve farms. The

first samples were collected when the calves were approximately seven to ten days old.

Calf faecal specimens were collected from an additional farm located in the Manawatu region during the same time period as well as during March 12 - April 1, 1998, the autumn calving season.

Jackson Farm

Reference number: C1

The calves were maintained in the same paddock for the first two samplings after which they were transferred to a new paddock for the August 28, September 5 and September 12, 1998 collection days.

Reid Farm

Reference number: C2

The calves were maintained in different paddocks located throughout the farm during the five week period. The calves were transferred between paddocks prior to collection on August 21, August 28, and September 5, 1998.

Montgomerie Farm

Reference number: C3

The calves were maintained in the same pen up to and including the August 28, 1998 collection day. The calves were then transferred and maintained on the same paddock for remainder of the study period.

Sing Farm (A)

Reference number: C4

The calves were maintained in four different paddocks over the five week period. The transfer of calves occurred prior to the August 21, August 28, and September 5, 1998 collection days. Prior to the September 12, 1998 collection day this group was combined with calves of the same age which had also been on the Sing farm. This second group of calves were being observed concurrently as reference number C5.

Sing Farm (B)

Reference number: C5

The calves were maintained in two different paddocks during the five week period. As noted above prior to the September 12, 1998 collection these calves were placed together with others of the same age that were being observed as reference number C4.

Sargent Farm

Reference number: C6

The calves were maintained in the same pen for the entire five week period.

Coles Farm

Reference number: C7

The calves were maintained in the same pen for the entire five week period.

Denton Farm

Reference number: C8

The calves were maintained in the same pen for the entire five week period.

Schierning Farm

Reference number: C9

The calves were maintained in three different paddocks during the five week period and were transferred prior to the August 21, and August 28, 1998 collection days.

Hook Farm

Reference number: C10

The calves were maintained in three different paddocks over the five week period with the transfers occurring prior to the August 21 and September 5, 1998 collection dates.

Hennessey Farm

Reference number: C11

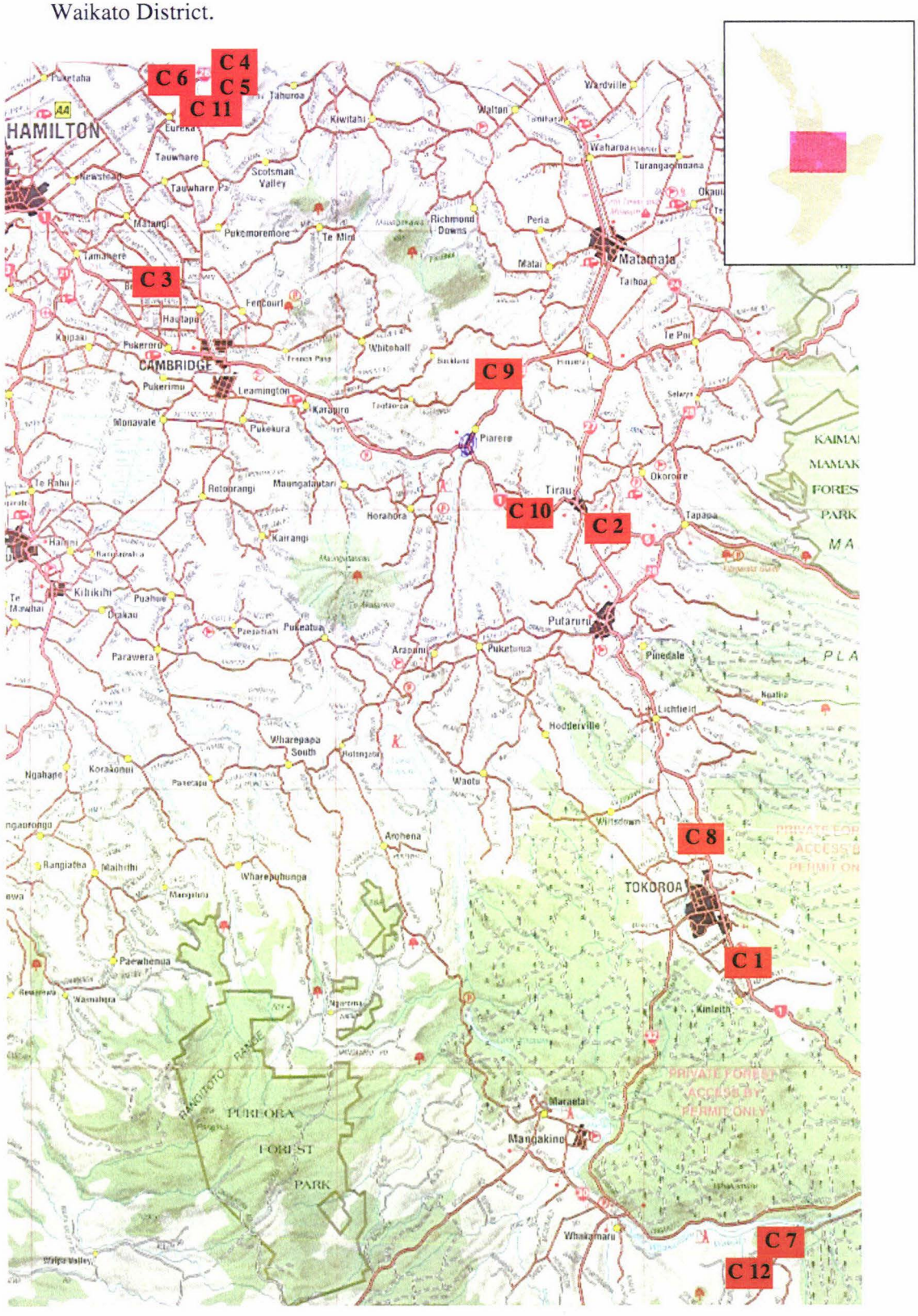
The calves were maintained in two pens during the five week study and were transferred prior to the August 28, 1998 collection day.

Rangi Farm

Reference number: C12

The calves were maintained in four different areas throughout the five week period. The calves were moved onto different paddocks prior to the August 21, August 28 and September 5, 1998 collection days.

Figure 2.1: The location of the twelve farm collection sites throughout the Waikato District.



2.1.6 Collection of human faecal specimens

Positive *Giardia* human faecal specimens were supplied by MedLab Waikato, a facility which services both residential and farming communities throughout the Waikato Region. Faecal specimens were collected between April 2 and October 10, 1998. The exact geographical location, sex, age and general occupation of each infected human host was unobtainable due to enforced legal restrictions. Faecal specimens were stored at 4°C without additional liquids, transported immediately to Massey University, Palmerston North and tested within twenty-four hours of receipt.

2.1.7 Detection of *Giardia* cysts by immunofluoresence antibody (IFA) test in faecal specimens

Materials

- Human or animal faecal specimens
- Sterile distilled H_2O
- Merifluor *Giardia* monoclonal antibody kit (Meridian Diagnostics, Inc)
- Fluoroprep mounting fluid (bioMerieux sa)
- PBS (see Section 2.1.4)
- Coated microscope slides (Waterborne, Inc)
- 1× Wash buffer

Method

A small sample of faecal matter, approximately 2–5g, was added to 1mL sterile distilled H_2O in a 1.6mL eppendorf tube. The sample was mixed by vortex and left to sit for up to five minutes at room temperature. This facilitated the settling of larger debris from the suspension. A 50μL aliquot was removed from each faecal specimen and spread over treated (coated to allow microorganisms to attach) microscope slides. A positive control was used on each microscope slide for later reference. The slide was then left to air dry at 37°C for two hours. A 50μl aliquot of Fluorscein Isothyiocyanate (FITC) labelled monoclonal antibody (Meridian Diagnostics, Inc) was diluted 10-fold in PBS and added to the dried

suspension. The slide was incubated in the dark for thirty minutes in a humidified container. The FITC labelled monoclonal antibody was removed by washing the slide with 1× wash buffer. A drop of fluoroprep mounting fluid was added to the well and overlaid with a coverslip. To eliminate coverslip movement over the slide the edges of the coverslip were sealed by applying clear nail polish. The faecal specimen preparation was viewed using an epifluorescent microscope with an excitation wavelength of 450-490nm at 400× magnification. The presence of *Giardia* cysts was detected by the apple-green fluorescent appearance of cysts approximately 5-9μm in size.

2.1.8 Sucrose gradient recovery of *Giardia* cysts from faecal specimens

Materials

- Positive *Giardia* faecal specimens
- 0.01% Polyoxyethylene sorbitan monolaurate (Tween 20, Sigma)
- 1M Sucrose (BDH)
- TE buffer (see Section 2.1.4)

Method

Faecal specimens containing high numbers (ten to fifteen cysts per field of view at 400× magnification) of *Giardia* cysts, as determined by IFA detection described in Section 2.1.7, were concentrated using a sucrose gradient procedure. Faecal specimens that contained high numbers of *Giardia* cysts provided sufficient DNA for further DNA manipulations. A faecal specimen of 5–10g was transferred into a 50mL conical centrifugation tube (NUNC Brand Products, Inc) and bought up to a volume of 45mL using 0.01% Tween 20. The centrifuge tube was vigourously mixed and centrifuged at 1000 × g for five minutes at room temperature. The supernatant was discarded, the pellet was washed using 45mL of 0.01% Tween 20 and centrifuged thrice. The pellet was resuspended in 35mL 0.01% Tween 20 and underlaid with 10mL 1M Sucrose, care was taken to avoid mixing the two solutions. The centrifuge tube was centrifuged at 1000 × g for ten minutes at room temperature with the brake off.

Fifteen millilitres of the interface solution present between the Sucrose and Tween 20 was collected and transferred to a fresh 50mL conical centrifugation tube. The volume was increased to 50mL with TE buffer. Following centrifugation at 1000 × g for five minutes the supernatant was discarded retaining only 2–5mL containing the *Giardia* cysts. This was repeated twice and the pellets were finally resuspended to 5mL in TE buffer. The concentration of isolated cysts were determined using a haemocytometer. Samples containing greater than 1 x 10^5 cysts per mL were then subjected to nucleic acid extraction.

2.2 NUCLEIC ACID EXTRACTION

2.2.1 DNA extraction from *Giardia* trophozoites

Materials

- *Giardia* trophozoite culture
- 10% sodium dodecyl sulphate (SDS, BDH)
- Pronase Type XIV 10mg/mL (Sigma)
- RNAse (Ribonuclease 1) 2mg/mL (Sigma)
- 5M sodium perchlorate (APS Ajax Finechem)
- 10× Saline Tris-EDTA buffer (STE)
- Phenol/chloroform/isoamyl alcohol (25:24:1) in saturated STE buffer
- Absolute Ethanol
- TE buffer (see Section 2.1.4)
- 3M Sodium acetate
- 70% Ethanol

RNAse

2mg/mL in Milli-Q water

The RNAse preparation was incubated at 90°C for ten minutes to remove any DNAse activity. The solution was stored at –20°C until required.

10× STE buffer

5.0M NaCl	20mL
1.0M Tris HCl (pH 7.5)	50mL
0.2M EDTA (pH 7.2)	5mL
Distilled water to	100mL

The solution was sterilised at 121°C and 103 kPa for fifteen minutes and then stored at room temperature until required.

Phenol/chloroform/isoamyl alcohol (25:24:1)

Phenol	2.5mL
Chloroform	2.4mL
Isoamyl alcohol	0.1mL
Saline Tris-EDTA	0.5mL

The solution was mixed by inversion then to aid mixing air was bubbled through the solution. This solution was prepared immediately prior to use.

Method

Giardia trophozoite cultures in the exponential growth phase, typically three to four days old, were harvested as described in Section 2.1.4. Alternatively, a previously frozen 5mL aliquot of *Giardia* trophozoites was thawed quickly at 37°C prior to centrifugation. The 5mL *Giardia* suspension was centrifuged at 500 × g for five minutes to pellet the trophozoites. The supernatant was discarded and the pellet resuspended in 1mL TE buffer. An aliquot of a 10% SDS solution was added to the 1mL cyst suspension to give a final concentration of 1% SDS. The solution was gently mixed and a volume of Pronase Type XIV (10mg/mL) was added to give a final concentration of 0.1mg/mL. Cyst lysis and protein digestion was facilitated by incubating the cyst suspension overnight at 50°C.

A 0.1mL aliquot of RNAse (2mg/mL) was added and the lysate incubated for one hour at 50°C. An aliquot of 5M sodium perchlorate was added to the suspension to give a final concentration of 1M and incubated for another hour at 50°C.

The digested proteins were separated from the extracted DNA using an equal volume of freshly prepared phenol/chloroform/isoamyl alcohol solution (25:24:1) saturated in one-tenth volume STE buffer. The lysate was mixed gently by inversion, left to stand for five minutes at room temperature and then centrifuged at 16,500 × g for five minutes. The upper aqueous layer of the lysate solution, containing the DNA, was transferred to a sterile 1.6mL eppendorf tube. This procedure was repeated thrice or until no white precipitant was present at the interface.

Precipitation of the extracted DNA involved the addition of one-tenth volume of 3M sodium acetate followed by gentle mixing prior to the addition of 2.5 volumes of cold absolute ethanol. The solution was gently mixed and placed at –20°C overnight. Collection of the precipitated DNA involved centrifugation at 5000 × g for ten minutes at room temperature. The supernatant was discarded and the pellet was washed briefly with 1mL 70% ethanol. The DNA pellet was dried under vacuum and resuspended in 500μl of sterile TE buffer. The *Giardia* DNA solution was stored at 4°C until required.

2.2.2 Nucleic acid extraction from *Giardia* cysts

Materials

- 10% Chelex 100™ (BioRad)
- *Giardia* cysts
- Phenol (Tris-buffered) pH 8.0 (APS Ajax Finechem)
- Chloroform/isoamyl (24:1)
- 3M Sodium acetate (pH 5.5)
- Absolute Ethanol
- 70 % Ethanol
- Sterile Milli-Q H_2O

10% Chelex 100™

Chelex 100™	1g
Milli-Q H_2O to	10mL

The suspension was stored at room temperature and mixed thoroughly immediately prior to use.

Method

A 5mL aliquot of *Giardia* cysts freshly isolated from faecal matter (see Section 2.1.8) was thoroughly mixed. A 100μL aliquot of this suspension was transferred to a fresh 1.6mL eppendorf tube and centrifuged at 1050 × g for two minutes. The supernatant was discarded and the pellet resuspended in 100μl of 10% Chelex 100™, taking care to ensure the Chelex solution was thoroughly mixed immediately prior to use. A small hole was made in the lid with a needle to prevent the eppendorf tube lid opening under pressure during the nucleic acid extraction.

The eppendorf tube was quickly frozen at –80°C for ten minutes in order to stress the cysts. The tube was immediately placed into boiling water and boiled for twenty minutes. The tube was occasionally agitated to disperse the Chelex granules which tended to sediment rapidly. After boiling, the tube was centrifuged at 1050 × g for five minutes at room temperature to pellet the cyst debris and chelex. The nucleic acid contained within the supernatant was transferred to a fresh sterile 1.6mL eppendorf ensuring that no Chelex granules were also transferred (Chelex inhibits DNA amplification, see Section 2.3.1). Extracted nucleic acid was left to stand at 4°C for at least two hours before further use.

An equal volume of Tris-buffered phenol was added to the extracted nucleic acid and the solution was mixed gently for two minutes. The tube was centrifuged at 1050 × g for four minutes. Following centrifugation the nucleic acid contained in the upper aqueous phase was transferred to a fresh sterile 1.6mL eppendorf tube taking care to leave the interface and phenol behind. An equal volume of chloroform/isoamyl (24:1) was added to the aqueous solution. Following two

minutes gentle mixing the eppendorf tube was centrifuged at 1050 × g for four minutes. The aqueous phase was transferred to a fresh sterile 1.6mL eppendorf tube. The solution containing the nucleic acid was concentrated by ethanol precipitation prior to further use.

The nucleic acid was concentrated using a one-tenth volume of 3M Sodium acetate followed by the addition of 2.5 volumes of absolute ethanol to the extracted nucleic acid solution. After gentle mixing the solution was left overnight at -20°C. Collection of the nucleic acid involved centrifugation at 1050 × g for thirty minutes at 4°C. The supernatant was discarded, 500μL of 70% ethanol was added to the eppendorf tube and gently mixed to wash the pellet containing the nucleic acid. The tube was then centrifuged at 1080 × g at 4°C for twenty minutes to pellet the nucleic acid. The supernatant was discarded immediately to avoid any loss of the pelleted nucleic acid. The nucleic acid was left to air dry after which it was resuspended in 10μL of sterile Milli-Q H_2O. The extracted nucleic acid was stored at –20°C until required.

2.3 POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION OF *GIARDIA* NUCLEIC ACID

2.3.1 *Giardia* nucleic acid amplification using PCR

Materials

- Nucleic acid extracted from *Giardia*
- PCR Reagents:
  - 10× PCR buffer (Tris-HCl, KCl, $[NH_4]_2SO_4$, 15mM $MgCl_2$, pH 8.7 at 20°C, QIAGEN™)
  - 5× concentrated Q-solution (QIAGEN™)
  - $MgCl_2$ solution (25mM, QIAGEN™)
  - *Taq* DNA Polymerase (5units/μL, QIAGEN™)
  - Ultrapure dNTP'S (2mM each of dATP, dCTP, dGTP, dTTP, Pharmacia Biotech)
  - Sterile Milli-Q H_2O

- Analytical grade Agarose (BioRad)
- 10× E buffer
- 1Kb Plus DNA or 1Kb DNA molecular marker ladder (Gibco BRL)
- Ethidium bromide (5μg/mL, BDH)
- 10× SDS loading dye

10× E Buffer

Tris (Sigma)	96.88g
EDTA	7.44g
Sodium acetate (BDH)	8.2g
Distilled H_2O to	2000mL

Adjust to pH 7.8 using glacial acetic acid and then dilute solution one-tenth in distilled H_2O for use.

10× SDS Loading Dye

20% Sucrose	4g
0.05M EDTA	0.2μL of 0.5M
10% SDS	2mL
Bromophenol blue dye (BDH)	40μg
Distilled H_2O to	20 mL

The solution was dispensed in 5mL aliquots and then stored at room temperature.

Bromophenol blue dye (BDH)

0.1 % w:v bromophenol blue	0.1g
80 % v:v glycerol	0.8mL
1× E Buffer	2.0mL

This was made up in 1× E Buffer and stored at room temperature.

Method

PCR was routinely carried out with nucleic acid extracted from *Giardia* cysts with a concentration of at least 10^5 cysts per gram faeces to provide sufficient template in a final amplification volume of 20μL. For each reaction a cocktail was prepared which contained 1× PCR buffer, 2× concentrated Q-solution, 250μmol of each dNTP, 10pmol of each primer, 1.25mM $MgCl_2$, 1 unit of *Taq* DNA polymerase and sterile Milli-Q H_2O to a total volume of 20μl per reaction. The PCR cocktail was dispensed as a 19μl aliquot to a sterile 0.2mL dome cap PCR reaction tube (Scientific Specialties, Inc). The 1μl aliquot of template DNA was added to the PCR reaction tube after the cocktail mix had been dispensed. In addition a negative control containing 1μl of sterile Milli-Q H_2O, instead of the nucleic acid, and a positive control containing 1μl DNA extracted from a cultured laboratory trophozoite strain of *Giardia* were amplified with each reaction set. The primers used for PCR amplifications are outlined in Section 2.3.2. The PCR protocol outlined below was used for all primer combinations as every primer had a calculated melting temperature (Tm) of Tm = 4(G+C)+2(A+T) of 62°C. High stringency conditions (Tm - 2°C) were applied to all reactions by use of an annealing temperature of 60°C.

All PCR amplifications were performed using a Perkin Elmer GeneAMP PCR System 9600 thermalcycler using Version 2.01 software. The first cycle began with two minutes at 98°C to ensure complete denaturation of the template DNA, this was of particular importance for targeting rDNA due to the high Guanine-Cytosine content, about 75%. The second temperature step involved one minute at 60°C for specific primer annealing. Generally the annealing temperatures used for all PCR amplifications was two degrees below the Tm of the primers, as calculated by the GC method. To complete the first cycle an extension period of one minute at 72°C was used. After the initial temperature cycle thirty-four repeats of the following was performed: 98°C for thirty seconds, 60°C for thirty seconds and 72°C for thirty seconds. To complete the PCR amplification a final extension period of 72°C for four and a half minutes was used. At the completion of the programme the reaction was held at 4°C until removed from the thermalcycler. Maximum ramping rates were used for all PCR amplification steps.

Each amplified PCR product was detected by gel electrophoresis using a 1.6% agarose/1× E buffer gel in a BRL Horizon 58 gel box run at 3-5V/cm. A 4μL aliquot of the PCR product was mixed with 1μL of 10× SDS loading dye and loaded into the gel. A 5μL aliquot of the 1Kb DNA or the 1Kb Plus DNA molecular marker ladder (see Appendix A) was also loaded in order to determine the size of the PCR fragment. Following electrophoresis the gel was stained with the addition of 0.5μg/mL ethidium bromide in 1× E buffer for twenty minutes and then visualised under short wave ultraviolet light.

2.3.2 PCR primers for *Giardia* nucleic acid amplification

Giardia specific PCR primer sequences

GspL: $^{5'}$CAT AAC GAC GCC ATC GCG GCT CTC AGG AA$^{3'}$
GspR: $^{5'}$ TTT GTG AGC GCT TCT GTC GTG GCA GCG CTA$^{3'}$

Giardia intestinalis specific PCR primer sequences

GIL: $^{5'}$AAG TGC GTC AAC GAG CAG CT$^{3'}$
GIR: $^{5'}$TTA GTG CTT TGT GAC CAT CGA$^{3'}$

Giardia muris specific PCR primer sequences

GmL: $^{5'}$GAG GAA TCA TCA GAA CCT CGC$^{3'}$
GmR: $^{5'}$ CAT AAA TCA GTG CAG TGT TTC TC$^{3'}$

Giardia intestinalis rDNA PCR primer sequences

Cyn 1: $^{5'}$CAG GAA TGT CTT GTA GGC GC$^{3'}$
Cyn 2: $^{5'}$CCC GGT TGG TTT CTC CTC C$^{3'}$
Hum: $^{5'}$GCG CCC CGG ACG CGC G$^{3'}$
Joli 1: $^{5'}$CGA TAG CAG GTC TGT GAT GC$^{3'}$
Joli 2: $^{5'}$GCT ACT GAT ATG CTT AAG TTC$^{3'}$
Pol: $^{5'}$CGG CGG GGC GTT CGG GG$^{3'}$

2.3.3 *Giardia* nucleic acid PCR primer combinations and expected product sizes

Gsp primers: 171 bp

GI primers: 218 bp

Gm primers: 306bp

Cyn 1 primer combinations:

Cyn 1-Cyn 2: 506bp

Cyn 1-Joli 2: 483bp

Cyn 1-Pol: 250bp

Joli 1 primer combinations:

Joli 1-Joli 2: 617bp

Joli 1-Cyn 2: 640bp

Joli 1-Pol: 374bp

Hum primer combinations:

Hum-Cyn 2: 406bp

Hum-Joli 2: 383bp

Hum-Pol: 150bp

2.3.4 Purification and determination of nucleic acid concentration of PCR products

Materials

- QIAquick PCR purification kit (QIAGEN™)
- Low Molecular Mass ladder (LMM, Gibco BRL)
- Sterile TE buffer (see Section 2.1.4)
- Agarose (BioRad Laboratories)
- 1× E buffer (see Section 2.3.1)

Method

G.intestinalis amplified PCR products specific to the rDNA were purified using a commercially available kit to remove unincorporated primers, dNTP's and other PCR reagents from amplified fragments. The QIAquick PCR purification kit (QIAGEN™) employs a spin column. The manufacture's instructions were followed for optimum results. Purified PCR products were quantified by comparing band intensities with known band concentrations using gel electrophoresis. Briefly, a 1μL aliquot of a purified rDNA PCR product was mixed with 2μL 10× SDS loading dye and 7μL sterile Milli-Q H_2O and loaded into a 1.6% agarose/1× E buffer gel in a BRL Horizon 58 gel box. A mixture containing 2μL Low Molecular Mass (LMM) ladder, 2μL SDS loading dye and 6μL sterile TE buffer was also loaded. The gel was electrophoresised at approximately 3–5V/cm. Following electrophoresis the gel was stained with the addition of 0.5mg/mL ethidium bromide in 1× E buffer for twenty minutes and then visualised under short wave ultraviolet light. Quantification of the rDNA PCR product concentrations were determined by comparing band intensities with those of the LMM ladder.

2.3.5 Automatic sequencing of *Giardia* ribosomal DNA (rDNA) PCR products

Materials

- *G.intestinalis* rDNA PCR products
- Sequencing primers (1pmol, see Section 2.3.2)
- Big Dye Termination reagent (Perkin Elmer)
- Sterile Milli-Q H_2O
- 3M NaOAc (pH 5.5)
- Absolute ethanol
- 70% ethanol

Method

An automatic sequencing reaction mixture was prepared containing 60–100ng template rDNA PCR product, 1.6pmol sequencing primer, 4μL Big Dye Termination reagent and sterile Milli-Q H_2O to a total volume of 10μL. The

reagents were mixed and pulse centrifuged to ensure the mixture was at the bottom of the tube.

All sequencing reactions were performed using a Perkin Elmer GeneAMP PCR System 9600 thermalcycler using Version 2.01 software. The sequencing reaction consisted of an initial denaturation of the sample at 98°C for ten seconds followed by twenty-five cycles of 98°C for ten seconds, 50°C for five seconds and 60°C for four minutes. All ramp rates were maintained at 1°C per second and following completion of the twenty-five cycles the reactions were held at 4°C.

Upon completion of the sequencing reaction the total reaction volume was transferred to a sterile 1.6mL eppendorf tube containing 10μL sterile Milli-Q H_2O, 2μL 3M NaOAc and 50μL absolute ethanol. The reaction tube was mixed, left on ice for five minutes and then centrifuged at 1050 × g and 4°C for fifteen minutes. Immediately following centrifugation the supernatant was discarded. A 500μL aliquot of 70% ethanol was added to the DNA, mixed thoroughly and centrifuged at 1050 × g and 4°C for ten minutes. The supernatant was again discarded immediately after centrifugation so as to ensure the DNA pellet did not detach from the eppendorf tube. The pellet was left to air dry.

Gel analysis of the sequencing reactions was performed at the Massey University DNA Analysis Service Sequencing Unit using a Perkin Elmer ABI Prism 377 Genetic Analyser (as shown in Figure 3.12).

2.4 DATA ANALYSIS

2.4.1 Generation of aligned *Giardia* rDNA sequences

The rDNA sequences obtained from human and animal isolates of *G.intestinalis* were aligned using the following computer software programmes:

- **DNA and Protein Sequence Analysis Programs, Version 5.07 (DM5, Genetics Software Centre, 1987)**

  DM5 was used to generate the reverse complimentary strand from sequence data.

- **Clustal X, Version 1.62b (Thompson *et al.*, 1994)**

  Clustal X was used to align the rDNA sequences for editing and comparison.

2.4.2 Analysis of aligned *Giardia* rDNA sequences

The following computer programmes were used in the analysis of the aligned rDNA sequence data from human and animal isolates of *G.intestinalis*:

- **Molecular Evolutionary Genetics Analysis, Version 1.02 (MEGA, Kumar *et al.*, 1993)**

  MEGA was used to process aligned sequences from different DNA sources over the same region and present them in a format used for publication. The programme displays sequences in six 10bp blocks per line and replaces identical bases in each region with a dot to indicate base similarity.

- **PAUP, Version 4.0d 64 (Swoftord, 1999)**

  PAUP was used to organise the aligned sequence data into an acceptable computer form to generate trees from the data. This format was also needed in order to generate enter data into the SplitsTree programme.

- **SplitsTree, Version 2.4 (Huson, 1998)**

  SplitsTree was used to generate relationship trees using the aligned data that was constructed in PAUP. SplitsTrees differs from Paup in that it does not assume there is a bifrucating nature inherent in the relationships of the data. Data is expressed as a network, strength for particular relationships as lines or boxes where the "boxiness" of data gives an indication as to the strength of the signal in the data for that particular relationship.

CHAPTER THREE: RESULTS

3.1 CALF ISOLATED *GIARDIA* CYSTS

During a five week period between August 16 and September 12, 1998 calf faecal specimens from each of the twelve farms were collected and examined for the presence of *Giardia* cysts. A total of 587 calf faecal specimens were collected and screened using the IFA test, as described in Section 2.1.7. The faecal specimens were categorised in one of three groups, no detectable *Giardia* cysts, one to nine *Giardia* cysts and greater than ten *Giardia* cysts as visualised per field of view at 400× magnification. Twenty-eight of the total 587 specimens collected (4.8%) were identified having ten or more *Giardia* cysts per field of view at 400× magnification. Two hundred and sixteen of the total 587 specimens collected (36.8%) were identified as having one to nine *Giardia* cysts per field of view at 400× magnification and the remaining 343 specimens (58.4%) were identified as containing no detectable *Giardia* cysts (as shown in Table 3.1). The twenty-eight calf faecal specimens that contained greater than ten *Giardia* cysts per field of view at 400× magnification were selected and the cysts were recovered using the sucrose gradient method, as described in Section 2.1.8. Due to the limitations of the sucrose gradient method, in particular poor cyst recovery, only specimens with greater than 10^5 cysts per gram faeces (greater than ten *Giardia* cysts detected per field of view) were subjected to nucleic acid extraction.

Using the IFA method to detect the presence or absence of *Giardia* cysts in the calf faecal specimens 244 out of 587 (41.6 %) specimens were positive for *Giardia* cysts.

Table 3.1: The distribution of *Giardia* cyst presence/ absence in calf faecal specimens collected from each farm in the Waikato region between August 16 and September 12, 1998. The calf faecal specimens from each farm were split into three groups; no detectable *Giardia* cysts, one to nine *Giardia* cysts and greater than ten *Giardia* cysts per field of view at 400× magnification. The percentage totals for each of the three groups on every farm was calculated (in brackets). The combined total of each group from all farms was also calculated.

Farm reference number	0 *Giardia* cysts detected (% of total)	1 – 9 *Giardia* cysts detected (% of total)	≥ 10 *Giardia* cysts detected (% of total)	Total faecal samples collected
C1	28 (57.1%)	16 (32.7%)	5 (10.2%)	49
C2	37 (78.7%)	8 (17%)	2 (4.3%)	47
C3	29 (69%)	12 (29%)	1 (2%)	42
C4	24 (49%)	24 (49%)	1 (2%)	49
C5	27 (54%)	21 (42%)	2 (4%)	50
C6	17 (38%)	24 (53%)	4 (9%)	45
C7	35 (70%)	13 (26%)	2 (4%)	50
C8	33 (67%)	16 (33%)	0 (0%)	49
C9	35 (57%)	23 (38%)	3 (5%)	61
C10	23 (49%)	23 (49%)	1(2%)	47
C11	31 (61%)	19 (37%)	1 (2%)	51
C12	24 (51%)	17 (36%)	6 (13%)	47
TOTAL	**343 (58.4%)**	**216 (36.8%)**	**28 (4.8%)**	**587**

3.2 HUMAN ISOLATED *GIARDIA* CYSTS

Human specimens of *Giardia* were collected between April 2 and October 10, 1998 from the Waikato region. A total of ninety-eight human specimens were screened for the presence of *Giardia* using the IFA test, as described in Section 2.1.7. Fifty-nine out of ninety-eight human faecal specimens (60%) were positive for *Giardia* cysts while the remaining thirty-nine human faecal specimens (40%) contained no detectable *Giardia* cysts (as shown in Table 3.2). As with calf faecal specimens human specimens with greater than ten *Giardia* cysts per field of view at 400× magnification (10^5 *Giardia* cysts per gram faeces) were selected and the cysts were recovered using the sucrose gradient method, as described in Section 2.1.8. A total of thirty-nine, out of the fifty-nine positive human faecal specimens, containing more than ten cysts per field of view at 400× magnification were subjected to sucrose gradients.

Table 3.2: The distribution of *Giardia* cyst presence/ absence in human faecal specimens collected from the Waikato region between April 2 and October 10, 1998. The faecal specimens were split into three groups; no detectable *Giardia* cysts, one to nine *Giardia* cysts and greater than ten *Giardia* cysts per field of view at 400× magnification. The percentage total for each group was calculated (in brackets).

	0 *Giardia* cysts detected (% of total)	**1 – 9 *Giardia* cysts detected (% of total)**	**≥ 10 *Giardia* cysts detected (% of total)**	**Total faecal samples collected**
Human faecal specimens	39 (40%)	20 (20%)	39 (40%)	98

3.3 OPTIMISATION OF *G.INTESTINALIS* NUCLEIC ACID PCR AMPLIFICATION USING rDNA PRIMERS

PCR optimisation of the *G.intestinalis* rDNA amplification was required due to the rich GC content of the target region. The PCR conditions optimised were the nucleic acid extraction method, the working concentrations of the Qiagen™ 5× concentrated Q-solution, $MgCl_2$, the denaturation temperature of the DNA templates targeted, the annealing temperatures of the specific rDNA primer sets and the number of PCR amplification cycles.

3.3.1 Optimisation of nucleic acid extraction

Nucleic acid extraction from *G.intestinalis* cysts using a chelating resin, Chelex 100™, was optimised to increase the recovery of nucleic acid from cysts and the simultaneously remove PCR inhibitors. Two methods were initially considered. de Lamballerie *et al.* (1992) reported the use of Chelex 100™ to extract bacterial and viral nucleic acid using SDS, Nonidet-P40 (NP40) and Tween 20. Mahbubani *et al.* (1998) reported the use of Chelex 100™ for the extraction of nucleic acid from *Giardia* cysts using two cycles of freezing and boiling (-78°C and 99°C).

Although both methods did result in the release of nucleic acid from *Giardia* cysts various modifications were found to increase the yield of DNA and improvement of PCR product results. The use of Tween 20, SDS and NP40 in the Chelex 100™ suspension resulted in the inhibition of PCR product amplification when extracted nucleic acid was both neat and diluted 10-fold. A suspension of 10% Chelex 100™ in distilled H_2O decreased the degree of inhibition of PCR product amplification. Figure 3.1 shows the PCR amplification using Genus specific (Gsp) primers of nucleic acid extracted from a laboratory strain of *G.intestinalis* and an environmental specimen of *G.intestinalis*. Two different nucleic acid extraction methods, Chelex-100™ in dH_2O and Chelex-100™ in SDS, Nonidet-P40 (NP40) and Tween 20, were performed using the same environmental specimen. Both samples were subjected to the same PCR amplification conditions. Correct PCR amplification of the extracted nucleic acid occurred from the sample extracted

using Chelex-100™ in dH_2O, lane 3, while the sample extracted using Chelex-100™ in SDS, Nonidet-P40 (NP40) and Tween 20 did not amplify, lane 4. Variations involving different temperature cycles using Chelex 100™ in dH_2O (data not shown) showed little or no benefits and were therefore not employed.

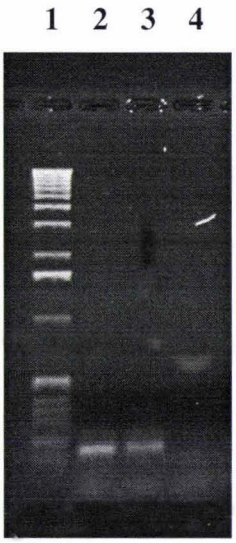


Figure 3.1: Optimisation of the nucleic acid extraction from *G.intestinalis* using 10% Chelex-100™ in two different suspensions followed by nucleic acid PCR amplification using Gsp primers (Section 2.3.2). Lane 1: 1 Kb DNA ladder (Gibco), lane 2: *Giardia* DNA extracted from a laboratory adapted trophozoite strain using the phenol/chloroform method as described in Section 2.2.1, lane 3: *Giardia* DNA extracted from an environmental strain using 10% Chelex 100™ in dH_2O (as described in Section 2.2.2), lane 4: *Giardia* DNA extracted from an environmental strain using 10% Chelex 100™ in 0.1% w/v SDS, 0.5% v/v Nonidet-P40 (NP40) and 0.5% v/v Tween 20.

Note: The amplified DNA fragment present (617bp) in lane 3 indicates that 10% Chelex-100™ can be used to extract nucleic acid from environmental cysts as effectively as the phenol/chloroform DNA extraction method used to extract DNA from laboratory adapted trophzoites.

3.3.2 Optimisation of 5× concentrated Q-solution (Qiagen™)

A comparision of 5× concentrated Q-solution (Qiagen™) was performed using zero, one, two and three volumes of the 5× concentrated Q-solution to determine the optimum concentration of Q-solution. The concentration per reaction of the Q-solution was determined to be important and aided with *Giardia* rDNA amplification. The optimum concentration was determined to be two volumes of the 5× concentrated Q-solution per PCR reaction, as shown by the amplification of a 617bp rDNA PCR product from a laboratory adapted trophozoite strain of *G.intestinalis* using the Joli primer set (Section 2.3.2) in figure 3.2 (lane 4).

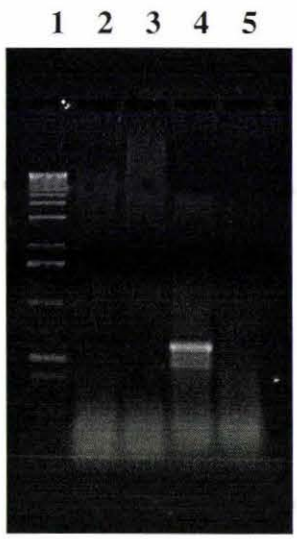


Figure 3.2: Optimisation of the 5× concentrated Q-solution using *G.intestinalis* rDNA primers (Joli primers) on a laboratory adapted trophozoite strain of *G.intestinalis*. Lane 1: 1 Kb DNA marker (Gibco BRL), lane 2: PCR reaction containing no 5× concentrated Q-solution, lane 3: PCR reaction containing one volume of 5× concentrated Q-solution, lane 4: PCR reaction containing two volumes of 5× concentrated Q-solution, lane 5: PCR reaction containing three volumes of 5× concentrated Q-solution. The 617bp PCR product was amplified only in the PCR reaction mixture containing two volumes of 5× concentrated Q-solution.

3.3.3 Optimisation of $MgCl_2$ concentration

A comparision of $MgCl_2$ concentration was performed using 0mM, 1.25mM, 2.5mM, 3.75mM, 5mM, 5.625mM, 6.25mM to determine the optimum concentration of $MgCl_2$. The concentration per reaction of $MgCl_2$ correlated directly to the degree of non-specific PCR products amplified. The optimum concentration was determined to be 1.25mM $MgCl_2$ per PCR reaction, as shown by the amplification of fewer non-specific bands close to the desired 617bp rDNA PCR product amplified from a laboratory adapted trophozoite strain of *G.intestinalis* using the Joli primer set (Section 2.3.2) in figure 3.3, (lane 3).

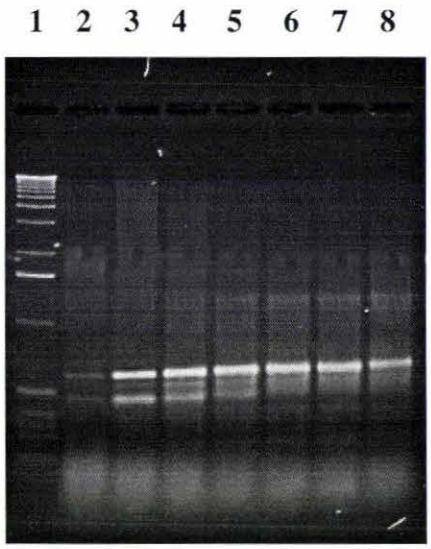


Figure 3.3: Optimisation of the $MgCl_2$ concentration using *G.intestinalis* rDNA primers (Joli primers) on a laboratory adapted trophozoite strain of *G.intestinalis*. Lane 1: 1 Kb DNA marker (Gibco BRL), lane 2: PCR reaction containing 0mM $MgCl_2$, lane 3: PCR reaction containing 1.25mM $MgCl_2$, lane 4: PCR reaction containing 2.5mM $MgCl_2$, lane 5: PCR reaction containing 3.75mM $MgCl_2$, lane 6: PCR reaction containing 5mM $MgCl_2$, lane 7: PCR reaction containing 5.625mM $MgCl_2$, lane 8: PCR reaction containing 6.25mM $MgCl_2$.

Note: The higher the concentration of $MgCl_2$ the closer the non-specific bands were to the desired PCR product when run on the gel. The PCR reaction containing 1.25mM $MgCl_2$ was chosen as the $MgCl_2$ concentration optimum as the desired band could be cut from the gel without the contamination of non-specific bands.

3.3.4 Optimisation of the denaturation temperature

A comparision of denaturation temperatures was performed at 95°C, 96°C, 97°C, 98°C. The denaturation temperature correlated directly to the PCR amplification of *Giardia* rDNA target sequence. The optimum denaturation temperature was determined to be 98°C, as shown by the amplification of the desired 617bp rDNA PCR product amplified from a laboratory adapted trophozoite strain of *G.intestinalis* using the Joli primer set (Section 2.3.2) in figure 3.4, lane 5.

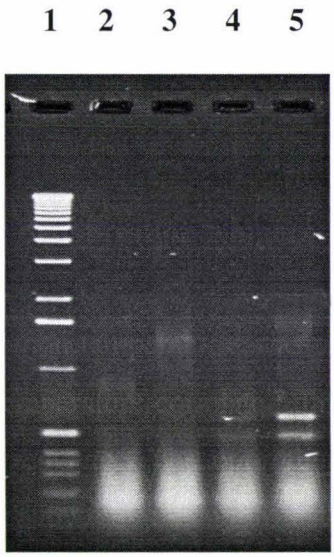


Figure 3.4: Optimisation of the denaturation temperature using *G.intestinalis* rDNA primers (Joli primers) on a laboratory adapted trophozoite strain of *G.intestinalis*. Lane 1: 1 Kb DNA marker (Gibco BRL), lane 2: PCR reaction amplified at 95°C, lane 3: PCR reaction amplified at 96°C, lane 4: PCR reaction amplified at 97°C, lane 5: PCR reaction amplified at 98°C. The denaturation temperature of 98°C resulted in the PCR amplification of the 617bp rDNA product from a laboratory adapted strain of *G.intestinalis*.

3.3.5 Optimisation of the primer annealing temperature

A comparision of annealing temperatures was performed using 59°C, 60°C, 61°C, 62°C, 62.5°C, 63°C, 63.5°C. The optimum annealing temperature was determined to be 60°C, as shown by the increased amount of the desired 617bp rDNA PCR product amplified from a laboratory adapted trophozoite strain of *G.intestinalis* using the Joli primer set (Section 2.3.2) in figure 3.5, (lane 3).

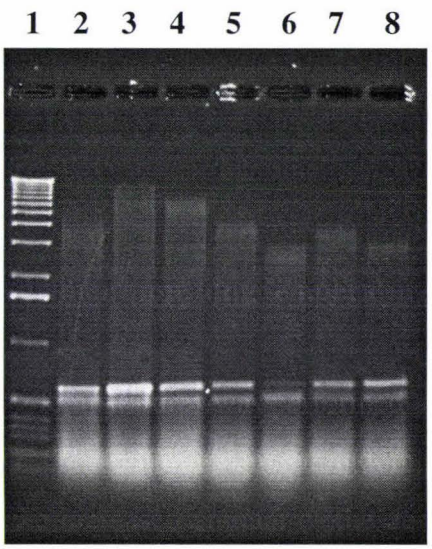


Figure 3.5: Optimisation of the annealing temperature using *G.intestinalis* rDNA primers (Joli primers) on a laboratory adapted trophozoite strain of *G.intestinalis*. Lane 1: 1 Kb DNA marker (Gibco BRL), lane 2: PCR reaction amplified at 59°C, lane 3: PCR reaction amplified at 60°C, lane 4: PCR reaction amplified at 61°C, lane 5: PCR reaction amplified at 62°C, lane 6: PCR reaction amplified at 62.5°C, lane 7: PCR reaction amplified at 63°C, lane 8: PCR reaction amplified at 63.5°C. The annealing temperature of 60°C (lane 3) resulted with the maximum PCR amplification of the 617bp rDNA product from a laboratory adapted trophozoite strain of *G.intestinalis*.

3.3.6 Optimisation of cycle number

The optimisation of the number of PCR amplification cycles was performed in five cycle increments between five and fifty-five. The optimum cycle number was determined to be thirty-five cycles as it produced a greater proportion of the desired 617bp rDNA PCR product, than the nonspecific bands, amplified from a laboratory adapted trophozoite strain of *G.intestinalis* using the Joli primers (Section 2.3.2), lane 8, Figure 3.6. The desired PCR product was subjected to fewer heating stresses in addition to the amplification of fewer non-specific PCR products.

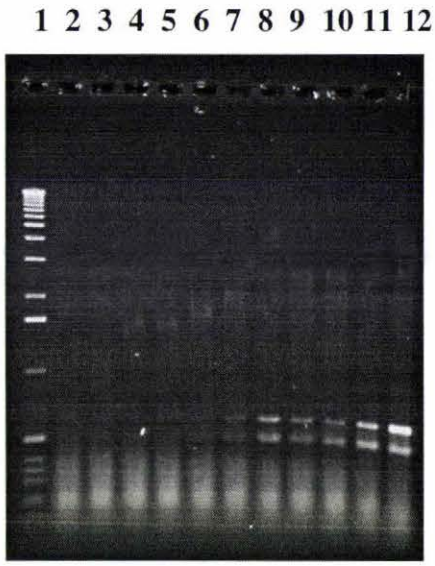


Figure 3.6: Optimisation of the number of PCR amplification cycles using *G.intestinalis* rDNA primers (Joli primers) on a laboratory adapted trophozoite strain of *G.intestinalis*. Lane 1: 1 Kb DNA marker (Gibco BRL), lane 2: 5 PCR amplification cycles, lane 3: 10 PCR amplification cycles, lane 4: 15 PCR amplification cycles, lane 5: 20 PCR amplification cycles, lane 6: 25 PCR amplification cycles, lane 7: 30 PCR amplification cycles, lane 8: 35 PCR amplification cycles, lane 9: 40 PCR amplification cycles, lane 10: 45 PCR amplification cycles, lane 11: 50 PCR amplification cycles, lane 12: 55 PCR amplification cycles. The optimum number of PCR amplification cycles was determined to be thirty-five cycles (lane 8) as shown by the PCR amplification of the 617bp rDNA product from a laboratory adapted strain of *G.intestinalis* with amplification of fewer non-specific bands.

3.3.7 Location of a new set of rDNA primers

In the latter part of the optimisation of the PCR conditions an investigation was under taken to examine the suitability of the Joli primers (Section 2.3.2) due to the close proximity of a non-specific PCR amplified fragment close to the desired product. A BLAST search of the *G.intestinalis* rDNA Joli 1/Joli 2 primer sequences revealed that the target sequence contained a portion of the telomeric region in the 23S rDNA gene and possibly accounted for the non-specific products. New primers, Cyn 1/Cyn 2 (Section 2.3.2), were designed 134 nucleotides closer to the 3' end of the 16S rRNA gene and 23 nucleotides further into the 23S rDNA gene (than the Joli 1/2 primers), and away from the telomeric region to eliminate the PCR amplification of the non-specific products. The Cyn primers amplified a PCR product of 506 bases and no non-specific products. All the PCR conditions optimised for the Joli primers were also determined to be the optimum conditions for the Cyn primers (data not shown). Figure 3.7b shows the removal of the non-specific products by the Cyn primers.

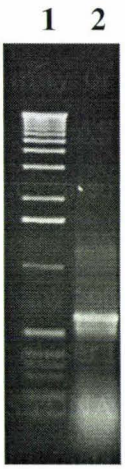


Figure 3.7a

Figure 3.7b

Figure 3.7a and 3.7b: A comparison of the Joli primers (Fig. 3.7a) and the new primer set, Cyn primers (Fig. 3.7b; located further into the 23S rDNA gene). Figure 3.7a lane 1: 1 Kb DNA ladder (Gibco BRL), lane 2: 617bp PCR product amplified using the Joli primers. Figure 3.7b lane 1: 1 Kb DNA Plus ladder (Gibco BRL), lane 2: 506bp PCR product amplified using the Cyn primers. The use of the Cyn primers removed the amplification of the non-specific PCR products, lane 2, Figure 3.7b.

3.4 ORIGINS OF SEQUENCED *G.INTESTINALIS* ISOLATES

Calf isolated sequences

The following *G.intestinalis* isolates obtained from calf faeces have been sequenced and the location of each farm has been outlined in Figure 2.1. Isolate C13H3 and C13H8 were obtained from calves located on Massey University Dairy Unit 1 and this farm has not been included in Figure 2.1. All of the calf isolates were obtained between August 16 and September 12 1998, except C13H3 which was collected during March, 1998.

McalfC13H3	Massey University Dairy Unit 1, Manawatu Region
McalfC13H8	Massey University Dairy Unit 1, Manawatu Region
WcalfC1H7	Jackson Farm, Waikato Region
WcalfC3H10	Montgomerie Farm, Waikato Region
WcalfC4H7	Sing Farm (A), Waikato Region
WcalfC5H1	Sing Farm (B), Waikato Region
WcalfC6H1	Sargent Farm, Waikato Region
WcalfC6H5	Sargent Farm, Waikato Region
WcalfC9H3	Schierning Farm, Waikato Region
WcalfC9H5	Schierning Farm, Waikato Region
WcalfC10H1	Hook Farm, Waikato Region
WcalfC11H5	Hennessey Farm, Waikato Region
WcalfC12H2	Rangi Farm, Waikato Region
WcalfC12H4	Rangi Farm, Waikato Region
WcalfC12H7	Rangi Farm, Waikato Region

Published Sequences

The following *Giardia* isolates have been sequenced and published by other researchers and were used as comparisions or out-groups.

Belgian	Human isolate, EMBL accession number – M73686
Polish	Human isolate, EMBL accession number – X52949
G.muris	EMBL accession number – X65063
G.ardeae	EMBL accession number – X58290
Giardia from ibis	GenBank – U20351
Giardia from vole	GenBank – AF006676

Human isolated sequences

The following *G.intestinalis* isolates obtained from human faeces have been sequenced in this study. The human isolates were obtained between April 2 and October 10, 1998 from a medical laboratory. The actual location of each isolate is unknown but presumed to have been obtained from throughout the Waikato Region.

Waihum64	Waikato Region
Waihum67	Waikato Region
Waihum70	Waikato Region
Waihum74	Waikato Region
Waihum89	Waikato Region
Waihum102	Waikato Region
Waihum106	Waikato Region
Waihum110	Waikato Region
Waihum136	Waikato Region

Laboratory adapted isolated sequences

The following isolates have been sequenced from laboratory adapted strains of *G.intestinalis* previously cultures and maintained *in vitro*.

Germhum1	Human isolate, Germany
Germhum2	Human isolate, Germany
Austhum	Human isolate, Australia
Sheep1	Sheep isolate, Canada
Sheep2	Sheep isolate, Canada
Beaver1	Beaver isolate, Canada
Beaver2	Beaver isolate, Canada
Beaver3	Beaver isolate, Canada
Ham	Hamster isolate, New Zealand

3.5 PCR ANALYSIS OF *G.INTESTINALIS* ISOLATES

3.5.1 PCR amplification of a laboratory strain of *G.intestinalis*

The laboratory isolate Germhum1, originally isolated from a human residing in Germany, was identified as *Giardia* using six different specific PCR primer pair reactions, Figure 3.8. Each of the six PCR primer pair reactions increased the specific identification of the isolate. The first PCR primer pair, Gsp primers, determined the genus of the isolate to be *Giardia.* The next two PCR primer pairs determined the species, whether the isolate was *G.intestinalis* (GI primers) or *G.muris* (Gm primers). The presence of amplification with the GI PCR primers (171bp product) and the absence of amplification with the Gm PCR primers (306bp product) indicates the isolate is *G.intestinalis* and not *G.muris.* The lab isolate Germhum1 was identified as being *G.intestinalis* 'Polish' human subgroup due to amplification with the Polish subgroup specific primers (Cyn1-Pol).

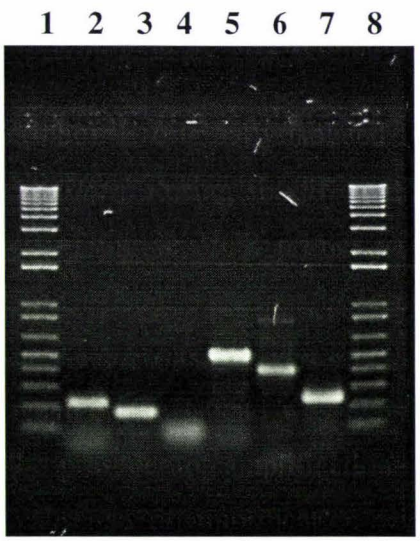


Figure 3.8: PCR amplification of the Germhum1 laboratory isolate with genus, species and sub-species primers. Lane 1: 1 Kb DNA Plus ladder, lane 2: *Giardia* specific primers (Gsp primers), lane 3: *G.intestinalis* specific primers (GI primers), lane 4: *G.muris* specific primers (Gm primers), lane 5: *G.intestinalis* rDNA specific primers (Cyn primers), lane 6: *G.intestinalis* from human origin specific primers (Hum-Cyn2), lane 7: 'Polish' human subgroup of *G.intestinalis* specific primers (Cyn1-Pol), lane 8: 1 Kb DNA Plus ladder. All eight laboratory isolates were examined with genus specific and species specific primers, only one of the eight isolates is shown as an example.

3.5.2 PCR amplification of a Waikato human isolate of *G.intestinalis*

The Waikato human isolate Waihum70 was isolated directly from a faecal specimen received during May, 1998, from the Waikato region. It was positively identified as *Giardia* using six different specific PCR primer pair reactions, Figure 3.9. As before each of the six PCR primer pair reactions increased the specific identification of the isolate. The first PCR primer pair, Gsp primers, determined the isolate to be *Giardia.* The next two PCR primer pairs determined that the isolate was *G.intestinalis* (GI primers) not *G.muris* (Gm primers). The Waikato human isolate Waihum70 was determined to not be of the *G.intestinalis* 'Polish' human subgroup as indicated by the absence of amplification by Cyn1-Pol primers, shown in lane 7 of Figure 3.9. Automatic sequencing identified the isolate to be of the *G.intestinalis* 'Belgian' human subgroup.

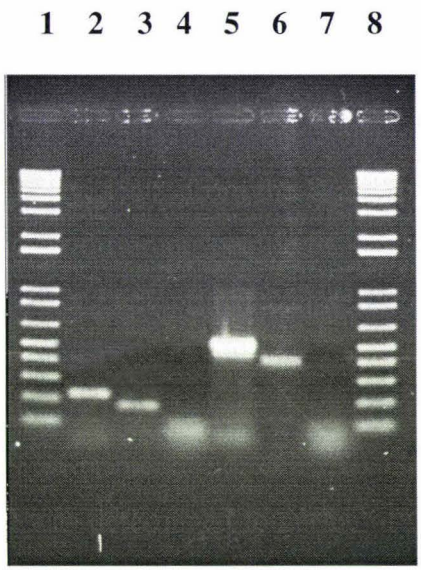


Figure 3.9: PCR amplification of a Waikato human isolate, Waihum70, with genus, species and sub-species primers. Lane 1: 1 Kb DNA Plus ladder, lane 2: *Giardia* specific primers (Gsp primers), lane 3: *G.intestinalis* specific primers (GI primers), lane 4: *G.muris* specific primers (Gm primers), lane 5: *G.intestinalis* rDNA specific primers (Cyn primers), lane 6: *G.intestinalis* from human origin specific primers (Hum-Cyn2), lane 7: 'Polish' human subgroup of *G.intestinalis* specific primers (Cyn1-Pol), lane 8: 1 Kb DNA Plus ladder. All nine human isolates were examined with genus specific and species specific primers, only one of the nine isolates is shown as an example.

3.5.3 PCR amplification of a Manawatu calf isolate of *G.intestinalis*

The calf isolate C13H3 was isolated directly from a faecal specimen collected during March, 1998, from the Manawatu region. It was positively identified as *Giardia* using six different specific PCR primer pairs, Figure 3.10. The first PCR primer pair, Gsp primers, determined the isolate to be *Giardia.* The next two PCR primers determined that the isolate was *G.intestinalis* (GI primers) not *G.muris* (Gm primers). The calf isolate C13H3 was determined to be *G.intestinalis*, but not to be of human origin. This is shown by lane 6 and 7, Figure 3.10, due to the absence of amplification using both the human specific PCR primers (Hum-Cyn2) and 'Polish' human subgroup specific PCR primers (C1-Pol).

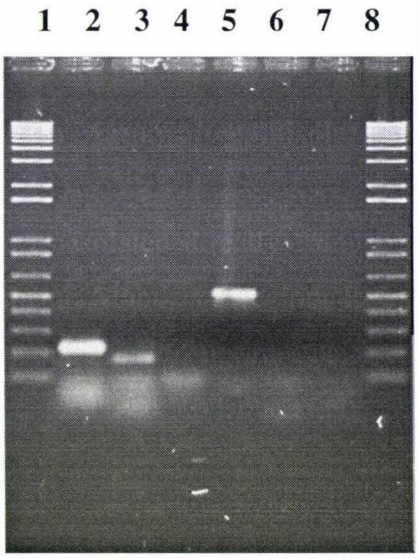


Figure 3.10: PCR amplification of a calf isolate, C13H3, with genus, species and sub-species primers. Lane 1: 1 Kb DNA Plus ladder, lane 2: *Giardia* specific primers (Gsp primers), lane 3: *G.intestinalis* specific primers (GI primers), lane 4: *G.muris* specific primers (Gm primers), lane 5: *G.intestinalis* rDNA specific primers (Cyn primers), lane 6: *G.intestinalis* from human origin specific primers (Hum-Cyn2), lane 7: 'Polish' human subgroup of *G.intestinalis* specific primers (Cyn1-Pol), lane 8: 1 Kb DNA Plus ladder. All fifteen calf isolates were examined with genus specific and species specific primers, only one of the fifteen isolates is shown as an example.

3.6 *G.INTESTINALIS* rDNA PCR PRIMER LOCATIONS

The position of the newly designed Cyn 1/ Cyn 2 (Section 2.3.2) primers, as opposed to the position of the Joli 1/ Joli 2 primers (Section 2.3.2), used to amplify the 5.8S rDNA gene and both flanking ITS regions is outlined below in Figure 3.11. Following the identification of host-specific sequences in humans and calves two additional primers were designed to distinguish human from calf isolated *G.intestinalis* (Hum primer, Section 2.3.2) as well as which human subtype was present (Pol primer, Section 2.3.2). The location and orientation of these two primers is also outlined below in Figure 3.11.

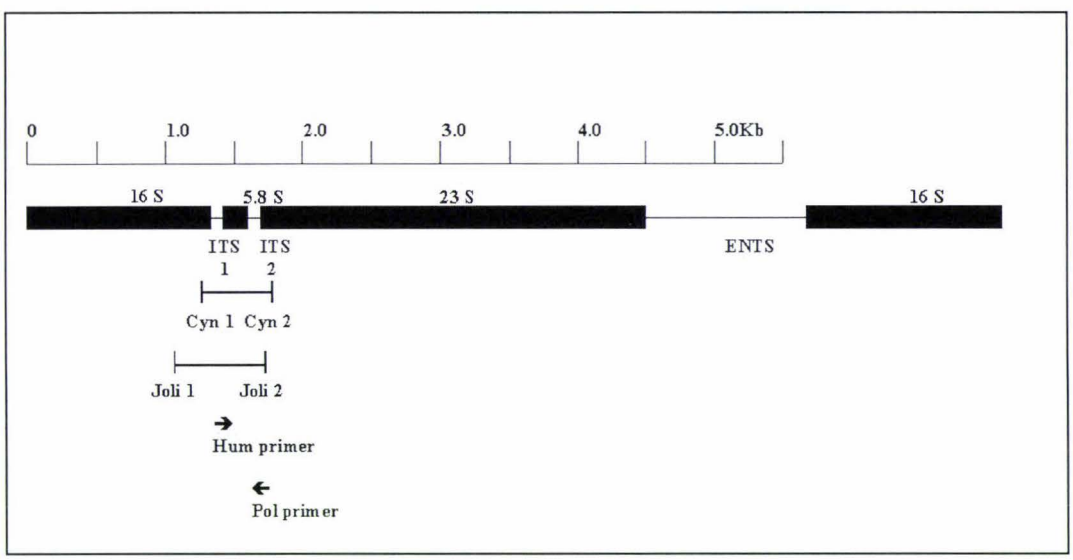


Figure 3.11: The location of the rDNA PCR primers used to amplify *G.intestinalis* rDNA from laboratory adapted isolates, human isolates and calf isolates.

3.7 AUTOMATIC SEQUENCING PRINTOUT OF *G.INTESTINALIS* rDNA

Figure 3.12: An electrophoretogram printout of a laboratory isolate of *G.intestinalis* sequenced using the Cyn-2 rDNA primer. (OVER PAGE →)

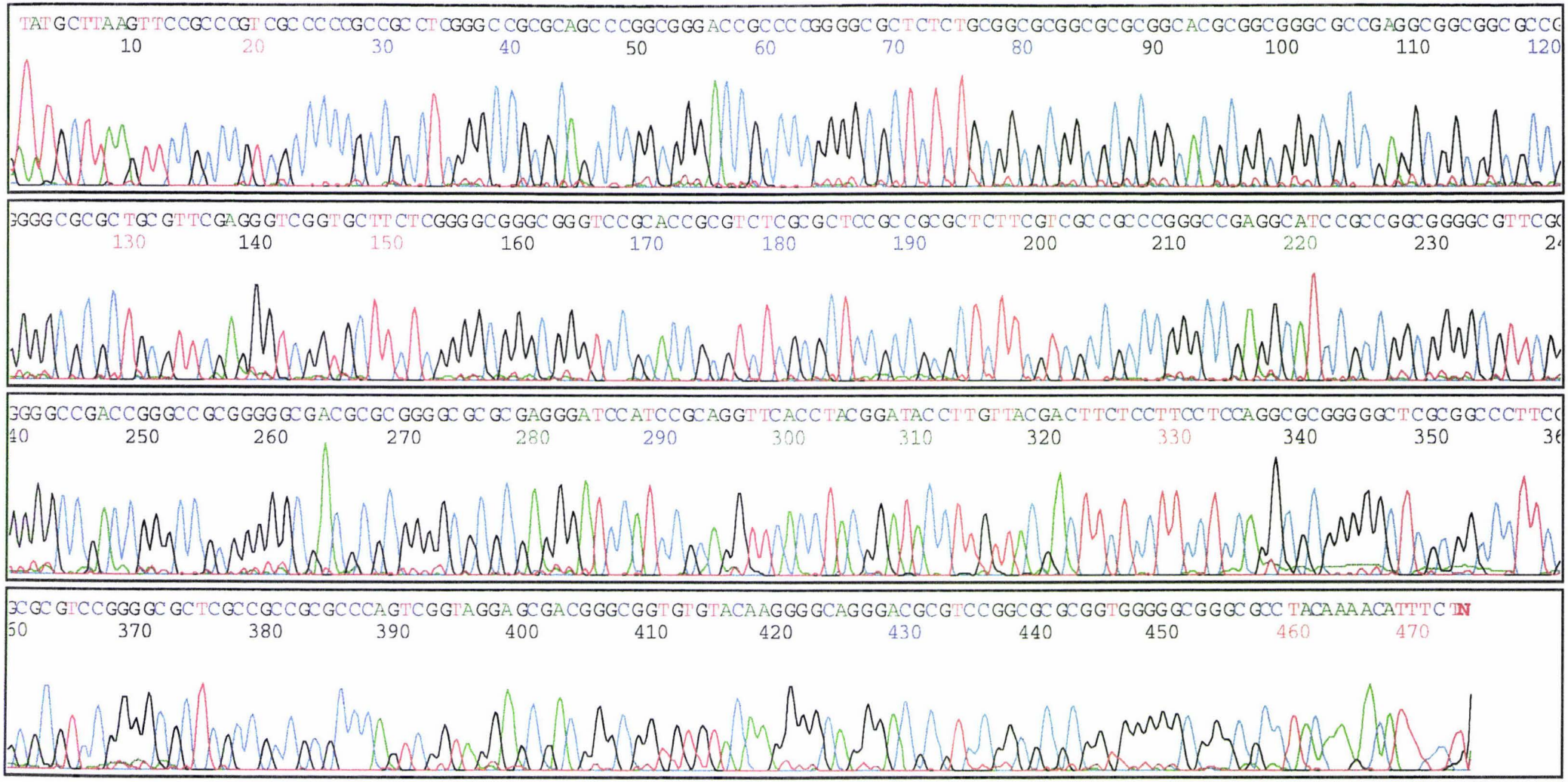
ABI PRISM™

Model 377
Version 3.3
ABI100
Version 3.2

03•S2.Cyn2
20ng(Temp 3.5ul,Primer 4ul) loaded 1.5ul
S2.Cyn2
Lane 3

Signal G:1321 A:374 T:412 C:1611
DT {BD Set Any-Primer}
BigDye48cm Matrix
Points 1320 to 6530 Pk 1 Loc: 1320

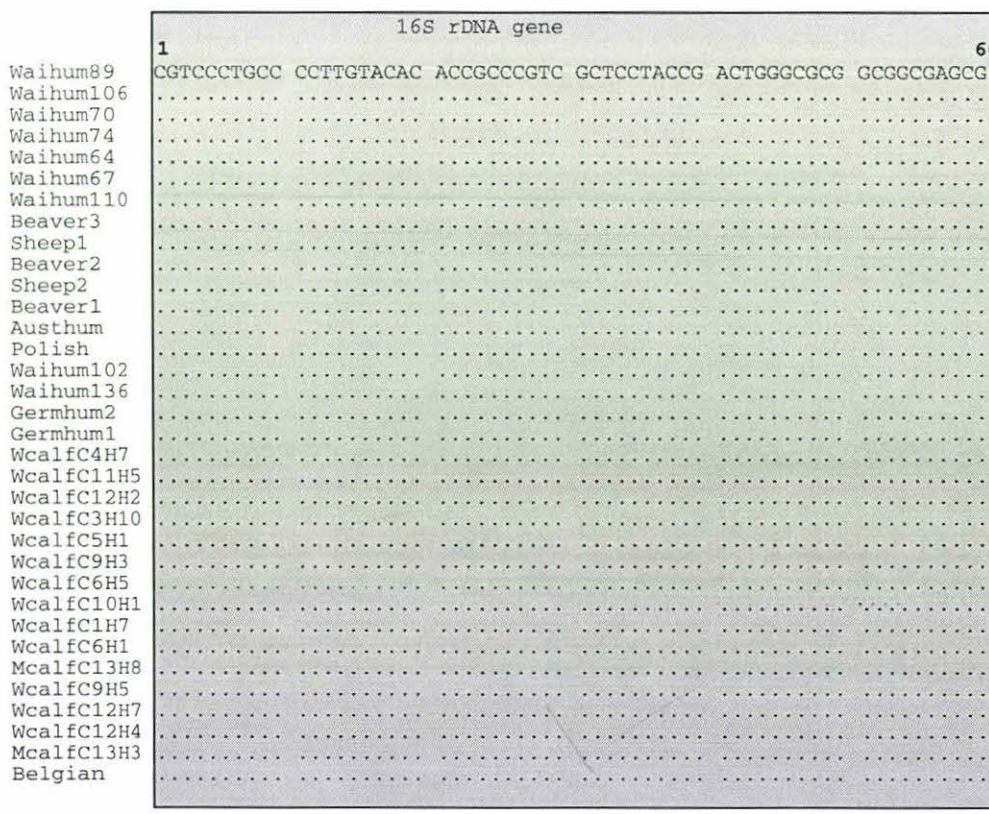
Sat, 13 Mar 1999 8:51 AM
Fri, 12 Mar 1999 12:10 PM
Spacing: 10.77{10.77}

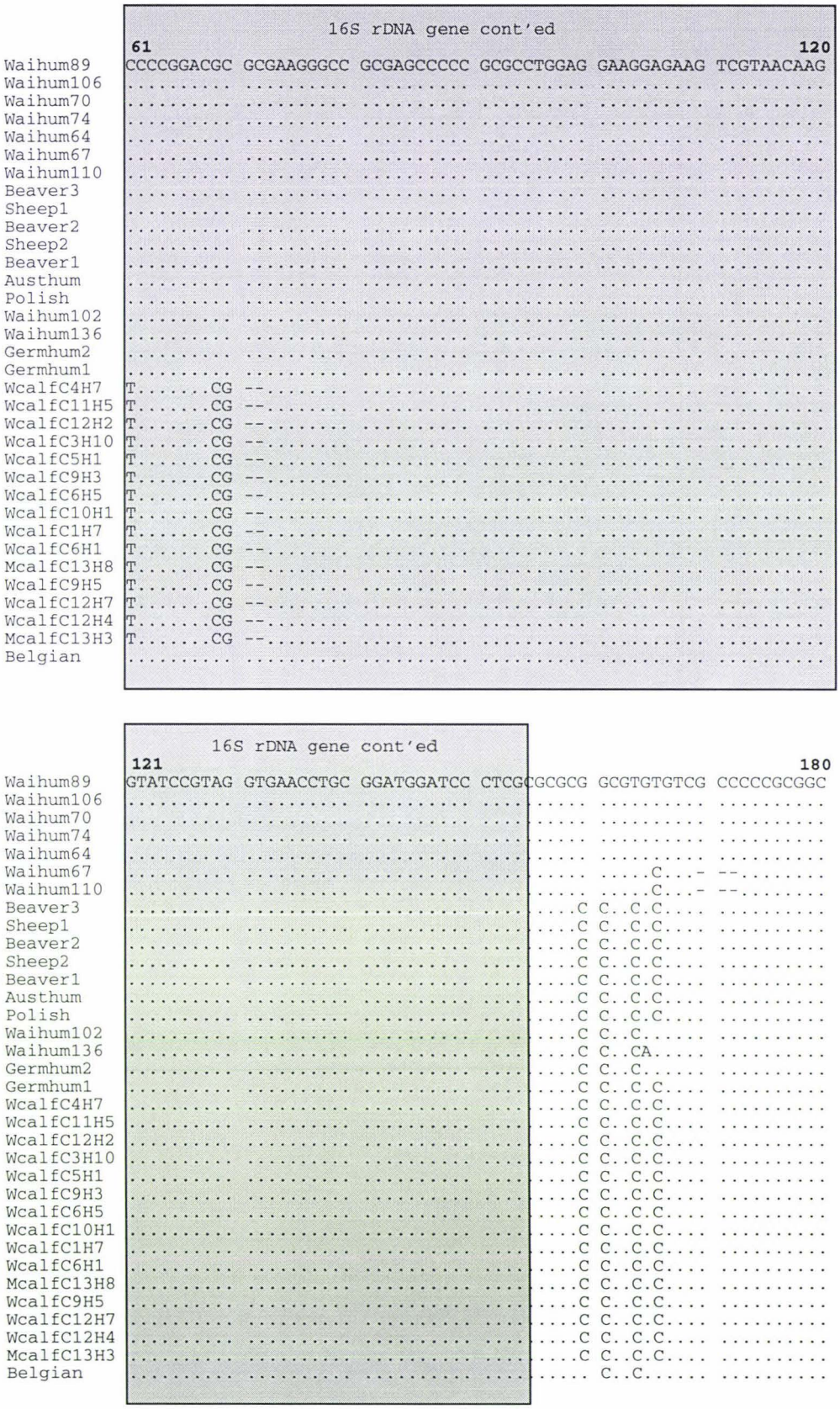


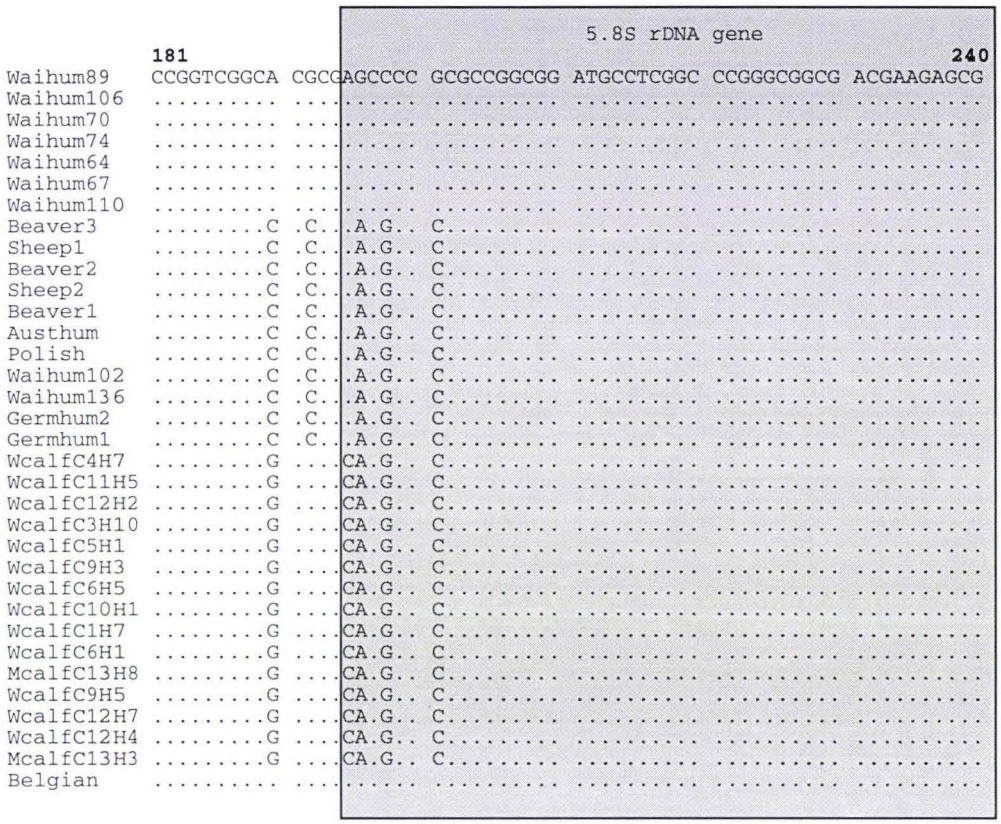
3.8 SEQUENCE ALIGNMENTS OF *G.INTESTINALIS* rDNA

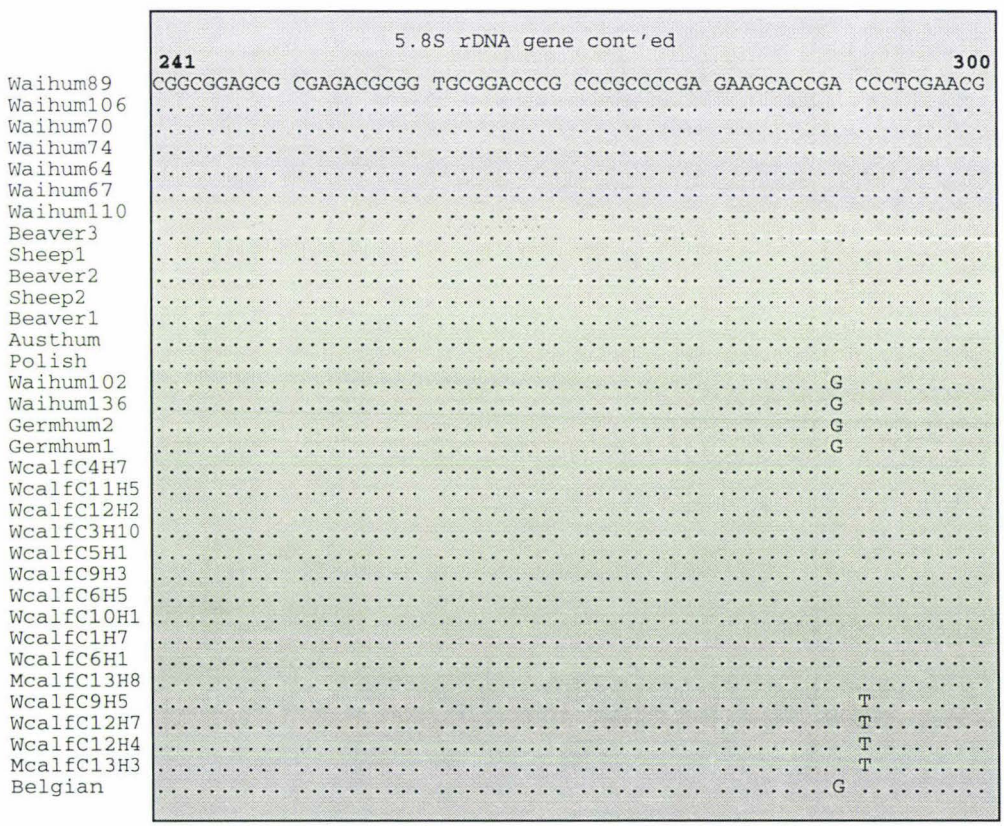
A total of thirty-two *G.intestinalis* rDNA isolates were sequenced using the rDNA primers. Two sets of primers, Cyn and Joli, were used to amplify the target sequence of rDNA containing the 5.8S rRNA gene and the two flanking ITS regions. Sequences were obtained from fifteen calf isolated *G.intestinalis* specimens, nine human isolated *G.intestinalis* specimens, and eight laboratory adapted *G.intestinalis* specimens. Two published *G.intestinalis* sequences, 'Polish' and 'Belgian', isolated from human hosts over the same region have been included for comparison. The host origin, location and codename of each isolate sequenced is detailed in Section 3.4. The sequences were aligned by Clustal X, Version 1.62b, and displayed using MEGA, Version 1.02. The top line displays the complete sequence, identical bases in the other sequences are displayed as dots while base changes are displayed as the base present in that particular sequence.

Figure 3.13: Alignment and comparsion of *G.intestinalis* rDNA sequences amplified from Waikato humans, Waikato and Manawatu calves and laboratory adapted isolates using rDNA primers as well as GenBank sequences.




16S rDNA gene cont'ed
61
120
Waihum89 CCCCGGACGC GCGAAGGGCC GCGAGCCCCC GCGCCTGGAG GAAGGAGAAG TCGTAACAAG
Waihum106
Waihum70
Waihum74
Waihum64
Waihum67
Waihum110
Beaver3
Sheep1
Beaver2
Sheep2
Beaver1
Austhum
Polish
Waihum102
Waihum136
Germhum2
Germhum1
WcalfC4H7 T.....CG --
WcalfC11H5 T.....CG --
WcalfC12H2 T.....CG --
WcalfC3H10 T.....CG --
WcalfC5H1 T.....CG --
WcalfC9H3 T.....CG --
WcalfC6H5 T.....CG --
WcalfC10H1 T.....CG --
WcalfC1H7 T.....CG --
WcalfC6H1 T.....CG --
McalfC13H8 T.....CG --
WcalfC9H5 T.....CG --
WcalfC12H7 T.....CG --
WcalfC12H4 T.....CG --
McalfC13H3 T.....CG --
Belgian
16S rDNA gene cont'ed
121
180
Waihum89 GTATCCGTAG GTGAACCTGC GGATGGATCC CTCGCGCGCG GCGTGTGTCG CCCCCGCGGC
Waihum106
Waihum70
Waihum74
Waihum64
Waihum67 .....C...- --
Waihum110 .....C...- --
Beaver3 C C..C.C.
Sheep1 C C..C.C.
Beaver2 C C..C.C.
Sheep2 C C..C.C.
Beaver1 C C..C.C.
Austhum C C..C.C.
Polish C C..C.C.
Waihum102 C C..C.
Waihum136 C C..CA.
Germhum2 C C..C.
Germhum1 C C..C.C.
WcalfC4H7 C C..C.C.
WcalfC11H5 C C..C.C.
WcalfC12H2 C C..C.C.
WcalfC3H10 C C..C.C.
WcalfC5H1 C C..C.C.
WcalfC9H3 C C..C.C.
WcalfC6H5 C C..C.C.
WcalfC10H1 C C..C.C.
WcalfC1H7 C C..C.C.
WcalfC6H1 C C..C.C.
McalfC13H8 C C..C.C.
WcalfC9H5 C C..C.C.
WcalfC12H7 C C..C.C.
WcalfC12H4 C C..C.C.
McalfC13H3 C C..C.C.
Belgian C..C.


5.8S rDNA gene
181
240
Waihum89 CCGGTCGGCA CGCCAGCCCC GCGCCGGCGG ATGCCTCGGC CCGGGCGGCG ACGAAGAGCG
Waihum106
Waihum70
Waihum74
Waihum64
Waihum67
Waihum110
Beaver3 .....C .C..A.G.. C.....
Sheep1 .....C .C..A.G.. C.....
Beaver2 .....C .C..A.G.. C.....
Sheep2 .....C .C..A.G.. C.....
Beaver1 .....C .C..A.G.. C.....
Austhum .....C .C..A.G.. C.....
Polish .....C .C..A.G.. C.....
Waihum102 .....C .C..A.G.. C.....
Waihum136 .....C .C..A.G.. C.....
Germhum2 .....C .C..A.G.. C.....
Germhum1 .....C .C..A.G.. C.....
WcalfC4H7 .....G ....CA.G.. C.....
WcalfC11H5 .....G ....CA.G.. C.....
WcalfC12H2 .....G ....CA.G.. C.....
WcalfC3H10 .....G ....CA.G.. C.....
WcalfC5H1 .....G ....CA.G.. C.....
WcalfC9H3 .....G ....CA.G.. C.....
WcalfC6H5 .....G ....CA.G.. C.....
WcalfC10H1 .....G ....CA.G.. C.....
WcalfC1H7 .....G ....CA.G.. C.....
WcalfC6H1 .....G ....CA.G.. C.....
McalfC13H8 .....G ....CA.G.. C.....
WcalfC9H5 .....G ....CA.G.. C.....
WcalfC12H7 .....G ....CA.G.. C.....
WcalfC12H4 .....G ....CA.G.. C.....
McalfC13H3 .....G ....CA.G.. C.....
Belgian


5.8S rDNA gene cont'ed
241
300
Waihum89 CGGCGGAGCG CGAGACGCGG TGCGGACCCG CCCGCCCCGA GAAGCACCGA CCCTCGAACG
Waihum106
Waihum70
Waihum74
Waihum64
Waihum67
Waihum110
Beaver3
Sheep1
Beaver2
Sheep2
Beaver1
Austhum
Polish
Waihum102 .....G
Waihum136 .....G
Germhum2 .....G
Germhum1 .....G
WcalfC4H7
WcalfC11H5
WcalfC12H2
WcalfC3H10
WcalfC5H1
WcalfC9H3
WcalfC6H5
WcalfC10H1
WcalfC1H7
WcalfC6H1
McalfC13H8
WcalfC9H5 T.....
WcalfC12H7 T.....
WcalfC12H4 T.....
McalfC13H3 T.....
Belgian .....G

```
                5.8S rDNA gene cont'ed
            301                                                        360
Waihum89    CAGCGCGCCC CGGCGCCGCC GCCTCGGCGC CCGCCGCGTG CCGCGCGCCG CGCCGCCGAG
Waihum106   .......... .......... .......... .......... .......... ..........
Waihum70    .......... .......... .......... .......... .......... ..........
Waihum74    .......... .......... .......... .......... .......... ..........
Waihum64    .......... .......... .......... .......... .......... ..........
Waihum67    .......... .......... .......... .......... .......... ..........
Waihum110   .......... .......... .......... .......... .......... ..........
Beaver3     .......... .......... .......... .......... .......... ......A...
Sheep1      .......... .......... .......... .......... .......... ......A...
Beaver2     .......... .......... .......... .......... .......... ......A...
Sheep2      .......... .......... .......... .......... .......... ......A...
Beaver1     .......... .......... .......... .......... .......... ......A...
Austhum     .......... .......... .......... .......... .......... ......A...
Polish      .......... .......... .......... .......... .......... ......A...
Waihum106   .......... .......... .......T.. .T........ .......... ......A...
Waihum136   .......... .......... .......T.. .T........ .......... ......A...
Germhum2    .......... .......... .......T.. .T........ .......... ......A...
Germhum1    .......... .......... .......... .......... .......... ......A...
WcalfC4H7   .......... .......... .......... .T........ .......... ......A...
WcalfC11H5  .......... .......... .......... .T........ .......... ......A...
WcalfC12H2  .......... .......... .......... .T........ .......... ......A...
WcalfC3H10  .......... .......... .......... .T........ .......... ......A...
WcalfC5H1   .......... .......... .......... .T........ .......... ......A...
WcalfC9H3   .......... .......... .......... .T........ .......... ......A...
WcalfC6H5   .......... .......... .......... .T........ .......... ......A...
WcalfC10H1  .......... .......... .......... .T........ .......... ......A...
WcalfC1H7   .......... .......... .......... .T........ .......... ......A...
WcalfC6H1   .......... .......... .......... .T........ .......... ......A...
McalfC13H8  .......... .......... .......... .T........ .......... ......A...
WcalfC9H5   .......... .......... .......... .T........ .......... ......A...
WcalfC12H7  .......... .......... .......... .T........ .......... ......A...
WcalfC12H4  .......... .......... .......... .T........ .......... ......A...
McalfC13H3  .......... .......... .......... .T........ .......... ......A...
Belgian     .......... .......... .......... .......... ....------ ----------
```

```
                                        23S rDNA gene
            361                      387
Waihum89    AGCCCCCCGC GGCGATCCCG CCGGGCT
Waihum106   .......... .......... .......
Waihum70    .......... ....G..... .......
Waihum74    .......... ....G..... .......
Waihum64    .......... ....G..... .......
Waihum67    .......... ....G..... .......
Waihum110   .......... ....G..... .......
Beaver3     ...G.....G ....G..... .......
Sheep1      ...G.....G ....G..... .......
Beaver2     ...G.....G ....G..... .......
Sheep2      ...G.....G ....G..... .......
Beaver1     ...G.....G ....G..... .......
Austhum     ...G.....G ....G..... .......
Polish      ...G.....G ....G..... .......
Waihum102   ...G.....G ....G..... .......
Waihum136   ...G.....G ....G..... .......
Germhum2    ...G.....G ....G..... .......
Germhum1    ...G.....G ....G..... .......
WcalfC4H7   ...G...... ....G..... .......
WcalfC11H5  ...G...... ....G..... .......
WcalfC12H2  ...G...... ....G..... .......
WcalfC3H10  ...G...... ....G..... .......
WcalfC5H1   ...G...... ....G..... .......
WcalfC9H3   ...G...... ....G..... .......
WcalfC6H5   ...G...... ....G..... .......
WcalfC10H1  ...G...... ....G..... .......
WcalfC1H7   ...G...... ....G..... .......
WcalfC6H1   ...G...... ....G..... .......
McalfC13H8  ...G...... ....G..... .......
WcalfC9H5   ...G...... ....G..... .......
WcalfC12H7  ...G...... ....G..... .......
WcalfC12H4  ...G...... ....G..... .......
McalfC13H3  ...G...... ....G..... .......
Belgian     ---------- ---------- -------
```

3.9 SEQUENCE VARIABILITY OF *G.INTESTINALIS* rDNA

The number of sequence differences observed and the percentage of sequence variability present within the *G.intestinalis* rDNA sequences from both humans and calves were calculated, Table 3.3. The human and calf isolated *G.intestinalis* rDNA sequences that were compared contained a total of 387 bases. Within the nine human isolated *G.intestinalis* rDNA sequences a total of twenty base differences were observed while only one base difference was observed within the fifteen calf isolated *G.intestinalis* rDNA sequences of 387 bases. The percentage of sequence variability observed within the human isolates of *G.intestinalis* rDNA was 5.2% while the calf isolates of *G.intestinalis* was 0.3% over the same region.

Table 3.3: The comparison of sequence differences and the degree of sequence variability within human and calf isolated *G.intestinalis* rDNA sequences.

***G.intestinalis* isolates**	**Number of Sequences**	**Number of sequence differences**	**Percentage of sequence variability**
Human	9	20	5.2%
Calf	15	1	0.3%

3.10 PHYLOGENETIC RELATIONSHIPS OF *GIARDIA* ISOLATES

Different isolates of *Giardia* were directly compared based upon rDNA sequence relationships. The region of *G.intestinalis* rDNA compared was that shown in Figure 3.13. For completeness two Genbank isolates representing the two human subgroups found worldwide, 'Polish' and 'Belgian', were included in the rDNA sequence comparisions. Five outgroups, *G.muris, G.ardeae* and *Giardia* from a hamster, vole and ibis, were included in the phylogenetic analysis, as shown in Figure 3.15, the sequence data used for the splitstree generation is shown in Appendix B.

The distance between each group indicates the degree of nucleotide differences while the physical orientation of each line is not in itself important. The distance between the *G.intestinalis* isolates and the outgroup (representing nucleotide changes) was quite substantial and as a result resolution between the thirty-five *G.intestinalis* isolates was lost therefore only the point at which they join the *G.intestinalis* isolates has been indicated in Figure 3.14. Figure 3.15 shows the five outgroups with one representative of each *G.intestinalis* isolate clusters (calf, laboratory adapted animal and human, Waikato human 'Polish' and 'Belgian' subtypes).

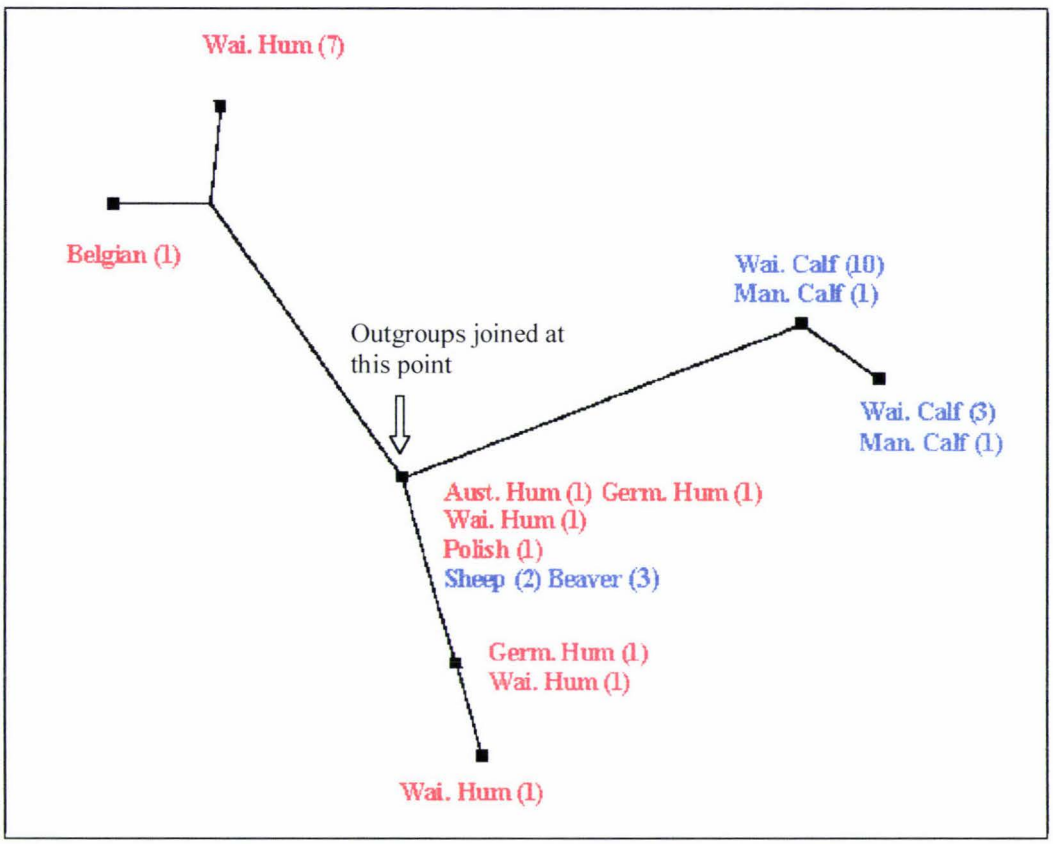


Figure 3.14: Splitstree showing the phylogenetic relationships between Waikato human isolates, Waikato and Manawatu calf isolates, laboratory isolates and GenBank isolates of *G.intestinalis* as represented within the 387 homologous nucleotide positions. *G.intestinalis* isolated from humans is represented in red while *G.intestinalis* isolated from animals is represented in blue. The number of isolates examined is in brackets.

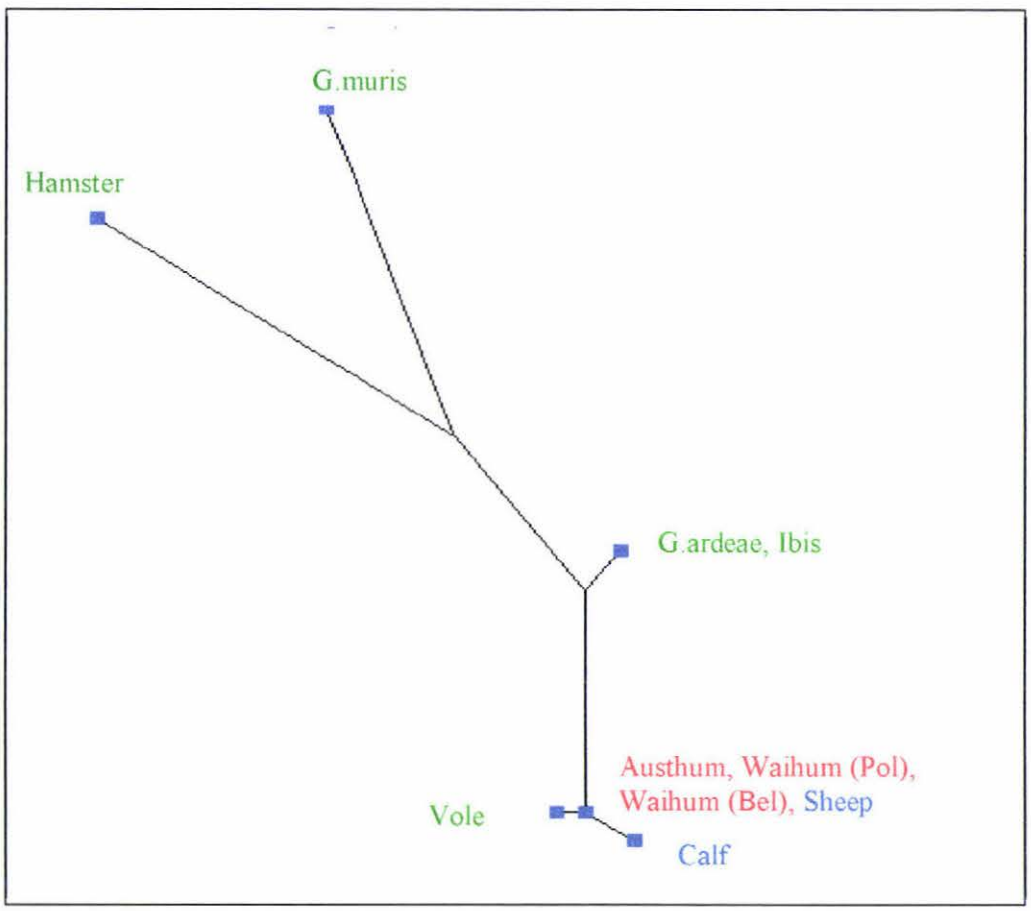


Figure 3.15: Splitstree showing the phylogenetic relationships between one representative of each *G.intestinalis* cluster (calf, sheep, 'Polish' and 'Belgian') and the five outgroups, *G.muris*, *G.ardeae* and *Giardia* from a hamster, vole and ibis. *G.intestinalis* isolated from humans is represented in red, *G.intestinalis* isolated from animals is blue while the *Giardia* outgroups are in green. The number of isolates examined is in brackets.

Note: Twenty-six nucleotide differences differentiated the ten *Giardia* isolates used to generate the phylogenetic relationships shown here (Appendix B contains the data set used).

3.11 DEVELOPMENT OF HUMAN SPECIFIC rDNA PRIMERS

Genetic analysis of the human and calf isolated *G.intestinalis* rDNA sequences identified regions in which calf and human sequences differed from each other considerably. Primers were designed to target these *G.intestinalis* rDNA regions. Two different primers were developed to work in combination with the original rDNA primers of Cyn 1/Cyn 2. The first primer, Hum, enables the identification of *G.intestinalis* strains previously shown to be present in naturally infected humans. The second primer, Pol, also amplifies *G.intestinalis* strains from humans only however, it only amplifies strains of the 'Polish' human subtype and not strains of the 'Belgian' human subtype. Both primers were designed to be used in combination with the existing Cyn rDNA primers, Cyn 1-Pol and Hum-Cyn 2. In addition to the specific nature of these two newly developed primers they could both be utilised for Nested PCR of *G.intestinalis* isolates.

The nucleotide sequences for both primers, Hum and Pol, are detailed in Section 2.3.2, while the PCR primer combinations and the expected product sizes are outlined in Section 2.3.3.

Table 3.4: The amplification specificities of the two newly developed *G.intestinalis* rDNA primers, Hum and Pol.

Primer name	Primer specificity
Hum	Amplifies human isolates only
Pol	Amplifies human isolates of Polish subtype only

CHAPTER FOUR: DISCUSSION

Studies, both overseas and in New Zealand, have reported that during the first three months of life, a high proportion of cattle become infected with *Giardia*. These infections are resolved naturally after one to two weeks, however during the course of infection the calves release a substantial number of *Giardia* cysts into the environment. During the current study, the impact of these calf *Giardia* cyst reservoirs on human giardiasis was investigated within the defined geographical region of the Waikato district. A comparative sequencing approach was adopted which involved direct sequencing of a portion of the rDNA locus of *Giardia* from cysts isolated from both humans and calves. This data was then used to investigate the relationships between *Giardia* from different host species.

This thesis had three major objectives:

1. To determine the prevalence of *Giardia* in the calf population of the Waikato district.
2. To determine rDNA sequences for *G.intestinalis* strains isolated from both humans and calves within the Waikato district.
3. To examine the possible phylogenetic relationships between human and calf isolates of *G.intestinalis* and determine the significance of genetic relationships with reference to cross-transmission between these host species.

4.1 THE PREVALENCE OF *GIARDIA* IN CALVES

The identification of a moderate *Giardia* infection rate in New Zealand calves as reported by Brown *et al.* (1997), following a relatively small survey, led to the current study. The much larger number of calves sampled in the current study enabled a more accurate assessment of the proportion of calves infected with *Giardia* in the Waikato district. For the current study a total of 587 calf faecal specimens were collected from the Waikato district during the spring calving season of 1998 and tested for the presence of *Giardia* cysts. The presence of *Giardia* was detected in faecal specimens from all Waikato farms studied with high concentrations of cysts detected in samples from all but one of these farms.

Overall it was found that 41.6% of the calf faecal specimens tested contained *Giardia* cysts, with almost 5% of positive specimens containing concentrations of greater than 10^5 *Giardia* cysts per gram of faecal matter. Similar studies in Canada have reported *Giardia* in as many as 73% of samples while studies in Spain have reported rates of only 38% in calves (Olson *et al.*, 1997; Quílez *et al.*, 1996). In comparison to those reported in other countries the occurrence of *Giardia* in calves within New Zealand does not appear to be particularly high, only 41.6%, nor warrant any reasonable concern for calf health.

The proportion of *Giardia* infected calves present on all farms were similar despite farms being widely distributed within the Waikato and different housing, feeding, and water management practices being employed on each farm. The overall similarity in the levels of *Giardia* on different farms was not found to correlate to a particular age or farming practice but rather to the point in time when calves were initially exposed to viable *Giardia* cysts already present in the environment. The earliest identification of *Giardia* cysts in calves on the majority of farms tended to occur following the transfer of the calves onto paddocks from the original holding pens. The identification of *Giardia* cysts in calves maintained in holding pens throughout the study indicated that in some cases exposure to *Giardia* had occurred immediately after birth prior to placement in the holding pens. The original source of *Giardia* in these instances was undetermined; however it was concluded that *Giardia* cysts were not introduced into the pens with the daily water or feed supplies or as a result of human contact.

4.2 STRUCTURE OF THE ITS REGION

All the amplified *G.intestinalis* rDNA sequences were 387bp in length and covered a homologous region. The sequences began 154 bases from the 3' end of the 16S rDNA gene and extended to the distal end of the ITS 2 region. Included within this locus was the entire ITS 1 non-coding region (40bp), the 5.8S rDNA gene (139bp) and the ITS 2 non-coding region (54 bp).

4.3 PHYLOGENETIC RELATIONSHIPS OF *G.INTESTINALIS* ISOLATED FROM THE SAME HOST SPECIES

Before phylogenetic comparisons were performed involving *G.intestinalis* rDNA isolated from different host species, in particular human and calf hosts, the rDNA sequences from each host species were compared. Such comparisons identified genetic structure within *G.intestinalis* rDNA isolated from the same host species.

4.3.1 Relationships between calf specific strains of *G.intestinalis*

Sequencing studies involving *G.intestinalis* isolated from naturally infected calves has, to date, remained limited despite the importance of these calves as reservoirs of cysts in the environment. In fact the analysis of *G.intestinalis* sequences obtained directly from naturally infected calves without subsequent *in vivo* culturing has not been previously reported. *Giardia* rDNA sequences were obtained from fifteen of the twenty-eight calf faecal specimens that contained greater than 10^5 cysts per gram of faeces. The fifteen *G.intestinalis* rDNA sequences from calf faecal matter were from ten different farms, one in the Manawatu region and nine widely distributed throughout the Waikato district. Two or more calf isolated *G.intestinalis* sequences were obtained from faecal matter collected at four farms, one in the Manawatu and three in the Waikato. In each case the sequences were determined from *G.intestinalis* cysts isolated from different faecal specimens. Each of the remaining six sequences obtained from calf faeces were from samples collected at six different Waikato farms.

Within the 387bp region one variable nucleotide position was observed between the calf isolates which corresponded to an overall sequence variability of 0.3% within the locus of interest. In the calf isolates the 3' end of the 16S rDNA gene contained no nucleotide substitutions while, in contrast, the 5.8S rDNA gene contained one nucleotide substitution. No nucleotide substitutions were observed in either of the ITS regions. The single nucleotide substitution (nt 291) split the calf isolates into two groups. The presence of the one nucleotide change within the 5.8S rDNA gene, a coding region of the rDNA locus, is unusual as most changes would be expected to be found in the non-coding regions. However, the

ITS regions and to a lesser extent the 5.8S rDNA gene, have been reported as rapidly evolving regions of the *Giardia* genome (Felleisen, 1997).

The two different groups of *G.intestinalis* from calves as separated by the one nucleotide variation were designated Calf Type I and Calf Type II and contained eleven and four isolates respectively. Of the eleven different isolates grouped as Calf Type I all but one was obtained from calves widely distributed throughout the Waikato, the one other isolate was obtained from a calf located in the Manawatu region. All of these isolates were collected during the natural spring calving season of 1998. The four calf isolates grouped as Calf Type II also consisted of specimens from both the Manawatu and Waikato. Three calf isolates from the Waikato were collected during the spring calving season of 1998 while the one Manawatu calf isolate was collected during the autumn calving season of 1998, a temporal difference of six months. The occurrence of identical sequences in calf isolates from different geographical regions as well as different calving seasons indicates no spatial or temporal correlation to a specific calf type. The presence of both Calf Type I and II on the same farm during the same calving season, as observed in two different farms located in the Waikato, supports the lack of spatial or temporal correlation to observed calf type. It is possible that the movement of cattle between different regions of New Zealand may account for the presence of homologous strains in both regions. However, that there may only be two strains present in New Zealand cattle can not be discounted.

As identified by the current research Calf Type I is almost three times more prevalent than Type II. The extensive variety of farming protocol observed throughout the Waikato may have led to the selection of a particular strain over the other. However, the homogeneity of the sequences collected suggests such type selection has not occurred in the Waikato and possibly within New Zealand as a whole.

4.3.2 Relationships between human specific strains of *G.intestinalis*

Ribosomal DNA sequences were collected from cysts isolated from nine human faecal specimens that contained greater than 10^5 *Giardia* cysts per gram faeces. These came from a total of thirty-nine human faecal specimens collected from the Waikato district that showed high levels of *Giardia* cysts. The specimens were from individuals living in the same geographical region as thirteen of the fifteen calf samples, although the exact locations, occupations and ages of the patients were unknown. In addition to the nine Waikato human specimens sequenced, a further three laboratory adapted cultures of *G.intestinalis*, previously isolated from humans, were also sequenced. Comparisons between human *G.intestinalis* isolates involved all Waikato human isolates and all laboratory adapted human isolates, however where applicable the distinction has been made between the different sources of human *G.intestinalis* isolates.

Within the 387bp region a total of twenty variable nucleotide positions were observed between the human isolates which corresponded to a sequence variability of 5.2% over this region. In all human isolates the 3' end of the 16S rDNA gene contained no nucleotide substitutions. In contrast, the coding region of the 5.8S rDNA gene contained six nucleotide substitutions which corresponds to 4.3% sequence variability (the number of nucleotide substitutions per 139bp of the 5.8S rDNA gene), 30% of the total sequence variability over this region. Most of the variation was located within the ITS regions, ITS 1 was found to contain ten nucleotide substitutions while ITS 2 contained four nucleotide substitutions, accounting for 50% and 20% of the total sequence variability respectively.

Of the twenty variable nucleotide patterns, eleven sites supported the separation of the human isolates into two distinct phylogenetic clusters. Although this separation was contradicted by one nucleotide site (nt 166), there is clearly strong evidence to support this genetic structure. Comparison of the current data set with previously reported human sequences found that this split corresponded to the 'Belgian' and 'Polish' subtypes of Homan *et al.* (1992). Seven of the twelve human isolates clustered together with the 'Belgian' human subtype while the remaining five human isolates, including all laboratory adapted human strains,

corresponded to the 'Polish' sequence. That the present study identified a larger proportion of the human isolates from the Waikato region to be of the 'Belgian' type is somewhat unusual. Previous studies have found the 'Polish' type to be more prevalent than 'Belgian' type isolates (Nash and Mowatt, 1992; Homan *et al.*, 1992; Andrews *et al.*, 1989). However these studies used *in vitro* culturing prior to molecular characterisation whereas the current study assayed the genetic variation between human isolates directly. This difference in methodology may explain the finding that the 'Belgian' type was the predominant strain within the Waikato district, in apparent contradiction with previous studies. Both Mayrhofer *et al.* (1995) and Hopkins *et al.* (1997) have reported that culturing biases are present when establishing *G.intestinalis* strains *in vitro* or *in vivo*. Such selection biases may result in one strain, in this case 'Polish', predominating under culture conditions and therefore being over-represented in molecular analyses. Directly assaying molecular variation without culturing the isolated strains may more accurately reflect the relative ratios of *Giardia* strains in the environment. Alternatively environmental differences and therefore differences in selective regimes, between the Waikato district and the study regions of previous workers (for example; Nash and Mowatt, 1992; Homan *et al.*, 1992; Andrews *et al.*, 1989) may be reflected in the relative ratios of the subtypes.

4.3.2.1 Phylogenetic relationships within 'Belgian' type *G.intestinalis*

Seven of the Waikato human isolates were identified to be of the 'Belgian' human subtype. Within the 'Belgian' cluster three different sequences were present; one type consisted of three isolates while the other two sequence types each occurred twice. The three groups were separated on the basis of three sequence differences; two nucleotide substitutions, one in each of the ITS regions, and a tri-nucleotide deletion located in ITS 1. The coding regions within the target locus were identical to the 'Belgian' type isolate supporting their identification as generally conserved regions of the rDNA.

4.3.2.2 Phylogenetic relationships within 'Polish' type *G.intestinalis*

The 'Polish' human subtype cluster consisted of two Waikato human isolates and all laboratory adapted human isolates. Within these isolates four sequence types were present, the only two identical isolates were a Waikato human and a laboratory adapted human isolate originating from Germany. The sequence types within the 'Polish' cluster were differentiated by five nucleotide differences; two nucleotide changes in ITS 1 and three nucleotide changes in the 5.8S rDNA gene. In contrast to the 'Belgian' sequences the 'Polish' subtype sequences were identical throughout the sequenced portion of the 16S rDNA gene and ITS 2.

4.3.3 Relationships between laboratory adapted animal specific strains of *G.intestinalis*

A collection of laboratory adapted isolates originally from naturally infected sheep and beaver hosts were also sequenced and added to the comparison. Three isolates were originally cultured from beavers, each collected during a different year and from widely distributed Canadian populations. Similarly the two sheep isolates were collected in different years and from different locations. The sheep and beaver *G.intestinalis* rDNA sequences were identical throughout the 387bp sequenced. It is possible that the similarity of these animal isolates was due to culturing biases. That is, in a pool of multiple strains of *G.intestinalis* a particular *G.intestinalis* isolate has a number of adaptations which minimise the effects of the culturing. Consequently it is this strain which has been attributed to a specific host as opposed to a type less tolerant to laboratory growth conditions. Difficulties in culturing *G.intestinalis* isolates *in vitro* have previously been reported for other mammalian host species, such as humans and dogs (Brown *et al.*, 1992; Hopkins *et al.*, 1997). These problems may be interpreted as supporting such a hypothesis which selects strains in culture. Given the host difference, as well as the geographical and temporal separation of these samples it seems unlikely these isolates would have originated from the same genetic pool and subsequently maintained the same sequence. The determination of the exact same rDNA sequence for all the laboratory adapted animal isolates is suggestive of sample contamination. Any inferences therefore, about the relationships of the laboratory

adapted animal strains to the "wild" strains are dubious because of the uncertainty surrounding the exact origins of the laboratory strains.

4.4 PHYLOGENETIC RELATIONSHIPS OF *G.INTESTINALIS* STRAINS ISOLATED FROM DIFFERENT HOST SPECIES

To investigate phylogenetic relationships between the four different groups of *G.intestinalis* isolates; calf, Waikato human and laboratory adapted animal and human isolates the rDNA regions sequenced were compared, as shown in Figure 3.14. Although the rDNA region sequenced in the current work had not been compared previously it is considered to be a rapidly evolving region in the *Giardia* genome and therefore suitable for phylogenetic analysis of different *G.intestinalis* isolates (Felleisen, 1997). In addition two isolates from GenBank which represent the 'Polish' and 'Belgian' human types were included in the comparisons of *G.intestinalis* isolates from different naturally infected host species. Sequences of the same rDNA region from five different outgroups, *G.muris*, *G.ardeae*, and three *Giardia* strains isolated from an ibis, a vole, and a hamster (Kakubayashi, 1998), were also included in the phylogenetic reconstruction's in order to calibrate the amount of variation within the study group (Figure 3.15).

Three distinct clusters of isolates were found among the thirty-five different *G.intestinalis* isolates; a) the calf isolates (fifteen sequences), b) human isolates with the 'Belgian' subtype motif (eight sequences), and c) human isolates with the 'Polish' subtype motif and laboratory adapted animal isolates (twelve sequences), shown in Figure 3.14. The five outgroups of *Giardia* were distinctly separate from all of the *G.intestinalis* isolates and grouped separately from each other according to the distinct *Giardia* species, as shown in Figure 3.15.

Within the 387bp region a total of twenty-eight nucleotide differences occurred between the Waikato human isolates, the laboratory adapted human and animal isolates, and the Waikato and Manawatu calf isolates, corresponding to an overall sequence variability of 7.2%. The sequences of Waikato human, laboratory adapted human and animal, and calf isolates differed throughout the rDNA locus

of interest, as shown in Figure 3.11. Within the 3' end of the relatively conserved 16S rDNA gene there were five nucleotide changes between the thirty-five different isolates, corresponding to 18% of the total sequence variability observed over the rDNA locus sequenced. The 5.8S rDNA gene, also a relatively conserved region of the rDNA repeat unit, accounted for 29% of the total sequence variability; eight nucleotide substitutions within the thirty-five isolates. Within the variable ITS 1 region, eleven nucleotide variations were observed which accounted for the largest proportion of sequence variability 39%. The second variable region, ITS 2, represented the smallest degree of sequence variability within the rDNA locus with only four nucleotide substitutions amounting to 14% total sequence variability. The total degree of sequence variation between all of the human isolates was 5.2% (as shown in Table 3.3) while between the human and calf isolates the total degree of sequence variation was 7.2%. The degree of sequence variability between the human and calf isolates was determined to be greater than that displayed from the human isolates as would be expected due to the *G.intestinalis* isolates originating from different host species. However, the degree of sequence variability present among the human isolates, only 2% lower than that seen from different host species, could be related to the clinical observation of different degrees of infections presented in humans. The same correlation may also occur in the calf isolates, such as the connection between a particular calf type and the observation of diarrhoea, however the current data can only speculate upon the possibility of such correlation's among specific rDNA sequences and clinical symptoms.

Based upon sequence differences located throughout the sequenced locus, the fifteen calf isolates of *G.intestinalis* clustered away from all of the human and laboratory adapted animal isolates. Despite the majority of the human and calf *G.intestinalis* isolates originating within the Waikato district they do not appear to interact in a fashion indicative of cross-transmission, for example the isolation of calf type sequences from humans and vice versa. However, the genetic similarity of the *G.intestinalis* isolates from these two host species suggests that they are closely related. This is supported by the distant association of the outgroup taxa to the *G.intestinalis* isolates. Similar studies investigating the relationship of *G.intestinalis* isolates from different host species living within the same

geographical location has also identified apparent host specific strains. Hopkins *et al.* (1997) compared DNA sequences from the 5' end of the 16S rDNA of isolates from thirteen human and nine dog specimens in an Aboriginal community in Australia. Four assemblages were identified; assemblage A and B contained *G.intestinalis* isolates from all the humans and a few dogs while assemblages C and D contained isolates only from dogs. As with the current study the sequence variability between these two host species was relatively low, less than 3% total sequence variability, again supporting the generalised finding that *G.intestinalis* is a closely related group. The identification of *G.intestinalis* host specific strains among humans and calves in the current study reflects the findings of Hopkins *et al.* (1997) despite the difference in the actual region of rDNA sequenced. However unlike Hopkins *et al.* (1997) no cross-transmission between humans and calves is apparent despite sequencing a similar number of human and calf specimens. Particular dog habits, such as eating human faeces, may account for the presence of 'human' strains in dogs however the distinct lack of similar habits in humans and cattle would act to reduce the likelihood of cyst transmission occurring between these two host species by similar means.

The finding that all of the laboratory adapted animal isolates had identical sequences to 'Polish' type human isolates was relatively unusual. Previous studies had found *G.intestinalis* isolates from sheep and cattle to associate together based upon sequences derived from the enzyme glutamate dehydrogenase loci and different variant-specific surface protein genes (Ey *et al.*, 1997). The separation of these two host species, in comparison to Ey *et al.* (1997), may have however resulted from culturing biases or the cross contamination of isolates during laboratory culture. Therefore little significance is placed on the position of the laboratory adapted animal isolates relative to the remaining sequences.

4.5 DEVELOPMENT OF HUMAN SPECIFIC rDNA PRIMER

The possibility of *Giardia* cyst transmission from animals to humans has been investigated for at least the last fifteen years (Nash *et al.*, 1985). Although the result of experimental infection has recently reported that humans are susceptible to infection by *Giardia* isolated from non-human hosts (Majewska, 1994), zoonotic transmission is difficult to demonstrate outside the laboratory environment. Although it appears that cross-transmission can occur, whether it does occur to a degree at which it poses a substantial human health risk remains questionable. The molecular characterisation of naturally acquired human and animal *G.intestinalis* infections has previously used either *in vivo* or *in vitro* techniques prior to molecular characterisation. Despite the previous identification of human specific, animal specific and human/animal strains of *G.intestinalis* the introduction of culturing biases and the effect of such selective pressures upon the results cannot be disregarded (Monis, 1998; Hopkins *et al.*, 1997). The current research identified human and calf specific strains of *G.intestinalis* from naturally infected hosts in the absence of such culturing biases. The development of the Hum rDNA PCR primer allows the differentiation of *Giardia* identified as infecting humans from those found in calves. Therefore this primer can identify naturally occurring reservoirs of *G.intestinalis* previously shown to be infectious to humans in the environment. In addition, use of the Pol rDNA PCR primer increases the level of identification, (in combination with either Cyn 1, Hum, or Joli 1 rDNA PCR primers), distinguishing between 'Polish' and 'Belgian' subtype sequences.

These PCR primers offer the advantage of rapidly identifying *G.intestinalis* strains isolated from a range of sources (human, animal, effluent or aquatic), without the need for nucleotide sequencing. The development of human specific *G.intestinalis* rDNA primers has also expanded the tools currently available for investigating *G.intestinalis* outbreaks in humans and identification of infection sources. It may be possible to detect natural reservoirs of *G.intestinalis* in the environment with the potential to infect humans and reduce the number of infections from such potential hot spots.

4.6 SUMMARY AND FUTURE DIRECTION

The current study identified *Giardia* in 41.6% of calves tested within in the Waikato district, a higher presence than reported by a similar New Zealand study (Brown *et al.*, 1992). Direct sequencing of a portion of the *G.intestinalis* rDNA loci isolated from naturally infected calves, without laboratory culture prior to sequencing, is the first reported. Two different sequence motifs were identified from the calf isolates, designated Calf Type I and II. A similar survey of humans naturally infected with *G.intestinalis* within the same geographical region identified two distinct sequence motifs, which corresponded to two recognised human types of 'Polish' and 'Belgian'. Transfer of viable *Giardia* cysts from calves to humans *via* either direct or indirect modes of transmission remains debatable however the current work identified what appear to be host specific strains in humans and calves throughout the Waikato region.

Despite a substantial proportion of calves excreting large numbers of *Giardia* cysts into natural waterways, *via* farm runoff, this study found no indications of cross-transmission between calves and humans. In contrast to previous studies which employed *in vivo* culturing methods the majority of the human isolates in the current study were of the 'Belgian' subtype. That identical rDNA sequences were found in human and laboratory adapted animal isolates may support the hypothesis that culturing of *G.intestinalis* isolates introduces selection biases. PCR primers developed to recognise human specific sequence motifs have been designed to enable the identification of natural reservoirs of these strains in the environment. It is proposed that the identification of such natural reservoirs will allow appropriate intervention and treatment prior to outbreaks of human giardiasis. Ultimately it is hoped that *G.intestinalis* rDNA sequences from other host species will enable the identification of other natural reservoirs of *G.intestinalis* strains infectious to humans as well as clarify which host species are potential zoonotic threats.

APPENDICES

APPENDIX A: REAGENTS

The DNA ladders used in this thesis to size and /or quantify the PCR fragments were supplied by Gibco BRL and are described below (taken from the Gibco BRL 1998-1999 Product Catalogue).

1Kb DNA Ladder (Gibco BRL)

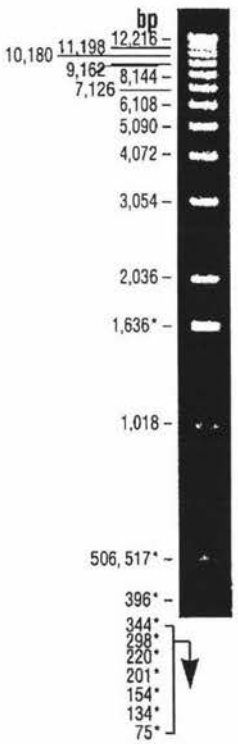
1 Kb DNA Ladder (U.S. Patent No. 4,403,036) is suitable for sizing linear double-stranded DNA fragments from 500 bp to 12 kb. Prepared from a plasmid containing repeats of a 1,018-bp DNA fragment (1), the ladder consists of 12 fragments ranging from 1,018 bp to 12,216 bp. In addition to these 12 bands, the ladder contains vector DNA fragments that range from 75 to 1,636 bp. The double-stranded ladder can be visualized on 0.5 to 1% agarose gels after ethidium bromide staining. This ladder may be radiolabeled using T4 polynucleotide kinase, T4 DNA polymerase, DNA polymerase I, or the large fragment of DNA polymerase I (Klenow fragment).

Concentration in storage buffer: 1 µg/µl in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA.

Recommended storage condition: −20°C.

Reference(s):

1. Hartley, J.L. and Donelson, J.E. (1980) *Nature (Lond.) 286*. 860.



0.5 µg/lane; 1.0% agarose gel stained with ethidium bromide.* fragments of the vector

1 Kb Plus DNA Ladder (Gibco BRL)

The 1 Kb PLUS DNA LADDER is suitable for sizing linear double-stranded DNA fragments from 100 bp to 12 kb. The ladder contains a total of twenty bands: twelve bands ranging in size from 1000 bp to 12,000 bp in 1000-bp increments and eight bands ranging in size from 100 to 1650 bp. The 1650-bp band contains approximately 8% of the mass applied to the gel. The ladder may be radioactively labeled by one of the following methods: (i) Partial exonucleolytic degradation and resynthesis with T4 DNA polymerase. This method is preferred because higher specific activity is achieved with less ^{32}P input; (ii) Labeling the 5′ ends with T4 polynucleotide kinase; (iii) Filling in the 3′ recessed ends with *E. coli* DNA polymerase I or the large fragment of DNA polymerase I.

Storage Buffer:
10 mM Tris-HCl (pH 7.5)
1 mM EDTA
50 mM NaCl

1 Kb PLUS DNA LADDER
0.7 µg/lane
0.9% agarose gel
stained with ethidium bromide

Low Molecular Mass Ladder (Gibco BRL)

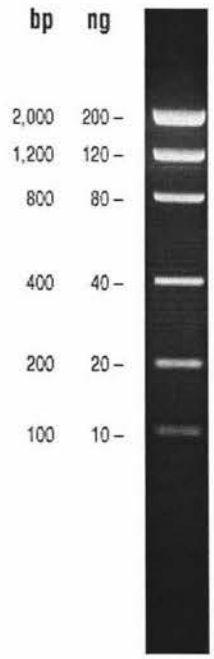
Low DNA MASS Ladder (patent pending) is suitable for estimating the mass of unknown DNA samples by ethidium bromide staining (1). Prepared from a specially constructed plasmid, the ladder consists of an equimolar mixture of six blunt-ended fragments from 100 to 2,000 bp. Electrophoresis of 4 µl of Low DNA MASS Ladder results in bands containing 200, 120, 80, 40, 20, and 10 ng (470 ng total) of DNA, respectively.

Concentration in storage buffer: 470 ng/4 µl in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA.

Recommended storage condition: −20°C.

Reference(s):

1. Hartley, J.L. and Xu, L. (1994) *Focus*® *16*, 52.



APPENDIX B: DATA

The data used for phylogenetic analysis and SplitsTree analysis of the four representatives of *G.intestinalis* isolates (calf, Waihum {Pol}, Waihum {Bel}, and Austhum) and the *Giardia* outgroups is outlined below. The 128 characters of the same rDNA region are outlined in the first two lists of sequences with the corresponding nucleotide positions noted. The twenty-six characters outlined in the third list of sequences details the nucleotide positions used to generate the phylogenetic relationships between the ten *Giardia* isolates shown in Figure 3.15.

```
Character-exclusion status changed: excluded missing and ambiguous sites

   505 characters excluded
   Total number of characters now excluded = 505
   Number of included characters = 128

Input data matrix:

            22222222222222222222222222222222222222222222222222222222222222233
            33333333344444444445555555556666666666777777777788888888899999999900
Taxon/Node  12345678901234567890123456789012345678901234567890123456780123456789

Austhum     CGTCCCTGCCCCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGCGCGGCGGCGAGGCCCCGGAGA
Waihum89    CGTCCCTGCCCCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGCGCGGCGGCGAGGCCCCGGAGA
Sheep1      CGTCCCTGCCCCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGCGCGGCGGCGAGGCCCCGGAGA
McalfC13H3  CGTCCCTGCCCCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGCGCGGCGGCGAGGTCCCGGAGA
Waihum136   CGTCCCTGCCCCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGCGCGGCGGCGAGGCCCCGGAGA
G.ardeae    CGTCCCTGCCCCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGCGCCGCGGCGAGCCCCGGGAGA
ibis        CGTCCCTGCCCCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGCGCCGCGGCGAGCCCCGGGAGA
vole        CGTCCCTGCCCCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGCGCGCGGGCGAGGCCCCGGAGA
G.muris     CGTCCCTGCCCCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGTCTTCTGGCGAGTCCTGGGAGA
hamster1    CGTCCCTGCCCCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGTGTCATGGCGAGGCCCTTGAGG

Input data matrix (continued):

            33333333333333333333333333333333333333333333333333333333333333333
            11111111112222222222333333333344444444445555555555666666666
Taxon/Node  01234567890123456789012345678901234567890123456789012345678901234567890123456789

Austhum     AGGGCCGCGAGCCCCCGCGCCTGGAGGAAGGAGAAGTCGTAACAAGGTATCCGTAGGTGA
Waihum89    AGGGCCGCGAGCCCCCGCGCCTGGAGGAAGGAGAAGTCGTAACAAGGTATCCGTAGGTGA
Sheep1      AGGGCCGCGAGCCCCCGCGCCTGGAGGAAGGAGAAGTCGTAACAAGGTATCCGTAGGTGA
McalfC13H3  AGGGCCGCGAGCCCCCGCGCCTGGAGGAAGGAGAAGTCGTAACAAGGTATCCGTAGGTGA
Waihum136   AGGGCCGCGAGCCCCCGCGCCTGGAGGAAGGAGAAGTCGTAACAAGGTATCCGTAGGTGA
G.ardeae    ACGGCGACGAGCCCTGGCGCCTGGAGGAAGGAGAAGTCGTAACAAGGTATCCGTAGGTGA
ibis        ACGGCGACGAGCCCTGGCGCTTGGAGGAAGGAGAAGTCGTAACAAGGTATCCGTAGGTGA
vole        AGGGCCGCGAGCCCCCGCGCCTGGAGGAAGGAGAAGTCGTAACAAGGTATCCGTAGGTGA
G.muris     ACAGGGACGAACCGCGAGGCTTGGAGGAAGGAGAAGTCGTAACAAGGTATCCGTAGGTGA
hamster1    ATAGGGACGAGCCATGGGACTTGAAGGAAGGAGAAGTCGTAACAAGGTATCCGTAGGTGA
```

```
Character-exclusion status changed: excluded constant

   102 characters excluded
   Total number of characters now excluded = 607
   Number of included characters = 26

Input data matrix:

            22222222222333333333333333
            77778899999011111222222233
Taxon/Node  78901201345912456034567803
-------------------------------------
Austhum     CGCGGCGCCCGAGGCCGGCCCGCGCG
Waihum89    CGCGGCGCCCGAGGCCGGCCCGCGCG
Sheep1      CGCGGCGCCCGAGGCCGGCCCGCGCG
McalfC13H3  CGCGGCGTCCGAGGCCGGCCCGCGCG
Waihum136   CGCGGCGCCCGAGGCCGGCCCGCGCG
G.ardeae    CGCCGCCCCCGGACGCGAGCTGGCGCG
ibis        CGCCGCCCCCGGACGCGAGCTGGCGTG
vole        CGCGCGGCCCGAGGCCGGCCCGCGCG
G.muris     TCTTCTTCTGGACAGGAAGCGAGGTG
hamster1    TGTCATGCCTTGTAGGAGATGGGATA
```

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