# Genotyping of Human and Animal Isolates of *Giardia intestinalis*

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# ABSTRACT

*Giardia intestinalis* is an important protozoan parasite that infects humans and animals. It has been suggested that cattle may be a major source of human *Giardia* infection so a dairy farming region of New Zealand was investigated. This thesis uses three molecular methods to genotype *G. intestinalis* isolates obtained from human and animal faecal specimens collected in the Waikato region of New Zealand, to determine if giardiasis is a zoonotic disease.

Random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) fingerprinting techniques were initially assessed for their ability to genotype *G. intestinalis* isolates. "Clear cut" evidence of zoonosis could not be established by either method, due to a low sample number.

To determine the stability of the *G. intestinalis* genome an axenic culture of *G. intestinalis* trophozoites was stressed with toxic levels of metronidazole and the survivors, following a number of passages, were examined using AFLP and RAPD analysis. The DNA fingerprints were compared to those of the original wild-type with the results being indicative of an unstable *G. intestinalis* genome.

A third molecular method was employed, which amplifies a portion of the tandemly repeated ribosomal DNA (rDNA). Each cyst contains 512 head to tail tandem repeat copies of the *rRNA* gene made up of both conserved and variable regions. The use of nested primers increased the sensitivity and specificity of the PCR reaction allowing the amplification of a 505bp rDNA fragment. DNA sequence analysis and alignment of the amplified products facilitated the comparison of *G. intestinalis* isolates. The relationship of the sequence data was generated and displayed using Splitstree software indicating that zoonosis did occur.

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# CHAPTER 1: REVIEW OF GIARDIA AND GIARDIASIS

## 1.1 History

*Giardia* is a parasitic protozoan organism found to infect the intestine of mammals, birds, reptiles, and amphibians, and is the causative agent of giardiasis (Adam, 1991). Antony van Leeuwenhoek is attributed to being the first to have discovered *Giardia* when examining his own diarrhoeic stool and described structures resembling those found in *Giardia*. But in 1859 a Czech physician, Vilem Lambl, isolated *Giardia* from children with dysenteric diarrhoea and is credited with the discovery of the organism, which now bears his name, *Giardia lamblia* (Adam, 1991; Boreham, 1990; Rose, 1999), also known as *G. duodenalis* and *G. intestinalis*. In this thesis *Giardia* capable of infecting human and other mammalian hosts will be referred to as *G. intestinalis*.

## 1.2 Biology

### 1.2.1 Morphology

*Giardia* has a two-stage life cycle, existing as a reproductive trophozoite form found in the intestine of the infected host, and a resistant cyst stage found in the faeces excreted by the host. The trophozoites are bi-laterally symmetrical with a pear shaped appearance, measuring 10 – 12 μm in length and 5 – 7 μm wide, with four pairs of flagella, and a pair of nuclei (Adam, 1991; Farthing, 1997; Ortega, 1997). The two nuclei of the trophozoite both contain the same amount of DNA, are synchronised for mitosis, and transcriptionally active (Kabnick, 1990). The posterior half of the organism contains median bodies that consist of microtubules, and are described as 'claw shaped' in *G. intestinalis* and 'round' in *G. muris*. The anterior of

the ventral surface of the trophozoite contains an ovoid 'sucking' disc, by which the organism attaches to the microvilli of the small intestine (Adam, 1991). Dorsally the organism is convex, and ventrally they are concave.

The cysts of *G. intestinalis* are oval shaped with a smooth surface and measure approximately 8 μm long, 5 μm wide with a cyst wall thickness of 0.3 μm. The cyst wall composes of two components; an inner cyst wall of two membranes, and an outer cyst wall that is constructed from a network of tightly packed filaments (Erlandsen, 1989; Sheffield, 1977).

### 1.2.2 Taxonomy

The taxonomy of *Giardia* has been limited by classical techniques due to the similar morphology of the cysts. Originally the genus was divided into three species based upon the morphological of the trophozoite media bodies by Filice (1952). The three species are:

1. *G. muris* which infects rodents, birds, and reptiles, has rounded median bodies found parallel to the long axis of the trophozoite.
2. *G. intestinalis* that infects a variety of mammals, including humans, and also birds and reptiles (Adam, 1991; Farthing, 1997; Marshall, 1997), has median bodies described as 'claw' shaped.
3. *G. agilis* was isolated from tadpoles, has median bodies fused into a single, club-shaped rod. The trophozoites are also more slender than *G. muris* and *G. intestinalis* with similar morphology but differ slightly due to their elongated shape (Feely, 1985).

Other species have been identified recently where an axenic culture of *G. ardeae* was established from the great blue heron (*Ardia herodias*) (Erlandsen, 1990a) and *G. psittaci* was identified by scanning electron microscopic examination of trophozoites isolated from budgerigars (Erlandsen, 1987). Molecular analysis by DNA sequence analysis of the small rRNA subunit of *Giardia* isolates from voles and muskrats identified the species *G. microti* (van Keulen, 1998). Examination of the rDNA

operon sequence has shown *G. intestinalis* and *G. ardeae* to be more closely related to each other than to *G. muris* (van Keulen *et al.*, 1993).

*Giardia* has been classified as follows (Cox, 1981; Levine, 1980):

Subkingdom: **Protozoa**

Phylum: **Sarcomastigophora**, with flagella, pseudophodia or both; with a single type of nucleus

Subphylum: **Mastigophora**, one or more flagella present in trophozoites: asexual reproduction by binary fission, and sexual reproduction in some groups.

Class: **Zoomastogophorea**, chloroplasts absent; one to many flagella.

Order: **Diplomonadida**, mirror symmetry; mitochondria and golgi apparatus absent; cysts present; free living or parasitic.

Suborder: **Diplomonadina**, body with 2-fold rotations symmetry or bilateral symmetry in one genus; cysts present; free-living or parasitic.

*Giardia* is believed to be a primitive eukaryotic organism, due to features such as a highly developed cytoskeleton, bound nuclei, chromosomes capped with telomeric repeats, and microtubules that make up the ventral disc, flagella, and median bodies, which are all features associated with eukaryotes (Upcroft, 1998; Vellai, 1998). Phylogenetic analysis based on rDNA sequences also supports the idea of *Giardia* being one of the most ancient representatives of eukaryotes (Vellai, 1998). However the organism also has prokaryotic features such as anaerobic metabolism and fermentative metabolism, alanine production, arginine utilisation, and the presence of plasmids, and shown to contain the bacterial enzyme pyruvate ferredoxin oxidoreductase (PFOR) and not the eukaryotic pyruvate dehydrogenase (Upcroft, 1998; Vellai, 1998). Also the *Giardia* genome contains no apparent introns and extremely short intergenic regions, and sequence analysis of the 16S rRNA shows many similar features to that of prokaryotes. Sequence analysis also shows high identity to Archeabacteria and Eubacteria (Sogin, 1989). These prokaryotic features suggest the primitive nature of the organism that represents an early branch of the eukaryotes on the evolutionary tree.

### 1.2.3 Life Cycle

The organism has a two stage life cycle, in the intestine *G. intestinalis* exists in its trophozoite form where the ventral disk is the site for attachment to the intestinal mucosa of the duodenum and upper jejunum of the host (Adam, 1991; Farthing, 1997; Teoh, 2000). Here the trophozoites divide by binary fission and as they move towards the colon and into the ileum they begin to form into cysts – encystation – where they are excreted in the faeces (Adam, 1991; Ortega, 1997). Shedding of the cysts can be intermittent or at low levels and difficult to detect, making diagnosis more difficult (Gardner, 2001).

The cyst is designed to allow *Giardia* to survive in the environment and is the infective stage of the life cycle where they enter the host by ingestion (Adam, 2001). After ingestion, the cysts are exposed to the acidic environment of the hosts stomach and then pass into the duodenum where they begin to excyst and release two trophozoites that colonise the intestine *via* their 'sucking' discs (Adam, 2001; Marshall, 1997; Ortega, 1997) thereby completing their life-cycle.

## 1.3 Giardiasis (The Disease)

Before the 1960's *G. intestinalis* was not acknowledged as a cause of illness in humans until a large numbers of people became ill after drinking water that had been contaminated with sewage or improperly filtered water in several United States communities (Logsdon, 1988). *G. intestinalis* is one of the most common enteric protozoan parasites of man, especially in children of developing countries (Erlandsen and Meyer, 1984). Infection can occur from the ingestion of 10 to 100 cysts with the incubation period varying from 1 to 45 days (Adam, 1991).

### 1.3.1 Symptoms

Giardiasis can have a wide range of symptoms from asymptomatic infections to chronic diarrhoea with malabsorption (Adam, 1991; Mejewska, 1998; Rose, 1999). The sheer number of trophozoites colonising the intestine cause a physical barrier to absorption but there have also been indications of malabsorption of fat (steathorea), lactose, and vitamins A and $B_{12}$ from this disease (Gardner, 2001).

Typical symptoms usually appear one to three weeks after ingestion of cysts with diarrhoea of loose, foul smelling stools, but may also include abdominal cramps, bloating, nausea, and decreased appetite (Marshall, 1997; Ortega, 1997) (Adam, 1991; Gardner, 2001; Jakubowski, 1988). Patients may also experience anorexia, a low-grade fever, chills, weight-loss, and belching with a foul taste (Marshall, 1997). The presence of mucus or blood in the stool is rare and any blood is indicative of anal irritation from the diarrhoea.

Patients with chronic giardiasis can experience profound malaise, diffuse epigastric and abdominal discomfort, and diarrhoea may persist, constipation may develop or could return to normal. The acute stage of the disease occurs usually three to four days after the first onset of symptoms, possibly resembling other causes of travellers' diarrhoea and not recognised as giardiasis.

If left untreated the disease can resolve itself spontaneously but could last for several months (Gardner, 2001).

### 1.3.2 Pathophysiology

Trophozoites are detected in the duodenum of the infected host and seen to not invade the cells unless there is necrosis or mechanical trauma of the mucosa (Owen, 1979). The attachment of the trophozoites to the intestinal mucosa has shown the shortening of the microvilli and a decrease in the ratio of villous to crypt cells (Buret *et al.*, 1992, Erlandsen, 1974; Solomons, 1982).

An animal model using gerbils infected with a human isolate of *G. intestinalis* was used by Buret *et al.* (1992) and showed the shortening of the microvilli and the decrease in the brush border surface area of the jejunum and ileum. This causes impaired digestion and absorption resulting in disaccharidase deficiencies and malabsorption of electrolytes, water and nutrients (Farthing, 1997; Teoh, 2000).

The use of a murine model of *G. muris* to study giardiasis has shown a decrease in the ratio of villous to crypt cells. Lesions that matched the size and shape of the sucking disc of the trophozoites were also seen on the microvillous border of villous epithelial cells (Erlandsen, 1974; Solomons, 1982).

### 1.3.3 Transmission

Most human infections occur by the ingestion of contamination water or direct faecal-oral transmission (Ortega, 1997). Transmission can also occur by, the ingestion of contaminated food, direct person-person contact and certain sexual practices (Jakubowski, 1988).

The cyst have a relatively high resistance to chlorination and UV treatments, which aids in its transmission *via* water along with its ability to survive long time periods in water (Adam, 2001; DeRegnier, 1989; Isaac-Renton, 1993; Kakubayashi, 1998; Ortega, 1997; Rose, 1999). A study by DeRegnier (1989) found *G. intestinalis* to be capable of surviving for one to three months in lake water <10°C. The introduction of poorly sanitised wastewater or recently excreted human or animal faecal matter containing *G. intestinalis* cysts into recreational aquatic environments has been reported to cause human giardiasis (Ortega, 1997).

The cysts are found widely distributed in the environmental waterways and in higher densities in water receiving sewage effluent (LeChevallier, 1991). It was recognised in 1970 that water can be a medium for transmission after American travellers from Leningrad had drunk water and became infected (Boreham, 1990). This disease is found worldwide but more common in areas of poor sanitary conditions and water

treatment (Gilman, 1988; Ortega, 1997). The Centre for Disease Control and Prevention estimates that 60% of all giardiasis cases stem from contaminated water (Rose, 1999). In New Zealand over a six year period, 320 out of 1669 (19.2%) waterways tested were found to be positive for *Giardia* cysts (Brown, 1998). A high proportion of the positive locations were extensively populated areas, both urban and rural.

Direct faecal-oral transmission generally occurs in populated areas with inadequate sanitation, or at congregations of young children in a community, such as child-care centres, pre-schools, and kindergartens (Mejewska, 1998). The increase in the number of young children in group-care outside of the family is a major source of children being exposed to infections, at a stage in life of having poor personal hygiene and being more susceptible to infections (Thompson, 2000). Another means of direct faecal-oral transmission can occur in sexual practices involving direct oral-anal contact (Gardner, 2001; Jakubowski, 1988).

Food-borne transmission was first reported in 1979 but is rarely seen, and usually traces to contamination by food handlers and people preparing the food (Adam, 1991; Boreham, 1990; Mejewska, 1998; Rose, 1999).

The zoonotic potential of *G. intestinalis* remains unclear but transmission studies using cysts isolated from infected humans have been shown to be capable of infecting beavers and muskrats (Erlandsen, 1988). Domestic and feral animals worldwide are infected with *G. intestinalis* and the risk these reservoirs pose to humans is unclear. Large numbers of animal reservoirs in New Zealand have been identified (Chivlers, 1998). The occurrence rates of *G. intestinalis* in cattle is 28.4%, sheep 30%, black rats 57.8%, mice 22%, opossums 15.7%, blackbirds 34% and chaffinches 61% (Brown, 1997). The indication of *G. intestinalis* being a zoonotic organism and the large number of animal reservoirs raises the concern of young livestock in large farming communities, and companion animals, like cats and dogs, being an important source for human giardiasis (Buret, 1990; Thompson, 2000). In New Zealand two cities, Palmerston North and Hamilton, showed in 3 to 25% of cats

and dogs excreting faeces containing *G. intestinalis* cysts (Tonks, 1991). In an Australia aboriginal community the genetic characterisation, using the sequence of the 5' end of the 16S rDNA gene, of *G. intestinalis* from humans and dogs inhabiting the same local environment identified four genetically distinct groups (Hopkins, 1997). Two groups, containing human and canine isolates, corresponded to the 'Polish' and 'Belgian' groups (Homan, 1992) respectively while the remaining groups contained only canine isolates. This genetic similarity of some human and dog isolates indicates cross-transmission of *G. intestinalis* between different host species. This division of human and canine *G. intestinalis* isolates by analysis of the glutamate dehydrogenase locus confirmed the division into four groups (Monis, 1998b), termed Assemblages A, B, C and D, identical to those identified by Hopkins *et al.* (1997) supporting the indication of cross-transmission.

Waterborne transmission of *G. intestinalis* between human and animals has been investigated. Using isoenzyme and pulsed-field gel electrophoresis, *G. intestinalis* isolates from water, animal and human sources during a waterborne outbreak of giardiasis, Isaac-Renton *et al.* (1993) determined the isolates to be genetically identical and the source traced upstream of the municipal water supply intake, to beavers.

### 1.3.4 Treatment

There are effective but a limited range of drugs used in the treatment of giardiasis, which raises the concern of the development of drug resistance. Another concern is the adverse effects of the currently available agents (Gardner, 2001).

Quinacrine was initially used as an antimalarial agent in 1930 that was discovered to be effective in the treatment of giardiasis (Gardner, 2001; Upcroft, 2001). Nausea, vomiting, dizziness, headache, skin and mucous membrane discolouration, and patients with a glucose-6-phosphate dehydrogenase deficiency sometimes show toxic psychosis and hemolysis are side effects that have been noted with the use of this agent (Gardner, 2001; Ortega, 1997). This agent also has a possible link with spina

bifida and renal agenesis and is therefore not used on pregnant patients (Gardner, 2001). Quinacrine is effective when given in a 5-10 day course with dosage usually 100 mg three times daily for adults and 6 mg/kg/day in three doses for children (Gardner, 2001).

The nitroimidazoles are a class of antibiotic agents including metronidazole, tinidazole, ornidazole, and secnidazole and are very effective against *G intestinalis* (Gardner, 2001). Metronidazole is activated when it is reduced by the trophozoite pyruvate ferredoxin oxidoreductase and the reduced metronidazole binds to DNA resulting in the loss of the helical structure, strand breakage and trophozoite death (Adam, 2001; Gardner, 2001). Metronidazole can be used in place of quinacrine and is found to be absorbed into the patient's body. Metronidazole has been shown to have carcinogenic properties with bacteria and in rodents, along with the rapid absorption into the circulation of a foetus and with its mutagenic properties, it is a treatment not recommended for use on pregnant patients. (Adam, 2001; Gardner, 2001; Ortega, 1997). Treatment is highly effective when given over 5-10 days with doses of 250 mg/dose, two to three times a day, or as a single dose therapy of 2 g/dose for one to three days and most patients respond to a single course of treatment (Gardner, 2001; Upcroft, 2001).

Furazolidine is a less effective treatment but is commonly used in children (Ortega, 1997). However furazolidine has been shown to be mutagenic in bacteria and causes mammary tumors in rats, and pulmonary tumours in mice when given in high doses (Gardner, 2001).

Paromomycin is poorly absorbed and almost all is excreted in the faeces, so a foetus is likely to not be exposed to this drug (Gardner, 2001). Paromomycin works by inhibiting protein synthesis in *G. intestinalis* by interference with the 50s and 30s ribosomal sub-units, resulting in mis-reading of mRNA (Gardner, 2001). The efficacy of the drug is less than that seen with metronidazole but because of the possible teratogenic effects of the other drugs, paromomycin is commonly used in pregnant patients (Ortega, 1997).

## 1.4 Molecular Analysis

Due to the difficulties of taxonomy and species analysis by classical techniques, research using molecular techniques will hopefully shed light on these questions. *G. intestinalis* isolates have been separated into two major groups, by molecular analysis, named "Assemblage A" and "Assemblage B", which encompasses four genetic groups (Lu, 1998; Paintlia, 1998; Thompson, 2000). Assemblage A also known as "Polish" by the European community, is equivalent to isoenzyme groups I and II, and small subunit rRNA sequence groups 1 and 2, while assemblage B, also known as "Belgian", equates to isoenzyme groups III an IV, and small subunit rRNA sequence group 3 (Paintlia, 1998; Paintlia, 1999).

The first molecular study of *G. intestinalis* was the analysis of the migration of six metabolic enzymes through a starch gel in the presence of an electric field (Adam, 2001). This isoenzyme analysis is based on the primary amino acid sequence of the enzymes, which effects the migration in the gel. The generation of different banding patterns, or zymodemes, would be due to differences in the genes of the enzymes. An experiment examining 27 enzyme loci by electrophoresis in a cellulose acetate gel has shown two major groups, each comprising of two genetic groups (Andrews, 1989; Mayrhofer, 1995). Isoenzyme analysis has shown heterogeneity among *G. intestinalis* isolates, with no relation to geographical location or host species (Isaac-Renton, 1988). The implication that the species is extremely variable, or different species within the same complex has been proposed but not confirmed (Upcroft, 1998).

Pulse field gel electrophoresis (PFGE) has karyotyped protozoa at the species and sub-species level and analysis of *G. intestinalis* has detected five chromosomes/nuclei and a genome size of 1.2 x $10^7$ bp (Adam, 1991; Farthing, 1997). Field inversion gel electrophoresis (FIGE) has demonstrated the presence of seven chromosomes (Ionas, 1994) but the use of these techniques in the classification of the organism has limited value due to the occurrence of frequent chromosome rearrangements that have been reported (Adam, 2001; Le Blancq, 1991). A study by Sarafis and Issac-Renton (1993) using PFGE to biotype *G. intestinalis* of 20 isolates grouped eleven from a single waterborne outbreak into one biotype which included one non-outbreak isolate, while the remaining isolates showed no correlation between geographical location and biotype.

Sequence analysis of the 16S rDNA has shown a distinct three-nucleotide sequence that separates isolates into two major genotypes; at position 22 of the rRNA. The sequence "GCG" indicates the "Polish" genotype, and "AUC" the "Belgian" genotype (van Keulen, 1995). Sequencing of the ribosomal DNA (rDNA) has occurred using isolates from around the world and analysis of this has divided the species into the two groups recognised by other studies (Monis, 1998a; van Keulen, 1995; Yong, 2000). The sequence of a 292 bp region of the 5' end of the small subunit rRNA gene of human and canine faecal samples that separated the isolates into four groups (Hopkins, 1997) that make up the two major groups (Polish and Belgian) proposed by van Keulen *et al.* (1995).

Sequencing of the glutamate dehydrogenase gene shows 89% nucleic acid identity between isolates from the two major assemblages, A and B, and 81% for triose phosphate isomerise gene, which are less than those identities seen in homologous genes from two different species of another protozoan parasite, *Entamoeba* (Paintlia, 1998). Weiss *et al.* (1992) showed that the equivalent of 1 organisms DNA was sufficient template for analysis and that a pure DNA sample was not required for PCR based systems making it very sensitive and specific.

Random amplification of polymorphic DNA (RAPD) analysis to group isolates of *G. intestinalis* has been shown to separate them into the two major groups seen by isoenzyme analysis studies (Paintlia, 1999). Although this is a PCR based system the requirement for a pure DNA sample is essential, as the primers used are non-specific.

A study using five axenic cultures of *G. intestinalis* from different hosts, by Archibald *et al.* (1991), that had previously been shown to be closely related from restriction enzyme analysis (REA), revealed genetic differences between the strains indicating the possibility of a species complex as seen with isoenzyme analysis.

A study by Nash *et al* (1985) using REA distinguished two major groups with ethidium bromide staining of eight of 15 isolates from a diverse range of hosts and geographical locations. Using southern blot analysis identified two major groups comprised of nine different banding patterns from the 15 isolates with no relationship to host or geographical location, except for two identical banding patterns from sisters.

The analysis of 5 isolates of *G. intestinalis* using REA revealed them to be identical, but using M13 DNA as a probe has been shown to be more discriminating than REA, finding the isolates were not genetically identical (Archibald, 1991). The use of random probes for restriction fragment length polymorphism (RFLP) analysis has identified three groups named group 1, group 2, and group 3 (Adam, 2001; Ey, 1992).

Isoenzyme analysis, REA, and RFLP analysis have showed human and animal isolates to be very similar or even identical, indicating that man and other mammals do not have their own unique isolates i.e. zoonotic potential of *G. intestinalis* (Farthing, 1997; Nash, 1985; Upcroft, 1990). The genetic diversity can be explained by the plasticity of the genome, as over time *in vitro* cultures have been shown to produce differences in their DNA fingerprints and isoenzyme zymodemes (Farthing, 1997; Stranden, 1990). This variation in the isolates has also been seen in an

infection study by Visversvara *et al.* (1988) that showed there were variations in human isolates in terms of their infectivity to gerbils.

The characterisation of *G. intestinalis* isolates by the above studies all made use of *in vitro* or *in vivo* culturing. The production of axenic cultures is difficult and time consuming, but also introduces selective bias for those isolates that perform better *in vitro*. In New Zealand Brown *et al.* (1992) reports establishing eight culture lines from 129 attempts, demonstrating the difficulty with establishing axenic cultures. Over a ten-year period study by Hopkins *et al.* (1997) no *in vitro* cultures of *G. intestinalis* isolated from canine hosts were established. It has been seen that greater genetic heterogeneity is found in *G. intestinalis* isolates not maintained by axenic culture than axenic isolates (Mayrhofer, 1995), however the isolates were inoculated into suckling mice, which introduces a selective bias for those isolates better adapted to survive in mice. Techniques that rely on *in vitro* or *in vivo* culturing limits the characterisation and comparison of genotypic attributes of *Giardia* and ideally molecular analysis would be best performed on *Giardia* cysts directly isolated from infected hosts.

## 1.5 Aims

As the zoonotic potential of *G. intestinalis* still remains unclear and the use of *in vitro* or *in vivo* culturing for current molecular analysis of *G. intestinalis* may not completely represent all isolates due to the difficulties associated with establishing cultures, this thesis has three major goals:

1 To examine the use of DNA fingerprinting techniques, amplified fragment length polymorphism (AFLP) and randomly amplified polymorphic DNA (RAPD) analysis, on DNA extracted directly from cysts of *G. intestinalis.* These fingerprinting methods will be examined for their use in the direct molecular analysis of cysts of *G. intestinalis* in order to eliminate the bias

selection placed on the research using axenically cultured or animal passaged isolates, which limits the number of isolates that can be analysed.

2 Axenic culturing of *G. intestinalis* may place another selection process on the genotyping of the isolates, so ability to alter the DNA fingerprints by growing axenic cultures under the selective pressure of metronidazole will be examined. Since metronidazole is the main treatment for giardiasis, use of this would be the most effective selective pressure for *Giardia*. AFLP and RAPD fingerprints will compare these trophozoites to the wild type trophozoites, to examine the effect of growth of *G. intestinalis* under selective pressure.

3 The rDNA sequence analysis performed by Hunt (1999) will be used to characterise a larger population of human and bovine isolates of *G. intestinalis* from the Waikato and Manawatu regions of New Zealand. Like most eukaryotes, *G. intestinalis* contains a set of tandemly repeated units containing the ribosomal DNA operon, with each trophozoite containing approximately 63 copies of the operon (Weiss, 1992). This tandem repetition made the PCR amplification of this operon more successful than other single copy loci.

These aims of the thesis examine the use of DNA fingerprinting techniques to aid in the understanding of the zoonotic potential of *G. intestinalis* and along with the rDNA sequence analysis utilise DNA extracted from the cysts to eliminate the use of cultures.

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 Collection of *Giardia* for DNA Extraction

### 2.1.1 Reviving Cryopreserved *Giardia intestinalis* Trophozoites

**Materials:**

- TY1-S-33 media (See Appendix A)
- Cryopreserved *G. intestinalis* culture

**Method:**

A cryopreservation tube was removed from liquid nitrogen storage, thawed quickly at 37°C, and the contents transferred into a tissue culture flask. The tissue culture flask was overfilled with pre-warmed TY1-S-33 media to ensure that all air bubbles were removed. The cryopreservation tube was washed with TY1-S-33 media and poured into a second tissue culture flask and both flasks were incubated at 37°C for 1 hour. The media was then replaced with fresh TY1-S-33 media to remove traces of Dimethyl sulphoxide (DMSO) and incubated at 37°C.

### 2.1.2 Maintenance of *Giardia intestinalis* Culture

**Materials:**

- *G. intestinalis* culture
- TY1-S-33 media

**Method:**

Every three to four days the media was replace with fresh media, pre-warmed to 37°C, and every week the trophozoites were sub-cultured by detaching the cells from the culture flasks by half filling the flask with cold TY1-S-33 media (4°C) and chilled on ice for 10 minutes. The culture was then divided into two new flasks,

completely filled with warm TY1-S-33 medium to ensure that all air bubbles were removed and incubated at 37°C.

### 2.1.3 Harvesting of *G. intestinalis* Trophozoites

**Materials:**

- *G. intestinalis* culture
- TY1-S-33 media
- 0.2 M EDTA (pH 7.2, see Appendix A)
- 1.0 M Tris HCl (pH 7.5, see Appendix A)
- Phosphate Buffered Saline (PBS, see Appendix A)
- Tris EDTA (TE) Buffer (See Appendix A)

**Method:**

A complete monolayer of trophozoites was harvested for DNA extraction, after approximately three to four days' growth.

The trophozoites were detached by half filling the flask with cold (4°C) TY1-S-33 media and it on ice for 10 minutes. A sharp firm tap on the flask facilitated detachment of the trophozoites and was monitored at 40x magnification using an inverted microscope. The trophozoites were transferred into a 50 mL conical centrifuge tube and pelleted by centrifugation at 1080x g for 10 minutes at room temperature. The supernatant was decanted and the pellet resuspended in 10 mL PBS. The trophozoites were twice more centrifuged and resuspended in PBS, to remove traces of growth media, and the pellet was resuspended in 1 mL of TE buffer for DNA extraction.

### 2.1.4 Cryopreservation of *G. intestinalis* Trophozoites

**Materials:**

- TY1-S-33 Growth media (pH 7.0)
- Dimethyl sulphoxide (DMSO) 15% (v/v) in TY1-S-33
- *G. intestinalis* culture

**Methods:**

Cultures of *G. intestinalis* trophozoites were harvested from tissue culture flasks by removing the medium, half filling the flask with fresh cold TY1-S-33 media (4°C), and placed on ice for 10 minutes to detach the trophozoites from the flask. The detached trophozoites were transferred into a 50 mL conical centrifuge tube and collected by centrifugation at 1080x g for 10 minutes. The supernatant was removed and the trophozoites were resuspended in 5 mL of fresh medium and 5 mL of 15% DMSO was slowly added to the cell suspension with constant shaking to give a final DMSO concentration of 7.5%. The trophozoites were then dispensed in 1 mL aliquots into 1.5 mL cryopreservation tubes (NUNC) and frozen slowly overnight at –80°C, then placed into liquid nitrogen storage for up to six months.

### 2.1.5 Screening of Faecal Samples for *G. intestinalis* Cysts

**Materials:**

- Distilled water
- Methanol
- Merifluor® Cryptosporidium/Giardia (Meridian Diagnostics, Inc.)
  - 20x Wash buffer
  - Detection reagent (FITC labelled anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies)
- Fluoprep Mounting Medium (bioMérieux)
- Three well SuperStick Slides (Waterborne, Inc.)

**Detection Reagent (Merifluor®)**

The monoclonal antibody was diluted 10 fold in sterile PBS and stored at 4°C.

**Method:**

A 2-5 g faecal specimen was emulsified in 1 mL of distilled water and was left to settle for two min at room temperature to remove the large debris. A 50 µl aliquot was placed on a SuperStick Slide (Waterborne, Inc.) and air dried at 37°C until dry (approximately 2 hours). A 100 µl aliquot of absolute methanol was placed on the slide to fix the specimen and left at room temperature until dry. A 20 µl aliquot of the diluted monoclonal antibody was spread over the sample and incubated at room temperature in a dark humidity chamber for 20 minutes. The slide was washed with 1x wash buffer to remove excess antibody and a drop of fluroprep was placed on the slide, covered with a coverslip, and sealed with nail polish to prevent movement of the coverslip. The sample was viewed on an epifluorescent microscope at 200x magnification for the appearance of *Giardia* cysts, characterised by an apple-green fluorescence approximately 5-9 µm in size.

### 2.1.6 Sucrose Flotation Recovery of Giardia from Faecal Specimens.

**Materials:**

- 0.01% (v/v) Tween 20
- 1.0 M sucrose (See Appendix A)
- PBS

**Method:**

A 10-15 g fecal specimen sample of faeces was emulsified in 45 mL of 0.01% Tween 20 and centrifuged at 1500x g for 10 minutes and the supernatant was removed and the process repeated four more times to remove debris other than the cysts. The washed pellet was then resuspended in 35 mL of 0.01% Tween 20 and underlaid with 10 mL of 1.0 M sucrose, taking care not to mix the two phases. The sample was centrifuged at 1000x g for 10 minutes at room temperature with the brake off. After centrifugation 15 mL was removed from the interface, containing the *Giardia* cysts, and placed into a new 50 mL conical tube. The cysts were then collected by centrifugation at 1500x g for 15 minutes at room temperature, resuspended in 1 mL of PBS and transferred to a 1.6 mL microcentrifuge tube for DNA extraction (Section 2.2.3).

### 2.1.7 Immuno-Magnetic Separation (IMS)

**Materials:**

- Dynabeads® G-C combo kit (Dynal®)
  - Dynabeads® Giardia-combo
  - 10x SL™-Buffer A
  - 10x SL™-Buffer B
- *G. intestinalis* positive faecal specimens

**Faecal Specimens for AFLP and RAPD Analysis:**

| Isolate Tested | Isolate Origin |
|---|---|
| H77 | Waikato Human Faecal Specimen. Received 6 December 1999. |
| H80 | Waikato Human Faecal Specimen. Received 21 January 2000. |
| C14 | Manawatu Calf Faecal Specimen. Collected 10 September 1999. |
| C16 | Manawatu Calf Faecal Sepcimen. Collected 10 September 1999. |

**Method:**

Approximately 15 g of faeces was resuspended in 7 mL of water to which 3 mL of diethyl ether was added and shaken for 30 secs, after 15 secs the lid was opened to release any pressure. The faecal specimen was centrifuged at 750x g for 1 minute at room temperature and the lipid plug that formed at the top of the tube was removed and discarded. The faecal specimen was placed at 37°C for 15 minutes (lid open) to remove excess diethyl ether as it interferes with the recovery of the immunomagnetic beads. Aliquots of 800 µl were placed into twelve 1.6 mL microcentrifuge tubes for AFLP analysis, and IMS was carried out using 20 µl of Dynabeads® (Dynal®) following the manufacturers protocol, with an additional washing step to remove faecal matter. Cyst isolation for RAPD analysis was conducted using the same procedure but only performed twice for each isolate. The cysts were detached from the immunomagnetic beads by incubating the cyst/bead complex in 0.1 M HCl for 10 minutes. The cysts from each faecal isolate were pooled and centrifuged at 10 000x g for 15 minutes at room temperature to collect the cysts for used for DNA extraction (Section 2.2.2).

## 2.2 DNA Extraction from *Giardia*

### 2.2.1 DNA Extraction from Trophozoites

**Materials:**

- Harvested *G. intestinalis* trophozoite
- 10% (w/v) Sodium Dodecyl Sulphate (SDS, BDH)
- Pronase Type XIV 10 mg/mL (Sigma )
- RNase (Ribonuclease A) 2 mg/mL (Sigma)
- 5.0 M Sodium Chloride (BDH)
- 5.0 M Sodium Perchlorate (APS Ajax Finechem)
- Saline Tris-EDTA (STE) Buffer (10x, See Appendix A)
- Phenol/Chloroform/Iso-amyl Alcohol (25:24:1)
- 3.0 M Sodium Acetate (pH 5.5)
- Absolute Ethanol

- 70% Ethanol
- TE Buffer

**RNase (2mg/mL)**

| | |
|---|---|
| Ribonuclease A | 2 mg |
| Sterile Milli-Q Water to | 1 mL |

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

**Phenol/Chloroform/Iso-amyl Alcohol**

| | |
|---|---|
| Phenol | 25 mL |
| Chloroform | 24 mL |
| Iso-amyl Alcohol | 1 mL |

The solution was prepared and saturated with 10x STE buffer by mixing by inversion and stored at 4°C.

**Method:**

The resuspended trophozoites (section 2.1.5) were incubated at 60°C in the presence of 0.1 mL SDS (10% w/v) and 0.1 mL Pronase Type XIV for 3 hours. A 0.1 mL aliquot of RNase was added to the lysate and incubated at 37°C for 1 hour, an aliquot of 5.0 M sodium percholate was added to give a final concentration of 1.0 M and incubated at 60°C for 30 minutes.

The DNA was isolated from the cellular debris by adding an equal volume of phenol/chloroform/isoamyl alcohol solution, mixing by inversion for 1 minute, followed by centrifugation at 10 000x g for 5 minutes at room temperature. The upper aqueous layer was removed with care not to disturb the interface and placed into a new tube. This isolation was repeated twice more or until the interface was clear.

The DNA was then precipitated by mixing with one-tenth the volume of 3.0 M sodium acetate (pH 5.5) and 2.5 volumes of cold (4°C) absolute ethanol, and incubated at –20°C overnight. The solution was centrifuged at 10 000x g for 30 minutes at 4°C to pellet the DNA and the supernatant was discarded. The pellet was washed with 1 mL of cold (4°C) 70% ethanol air dried at 37°C, and resuspended in 1 mL of sterile TE Buffer and stored at 4°C.

### 2.2.2 DNA Extraction from *Giardia* Cysts for AFLP and RAPD analysis

**Materials**:

- 2x Lysis Buffer (pH 7.5)
- RNase 2 mg/ml (Sigma)
- *G. intestinalis* cysts (Section 2.1.7)

**2x Lysis Buffer**

| | |
|---|---|
| 1.0 M Tris HCl | 0.4 mL |
| 0.2 M EDTA | 0.2 mL |
| 10% SDS | 2.0 mL |
| 5.0 M NaCl | 0.2 mL |
| Sterile Milli-Q Water up to | 10.0 mL |

The solution was adjusted to pH 7.5 with NaOH and stored at room temperature until required.

**Method:**

Cysts isolated from faeces by immunomagnetic separation (Section 2.1.7) were resuspended in 100 µl of distilled water and 100 µl of 2x SDS lysis buffer. The cysts were then frozen in liquid nitrogen for 1 minute and quickly thawed at 56°C for 1 minute to disrupt the cyst wall. This cycle was repeated twice more and then incubated at 56°C for 1 hour aid in the lysis of the cysts. After incubation 10 µl of RNase was added to the lysate and incubated at 37°C for 1 hour. The DNA was then isolated by adding an equal volume of phenol/chloroform/isoamyl alcohol solution,

mixing by inversion for 1 minute, and centrifuged at 10 000x g for 5 minutes at room temperature. The upper aqueous layer was removed with care not to disturb the interface and placed into a new tube. This phenol/chloroform/isoamyl alcohol isolation was repeated twice more or until the interface was clear.

The DNA was precipitated by adding one-tenth the volume of 3.0 M sodium acetate (pH 5.5), and 2.5 volumes of cold (4°C) absolute ethanol and incubated at –20°C overnight. The solution was centrifuged at 10 000x g for 30 minutes to pellet the DNA and the supernatant was discarded. The pellet was washed with 1 mL of cold (4°C) 70% ethanol, air dried at 37°C and resuspended in 5 µl of sterile Milli-Q water. The DNA was then dialysed on a 0.025 µm MF-Millipore™ Membrane Filter (Millipore) against sterile TE buffer to remove any inhibitors, and used for AFLP or RAPD analysis (Sections 2.3 and 2.5).

### 2.2.3 DNA Extraction from *Giardia* Cysts for rDNA PCR Analysis

**Materials:**

- 1% Nonidet P40 (BDH) in TE Buffer
- 20% (w/v) Chelex® 100 Resin (BioRad)

**Method:**

Cysts that had been isolated by sucrose flotation (Section 2.1.6) were collected by centrifugation at 10 000x g for 15 minutes, the supernatant discarded and the cysts resuspended in 100 µl of 1% Nonidet P40 and 20 µl of 20% Chelex. The cysts were frozen in liquid nitrogen for 1 minute and thawed in boiling water for 1 minute, this was repeated at least three times to release the DNA. After the DNA extraction the sample was centrifuged at 10 000x g for 2 minutes at room temperature and the supernatant was transferred to a new tube with care not to transfer any of the pellet as the Chelex inhibits PCR amplification. The DNA was diluted 10-fold for PCR amplification (section 2.4 and 2.7.2).

### 2.2.4 Trophozoite DNA Concentration Determination by Spectroscopy

**Materials:**

- *G. intestinalis* trophozoite DNA (Section 2.2.1)
- TE buffer (See Appendix A)
- Shimadzu UV-160A automatic scanning spectrophotometer

**Method:**

The concentration of genomic DNA from trophozoites was determined by measurement of the optical density of DNA diluted 1:100 in TE buffer using a quartz curvette in a Shimadzu UV-160A automatic scanning spectrophotometer.

Optical density was measured at 230nm, 258nm, 260nm, 270nm, 280nm, and 300nm. Ratios of 260/230 = 1.8 to 2.5 and 260/280 = 1.8 to 2.0 indicated a satisfactory level of purity of the DNA. If the optical density was greater at 270nm than at 260nm, then the DNA was ethanol precipitated a second time and re-assayed, as this reading indicated phenol contamination.

The concentration of the DNA was calculated using the following equation:

$$\text{Concentration (mg/mL)} = \frac{\text{Optical Density}_{(258nm)} - \text{Optical Density}_{(300nm)} \times \text{Dilution factor}}{20}$$

The DNA was then diluted to the concentration of 1 mg/mL and used for AFLP analysis.

## 2.3 PCR Amplification of *Giardia intestinalis* DNA with Giardia Specific (Gsp) and *Giardia intestinalis* (GI) Primers.

**Materials:**

- *Giardia* DNA (Section 2.2.2)
- Gsp Primers
- GI Primers

**GspL Primer**

$^{5'}$CAT AAC GAC GCC ATC GCG GCT CTC AGG AA

**GspR Primer**

$^{5'}$TTT GTG AGC GCT TCT GTC GTG GCA GCG CTA

**GIL Primer**

$^{5'}$AAG TGC GTC AAC GAG CAG CT

**GIR Primer**

$^{5'}$TTA GTG CTT TGT GAC CAT CGA

**Method:**

Each DNA sample was amplified using a 2 µl aliquot of DNA extracted from cysts of *G. intestinalis* (section 2.2.3). The DNA was amplified using 1x Taq polymerase reaction buffer, 0.1 mM of each dNTP, 1 pmol each of each primer, 1.25 mM $MgCl_2$, 2x Q solution, and 1 unit of DNA Taq polymerase (Qiagen$^{TM}$) and made to a final volume of 20 µl with sterile Milli-Q water.

The PCR reaction was performed in a Perkin Elmer GeneAmp® PCR System 9700 programmed for 2 minutes at 98°C, 30 seconds at 60°C, 30 seconds at 72°C; then 40 cycles of: 30 seconds at 98°C, 30 seconds at 60°C, and 30 seconds at 72°C; To complete the PCR amplification a final extension of 5 minutes at 72°C was used and then held at 4°C until required using the 9600 ramping speed.

The amplified products were examined on a 1.6% (w/v) agarose gel/1x E buffer as described in Appendix B.

## 2.4 Amplified Fragment Length Polymorphism (AFLP) Analysis

(See Appedix A, Figure 3.37)

### 2.4.1 Digestion and Adaptor Ligation

**Materials:**

- *Eco*RI (Boehringer Mannheim)
- *Mse*I (GibcoBRL)
- *Giardia* Genomic DNA (Section 2.2.1 and 2.2.2)
- T4 DNA Ligase (GibcoBRL)
- EcoRI Adaptor
- MseI Adaptor

**EcoRI Adaptor**

EcoRI Oligonucleotide I

$^{5'}$CTCGTAGACTGCGTACC

EcoRI Oligonucleotide II

$^{5'}$AATTGGTACGCAGTCTAC

EcoRI Adaptor

$^{5'}$CTCGTAGACTGCGTACC

CATCTGACGCATGGTTAA

The EcoRI adaptor was prepared by adding 500 pmol of each oligonucleotide to 45 µl of sterile TE buffer and made up to a final volume of 100 µl with sterile Milli-

Q water. The solution was heated at 95°C for 4 minutes and then left to cool to room temperature on the bench.

**MseI Adaptor**

MseI Oligonucleotide I

$^{5'}$GACGATGAGTCCTGAG

MseI Oligonucleotide II

$^{5'}$ TACTCAGGACTCAT

MseI Adaptor

$^{5'}$GACGATGAGTCCTGAG

TACTCAGGACTCAT

The MseI adaptor was prepared by adding 5 nmol of each oligonucleotide to 45 µl of sterile TE buffer and made up to a final volume of 100 µl with sterile Milli-Q water. The solutions were heated at 95°C for 4 minutes and then left to cool to room temperature on the bench.

**Method:**

The genomic DNA from trophozoites (Section 2.2.1) was used to prepare three digestion reactions, one using 5 units of *Mse*I, another with 5 units *Eco*RI, and the third with 5 units of *Eco*RI and 5 units of *Mse*I, with each reaction digesting 3 µg of DNA. The digestions were performed in 1x reaction buffer and made to a final volume of 10 µl with sterile Milli-Q water and incubated at 37°C for 3 hours. An aliquot of 5 µl of the digestion reaction was then used for the ligation reaction.

Aliquots of 1 µl of the appropriate adaptor(s) was ligated to 5 µl of the restriction fragment solution in 1x reaction buffer and 1 unit of T4 DNA ligase in a final volume of 10 µl and incubated at 37°C for 3 hours.

### 2.4.2 Pre-amplification

**Materials:**

- PCR Reagents:
  - 10x Taq Polymerase Reaction Buffer (Qiagen™)
  - 5x Concentrated Q-Solution (Qiagen™)
  - Taq DNA Polymerase (5 units/$\mu$l, Qiagen™)
  - Ultrapure dNTP's (2 mM each of dATP, dTTP, dCTP, dGTP, Pharmacia Biotech)
- Sterile Milli-Q Water
- Pre-amplification Primers

**MseI Pre-amplification primers**

$^{5'}$GATGAGTCCTGAGTAAN

N = Nucleotide Base (A, T, C, or G)

**EcoRI Pre-amplification primers**

$^{5'}$GACTGCGTACCAATTCN

N = Nucleotide Base (A, T, C, or G)

**Method:**

The pre-amplification reaction contained 1x Taq polymerase reaction buffer (containing 15 mM $MgCl_2$), 0.25 mM of each dNTP, 1 pmol of each primer, 1 µl of adaptor ligated restriction fragments, 1x Q solution, and 1 unit of DNA Taq polymerase (Qiagen™), made to a final volume of 20 $\mu$l with sterile Milli-Q water.

The pre-amplification was performed in a Perkin Elmer GeneAmp® PCR System 9700 programmed for 19 cycles of: 30 seconds at 94°C, 1 minute at 56°C, and 1 minute at 72°C, then held at 4°C until required, using 9600 ramping speed. The PCR amplified products were then diluted 10 fold with sterile distilled water and used for the selective amplification.

### 2.4.3 Selective amplification

**Materials:**

- PCR Reagents:
  - 10x Taq Polymerase Reaction Buffer (Qiagen$^{TM}$)
  - $MgCl_2$ Solution (25 mM, Qiagen$^{TM}$)
  - Taq DNA Polymerase (5 units/$\mu$l, Qiagen$^{TM}$)
  - Ultrapure dNTP's (2 mM each of dATP, dTTP, dCTP, dGTP, Pharmacia Biotech)
- Sterile Milli-Q Water
- Selective Amplification Primers

**MseI Selective Amplification Primers**

$^{5'}$GATGAGTCCTGAGTAAN$_1$($N_2N_3$)

$^{5'}$GATGAGTCCTGAGTAAN$_4$($N_5N_6N_7$)

$N_1$, $N_2$, $N_3$, $N_4$, $N_5$, $N_6$, and $N_7$ = Nucleotide Bases (A, T, C, or G)

**EcoRI Selective Amplification Primers**

$^{5'}$GACTGCGTACCAATTCN$_a$($N_bN_c$)

$^{5'}$GACTGCGTACCAATTCN$_d$($N_eN_fN_g$)

$N_a$, $N_b$, $N_c$, $N_d$, $N_e$, $N_f$, and $N_g$ = Nucleotide Bases (A, T, C, or G)

**Method:**

The pre-amplified fragments were amplified using the primers identical to those used in the pre-amplification reaction with the addition of two or three additional nucleotides to the 3' end.

An aliquot of 1 µl of the diluted pre-amplified solution was amplified using 1x Taq polymerase reaction buffer (containing 15 mM $MgCl_2$), 0.25 mM of each dNTP, 1 pmol of each primer, 3.125 mM $MgCl_2$, and 1 unit of DNA Taq polymerase (Qiagen$^{TM}$), made to a final volume of 20 $\mu$l with sterile Milli-Q water.

The amplifications were performed in a Perkin Elmer GeneAmp® PCR System 9700 programmed for 94°C for 2 minutes, then 6 cycles of: 30 sec at 94°C, 30 sec at 65°C, and 1 minute at 72°C; 6 cycles of: 30 sec at 94°C, 30 sec at 60°C, and 1 minute at 72°C; 24 cycles of: 30 sec at 94°C, 30 sec at 56°C, and 1 minute at 72°C; then a final elongation step of 72°C for 5 minutes and held at 4°C until required, using 9600 ramping speed.

The selectively amplified products were examined on a 2% (w/v) agarose gel/1x E buffer as described in Appendix B.

### 2.4.4 Polyacrylamide Gel Electrophoresis

**Materials:**

- Formamide Loading Buffer
- 10x Tris Borate EDTA (TBE) Buffer
- 5% Polyacrylamide Gel

**Formamide Loading Buffer**

| | |
|---|---|
| Bromophenol Blue | 0.005 g |
| Xylene Cyanol | 0.005 g |
| Formamide | 9.8 mL |
| 0.5 M EDTA | 0.2 mL |

**10x TBE Buffer**

| | |
|---|---|
| Tris | 108.0 g |
| Boric acid | 54.0 g |
| 0.05 M EDTA (pH 8.0) | 20 mL |
| Milli-Q Water to | 1 000 mL |

**5% Polyacrylamide Gel**

| | |
|---|---|
| Urea (BDH) | 29.4 g |
| Long Ranger Gel Solution (FMC bioproducts) | 7 mL |
| 10x TBE | 7 mL |
| Milli-Q Water to | 70 mL |
| 10% Ammonium Persulphate (GibcoBRL) | 0.35 mL |
| NNN'N'-Tetramethylethylenediamine (TEMED) (GibcoBRL) | 0.035 mLl |

The polyacrylamide gel was prepared by dissolving the urea in 30 mL of water, adding 10x TBE and Long Ranger Gel Solution (FMC Bioproducts), filtered through two pieces of Whatman 1 filter paper and made to a final volume of 70mL with Milli-Q water. The 10% ammonium persulphate and TEMED were added immediately prior to casting the gel. The gel was left overnight to polymerise and pre-electrophoresed in 1x TBE buffer at 55 W for 30 minutes immediately prior to use.

**Method:**

While the gel was being pre-electrophoresed, 6 µl aliquots of each amplified product was denatured with 1 µl of formamide loading buffer by heating in a Perkin Elmer GeneAmp® PCR System 9700 for 4 minutes at 94°C and held at 4°C until required. The samples were loaded and electrophoresed at 45 W for 4 hours, or until the top dye front was approximately 10 cm from the base of the gel, after which the plates were separated for silver staining.

### 2.4.5 Silver Staining and Developing the Polyacrylamide Gel

**Materials:**

- Acetic Acid (10%)
- Staining Solution
- Developing Solution
- Milli-Q Water

**10% Acetic Acid**

| | |
|---|---|
| Concentrated Acetic Acid (BDH) | 400 mL |
| Milli-Q Water to | 4000 mL |

**Staining Solution**

| | |
|---|---|
| Silver Nitrate (BDH) | 3.0 g |
| 37% Formaldehyde (BDH) | 4.5 mL |
| Milli-Q Water to | 3000 mL |

This solution was prepared immediately prior to use.

**Developing Solution**

| | |
|---|---|
| Sodium Carbonate (BDH) | 120 g |
| 37% Formaldehyde (BDH) | 6.5 mL |
| Sodium Thiosulphate 10 mg/mL (BDH) | 0.8 mL |
| Milli-Q water to | 4000 mL |

The sodium carbonate was dissolved in the water and chilled in the freezer while the gel was in the acetic acid. The formaldehyde and sodium thiosulphate were added immediately prior to use.

**Method:**

The glass casting plates were separated and the plate containing the polyacrylamide gel was placed into 4 L of 10% acetic acid and agitated for 2 hours, and rinsed in 2 L of Milli-Q water three times to remove traces of the acetic acid. Half of the acetic acid was retained and placed in the freezer to chill. The polyacrylamide gel was placed in 3 L of the stain solution and agitated for 1 hour. The gel was rinsed briefly by passing it through chilled Milli-Q water, approximately 3 secs, and then placing the gel into half (2 L) of the developing solution. The polyacrylamide gel was agitated in the developing solution until bands began to appear, at which point the gel was transferred into the remaining half of the developing solution and agitated by hand until the gel began to darken. The developing reaction was then stopped by

adding the reserved acetic acid, and left until the effervescence had stopped and was then rinsed in 2 L of Milli-Q water for 5 minutes.

The gel was examined on a white light box and each of the amplified DNA fragments that were excised from the polyacrylamide gel were placed in 50 µl of sterile distilled water and left overnight at 4°C prior to DNA re-amplification.

### 2.4.6 Re-amplification

**Materials:**

- PCR Reagents:
  - 10x Taq Polymerase Reaction Buffer (Qiagen™)
  - 5x Concentrated Q-Solution (Qiagen™)
  - Taq DNA Polymerase (5 units/µl, Qiagen™)
  - Ultrapure dNTP's (2 mM each of dATP, dTTP, dCTP, dGTP, Pharmacia Biotech)
- Sterile Milli-Q Water
- Selective amplification primers

**Method:**

The extracted DNA was re-amplified using the selective amplification primers. Each reaction contained a 1 µl aliquot of DNA extracted from the polyacrylamide gel, 1x Taq polymerase reaction buffer, 0.1 mM of each dNTP, 1 pmol of each primer, and 1 unit of DNA Taq polymerase (Qiagen™), and made to a final volume of 20 µl with sterile distilled water.

The amplifications were performed in a Perkin Elmer GeneAmp® PCR System 9700 programmed for 30 cycles of: 94°C for 30 sec, 60°C for 1 minute, and 72°C for 1 minute; and then held at 4°C until required.

The concentration of the amplicon was determined on a 2% (w/v) agarose gel/1x E as described in Appendix B, prior to cloning.

### 2.4.7 Cloning

**Materials:**

- PGEM®-T EasyVector System I (Promega)
- Subcloning Efficiency™ DH5α Competent Cells (Gibco BRL)
- STET Buffer
- Lysozyme (10 mg/mL, Sigma)
- RNase (2 mg/mL)

**STET Buffer**

| | |
|---|---|
| 5M NaCl | 0.2 mL |
| 1M Tris HCl (pH 7.5) | 0.1 mL |
| 0.2M EDTA | 0.05 mL |
| Triton X-100 (BDH) | 0.5 mL |
| Distilled Water to | 10 mL |

**Method:**

The re-amplified fragments were ligated into the pGEM-T Easy Vector as per the suppliers' instructions to purify the DNA fragment for sequencing. The vectors containing the inserted fragments were then used to transform DH5α cells to amplify the insert. The transformation was carried out using Subcloning Efficiency™ DH5α Competent Cells (Gibco BRL) according to the supplier's details, using LB plates and broth.

A single white colony was selected from the plates and placed in 5 mL LB broth and 100 μg/mL ampicillin and grown overnight with shaking at 150 rpm. A 2.5 mL aliquot of the cell culture was collected by centrifugation at 1080x g for 5 minutes at room temperature, resuspended in 350 μl of STET buffer, 25 μl lysozyme and placed in boiling water for 40 secs. A 10 μl aliquot of RNase (2 mg/mL) was added to the boiled sample and incubated at 37°C for 1 hour. The samples was centrifuged at 10 000x g for 10 minutes at room temperature. The cell debris was removed from the bottom of the centrifuge tube with a sterile toothpick and 40 μl of sodium acetate

and 420 μl of isopropanol were added and incubated at room temperature for 5 minutes. The DNA was collected by centrifuge at 10 000x g for 10 minutes at room temperature. The pellet was washed in 1 mL of 70% ethanol, air dried at 37°C and resuspended in 100 μl of sterile distilled water for sequencing.

### 2.4.8 Sequencing

**Materials:**

- Plasmid Containing Insert (section 2.3.7)
- Forward Sequencing Primer
- Reverse Sequencing Primer

**Forward Sequencing Primer**

5'GCCAGTGAATTGTAATACGA

**Reverse Sequencing Primer**

5'ATGACCATGATTACGCCAAG

**Method:**

The cloned fragments were sequenced at Massey University DNA Analysis Service Sequencing Unit, using the Big Dye Termination Kit (Perking Elmer) in a Perkin Elmer ABI Prism 377 Genetic Analyser. The vectors containing the inserts were supplied at a concentration of 100-200 ng/μl and the primers at 1 pmol/μl.

## 2.5 Random Amplification of Polymorphic DNA (RAPD) Analysis of *G. intestinalis* Isolates

### 2.5.1 Pre-amplification

**Materials:**

- PCR Reagents:
  - 10x Taq Polymerase Reaction Buffer (Qiagen™)
  - Magnesium Chloride solution (25 mM, Qiagen™)
  - Taq DNA Polymerase (5 units/μl, Qiagen™)
  - Ultrapure dNTP's (2 mM each of dATP, dTTP, dCTP, dGTP, Pharmacia Biotech)
- Sterile Milli-Q Water
- RAPD Pre-amplification Primers

**RAPD Pre-amplification Primers**

| | |
|---|---|
| GC50 | $^{5'}$TGGTCACT |
| GC60 | $^{5'}$CGGTCACT |
| GC70 | $^{5'}$CGGCCACT |
| GC80 | $^{5'}$CGGCCCCT |
| GC90 | $^{5'}$CGGCCCCG |
| GC100 | $^{5'}$CGGCCCCG |

The pre-amplification reaction contained 1x Taq polymerase reaction buffer (containing 15 mM $MgCl_2$), 0.25 mM of each dNTP, 1 pmol of each primer, 2 μl of extracted DNA, 1.25 mM $MgCl_2$, and 1 unit of DNA Taq polymerase (Qiagen™), and made to a final volume of 20 μl with sterile Milli-Q water.

The pre-amplification was performed in a Perkin Elmer GeneAmp® PCR System 9700 programmed for 1 cycle of: 2 minutes seconds at 94°C, 30 seconds at 20°C, and 30 seconds at 72°C; 20 cycles of: 30 seconds at 94°C, 30 seconds at 20°C, and 30

seconds at 72°C: then 1 cycle of: 30 seconds at 94°C, 30 seconds at 20°C, and 5 minutes at 72°C and held at 4°C until required, using 9600 ramping speed. The PCR amplified products were then diluted 10 fold with sterile distilled water and used for selective amplification.

### 2.5.2 Selective amplification

**Materials:**

- PCR Reagents:
  - 10x Taq Polymerase Reaction Buffer (Qiagen™)
  - $MgCl_2$ Solution (25 mM, Qiagen™)
  - Taq DNA Polymerase (5 units/$\mu$l, Qiagen™)
  - Ultrapure dNTP's (2 mM each of dATP, dTTP, dCTP, dGTP, Pharmacia Biotech)
- Sterile Milli-Q Water
- RAPD Selective Amplification Primers

**RAPD Selective Amplification Primers**

| | |
|---|---|
| GC50+GT | 5'TGGTCACTGT |
| GC60+GT | 5'CGGTCACTGT |
| GC70+GT | 5'CGGCCACTGT |
| GC80+GT | 5'CGGCCCCTGT |
| GC90+GT | 5'CGGCCCCGGT |
| GC100+GC | 5'CGGCCCCGGC |

**Method:**

The selective primers used were identical to those used in the pre-amplification reaction with the addition of two extra nucleotide bases to the 3' end (eg. Primer GC50+GT is used for the pre-amplification products of primer GC50).

The selective amplification reaction contained 1x Taq polymerase reaction buffer (containing 15 mM $MgCl_2$), 0.25 mM of each dNTP, 1 pmol of each primer, 2 µl of

extracted DNA, 1.25 mM $MgCl_2$, and 1 unit of DNA Taq polymerase (Qiagen$^{TM}$), and made to a final volume of 20 $\mu$l with sterile Milli-Q water.

The selective amplification was performed in a Perkin Elmer GeneAmp® PCR System 9700 programmed for 1 cycle of: 2 minutes at 94°C, 30 seconds at 35°C, and 30 seconds at 72°C; 35 cycles of: 30 seconds at 94°C, 30 seconds at 35°C, and 30 seconds at 72°C: then 1 cycle of: 30 seconds at 94°C, 30 seconds at 35°C, and 5 minutes at 72°C and held at 4°C forever, using 9600 ramping speed.

A 5 $\mu$l aliquot of the PCR products was electrophoresed on a 2% (w/v) agarose/1x E buffer gel as described in Appendix B.

## 2.6 Selective Pressure on *in vitro G. intestinalis* trophozoites

**Materials:**

- *G. intestinalis* culture (See Section 2.1.2)
- TY1-S-33 Growth Media (See Section 2.1.1)
- Metronidazole (1 mg/mL)

**Metronidazole (1 mg/mL)**

| | |
|---|---|
| Metronidazole | 0.001 g |
| TY1-S-33 Growth Media to | 1 mL |

**Method:**

Concentrations of metronidazole; 0.01 mg/L, 0.05 mg/L, 0.1 mg/L, 0.5 mg/L, and 1 mg/l, were used to grow established cultures of *G. intestinalis* to determine a sub-lethal concentration to apply a selective pressure on the trophozoites. A culture that showed confluent growth in a 70 mL tissue culture flask was filled with the media containing the metronidazole and incubated at 37°C for four days, after which the

media was replaced with pre-warmed media lacking metronidazole and grown until confluent growth was seen

A concentration of 0.05 mg/L was initially selected, and the trophozoites were grown for four days, after which the media was replaced with no metronidazole and the cells left for two days or until there was confluent growth was seen. When the trophozoites had adapted to the concentration of metronidazole and confluent growth was detected, the concentration was increased and the method of exposure continued. When the trophozoites had adapted to 1 mg/L metronidazole and cells were harvested (section 2.1.3) and DNA was extracted (section 2.2.1) for AFLP (section 2.3) and RAPD (section 2.5) analysis to compare the "wild type" culture with the metronidazole treated culture.

## 2.7 Genotyping of Human and Bovine Isolates by rDNA Sequence Analysis

### 2.6.1 Faecal Specimens

Faecal specimens from bovine and human sources were collected from the Waikato area for the genotyping study, and screened for *G. intestinalis* as described in section 2.1.5. Positive samples were graded depending on the amount of cysts seen in the samples:

| Grade | Cysts per field of view at 200x magnification |
|---|---|
| 3 | >10 |
| 2 | 5-10 |
| 1 | 1-5 |
| 0 | 0 |

The cysts from the positive faecal specimens were isolated by sucrose floatation (Section 2.1.6) and DNA was extracted, as described in section 2.2.3, for rDNA PCR amplification (Section 2.7.2) and sequence analysis.

### 2.7.2 Amplification of the rDNA loci

**Materials:**

- PCR Reagents:
  - 10x Taq Polymerase Reaction Buffer (Qiagen$^{TM}$)
  - 5x Q Solution
  - $MgCl_2$ Solution (25 mM, Qiagen$^{TM}$)
  - Taq DNA Polymerase (5 units/$\mu$l, Qiagen$^{TM}$)
  - Ultrapure dNTP's (2 mM each of dATP, dTTP, dCTP, dGTP, Pharmacia Biotech)
- Sterile Milli-Q Water
- MAT1 Primer
  $^{5'}$GTCTGTGATGCCCTCAGACG
- MAT2 Primer
  $^{5'}$GGGGCGTATTTAGCCTTGGA
- Cyn0 Primer
  $^{5'}$CGCACCAGGAATGTCTTGTAG
- Cyn2 Primer
  $^{5'}$CCCGGTTGGTTTCTCCTCC
- *G. intestinalis* cyst DNA (Section 2.2.3)

**Method:**

The rDNA loci was amplified using nested primers, first using the MAT1/MAT2 primer combination to amplify a larger product that encompassed the sites for the Cyn0/Cyn2 primer combination. The PCR product was diluted 10-fold for PCR amplification using the Cyn0/Cyn2 primer combination developed by Hunt C. L. (1999).

Each isolate was amplified in duplicate using a 2 µl aliquot of DNA extracted from cysts of *G. intestinalis* (section 2.2.3). The DNA was amplified using 1x Taq polymerase reaction buffer, 0.1 mM of each dNTP, 1 pmol each of MAT 1 and MAT 2 primers, 2x Q solution, and 1 unit of DNA Taq polymerase (Qiagen™) and made to a final volume of 20 µl with sterile Milli-Q water.

The PCR reaction was performed in a Perkin Elmer GeneAmp® PCR System 9700 programmed for 98°C for 2 minutes, 30 seconds at 60.5°C, 60 seconds at 72°C; then 40 cycles of: 30 seconds at 94°C, 30 seconds at 60.5°C, and 1 minute at 72°C; then held at 4°C until required using the 9600 ramping speed. The PCR product was diluted 10 fold and before being used with the Cyn0/Cyn2 primer combination.

A 2 µl aliquot of the diluted MAT PCR product was amplified using 1x Taq polymerase reaction buffer, 0.1 mM of each dNTP, 1 pmol each of Cyn 0 and Cyn 2 primers, 2x Q solution, 1 unit of DNA Taq polymerase (Qiagen™) and made to a final volume of 20 µl with sterile Milli-Q water.

The PCR reaction was performed in a Perkin Elmer GeneAmp® PCR System 9700 programmed for 98°C for 2 minutes , 30 seconds at 60°C, 60 seconds at 72°C; then 45 cycles of: 30 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 72°C; then held at 4°C until required using the 9600 ramping speed

The amplified product was then examined on a 1.6% (w/v) agarose gel/1x E buffer (See Appendix B).

### 2.7.3 PCR Purification

**Materials:**

- rDNA PCR Product
- QIAquick™ Gel Extraction Kit (Qiagen)

**Method:**

The entire sample of the rDNA PCR product was run on a 2% agarose gel to separate the PCR product from other non-specific PCR products and the correct band was excised from the gel and purified through a QIAquick™ Gel Extraction Kit as per the manufacturers instructions. The concentration of the purified product was then determined as described in Appendix B.

### 2.7.4 rDNA Sequencing

**Materials:**

- Purified rDNA PCR Product
- Cyn 0 Primer
- Cyn 2 Primer

**Method:**

The Massey University DNA Analysis Service Sequencing Unit sequenced the purified PCR products using the Big Dye Termination Kit (Perkin Elmer) in a Perking Elmer ABI Prism 377 Genetic Analyser. The purified PCR product was supplied at a concentration of 10-20 ng/μl and the primers at 1 pmol/μl, as described in Appendix B.

### 2.7.5 DNA Sequence Analysis

#### 2.7.5.1 Alignment of rDNA Sequences

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

- MT Navigator, Version 1.0.2b3 (ABI Prism, Perkin Elmer)
  MT Navigator was used to edit the forward and reverse sequences to establish nucleotides that the sequencing equipment could not.
- ClustalX, Version 1.8 (Thompson, 1997)
  ClustalX was used to align all of the rDNA sequences for data analysis.

#### 2.7.5.2 Analysis of rDNA Sequences

The analysis of the rDNA sequences was performed using the following computer software programmes:

- Molecular Evolutionary Genetics Analysis, Version 1.02 (MEGA, Kumar *et al.* 1993)
- PAUP, Version 4.0b8 (Swoftord, 1999)
  PAUP was used to convert the aligned sequences into a format for SplitsTree analysis.
- SplitsTree, Version 2.4
  SplitsTree was used to generate and display the phylogenetic relationship of the isolates.

# CHAPTER 3: RESULTS

## 3.1 DNA Extraction from *G. intestinalis* cysts

A procedure to extract DNA from cysts was required from AFLP and RAPD analysis techniques. DNA extraction from *G. intestinalis* cysts was based on freezing cysts with liquid nitrogen and thawing quickly in a 56°C water bath to disrupt the cyst wall .

Optimisation of the cyst DNA extraction was performed and the addition of SDS and RNase was determined to be effective for the extraction of DNA from cysts that was seen by the presence of high molecular weight DNA with the absence of smearing and RNA (as shown in Figure 3.1).

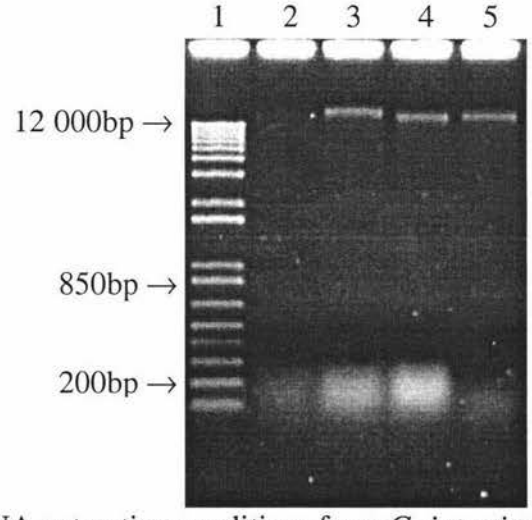



**Figure 3.1:** DNA extraction conditions from *G. intestinalis* cysts were optimised and the DNA was detected in a 1.6% agarose gel by ethidium bromide staining. Lane 1: 1 Kb plus DNA ladder (Gibco BRL); Lane 2: Five cycles of freezing cysts in liquid nitrogen and thawing at 56°C; Lane 3: As lane 2 with the addition of incubation in 1% SDS and 20 µg proteinase K (Sigma) for 3 hours at 56°C; Lane 4: As lane 3 with the removal of proteinase K (Sigma); Lane 5: As lane 4 followed by incubation at for 1 hour at 37°C in the presence of 10 µg RNase (Sigma). The extraction procedure for lane 5 was determined to be optimum for DNA extraction from *Giardia* cysts.

The DNA extracted from faecal specimens was confirmed to be from *G. intestinalis* using two sets of PCR primers. The *Giardia* genus specific (Gsp) primer pair which gives an expected product size of 218bp and *Giardia intestinalis* specific (GI) primers that produces a product of 171bp (as shown in figure 3.2) indicating the successful extraction of DNA from the cysts of *G. intestinalis*. The successful DNA extraction technique for *G. intestinalis* cysts was used to extract DNA from further analysis techniques.

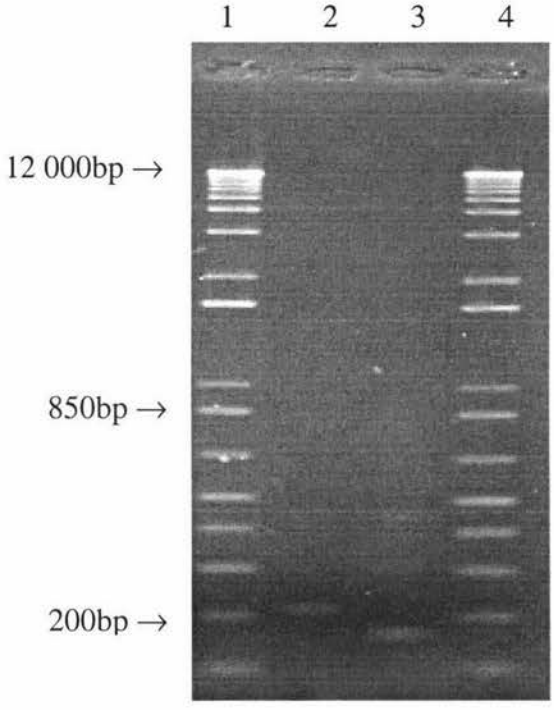



**Figure 3.2:** Confirmation of DNA extraction from *G. intestinalis* using *Giardia* genus specific (Gsp) and *Giardia intestinalis* specific (GI) primers, and visualisation of the PCR products on a 1.6% agarose gel. Lanes 1 and 4: 1 Kb plus DNA ladder (Gibco BRL); Lane 2: PCR amplification of *G. intestinalis* cyst DNA using Gsp primers; Lane 3: PCR amplification of *G. intestinalis* cyst DNA using GI primers. Both primer sets gave the expected band size indicating the DNA extracted was from *Giardia* and specifically *G. intestinalis*.

## 3.2 AFLP Analysis of *G. intestinalis*

AFLP analysis of DNA extracted from five axenic cultures and the cysts of *G. intestinalis* was performed using various different primer combinations to identify polymorphic bands between human and animal isolates. Each sample was performed in duplicate to ensure each sample was producing reproducible banding patterns, and AFLP analysis of *G. intestinalis* cysts was performed on duplicate DNA templates to ensure any polymorphic differences was not due to the analysis procedure itself but differences in the genomic DNA sequences.

No polymorphic differences were detected between the five axenic cultures examined on 2% agarose and 5% polyacrylamide gels, but the DNA extracted from *G. intestinalis* cysts from two human and two bovine isolates, revealed polymorphic differences between each isolate examined. Polymorphic bands were extracted from 5% polyacrylamide gels for re-amplification, cloning and sequencing.

### 3.2.1 Differentiation of in vitro G. intestinalis Cultures by AFLP Analysis

AFLP analysis of *Eco*RI restriction fragments performed on three human and two animal *G. intestinalis* axenic cultures using the Eco-ACG and Eco-ATA selective primers (Figure 3.3). The AFLP banding patterns were found to be identical for each isolate using these selective primers.

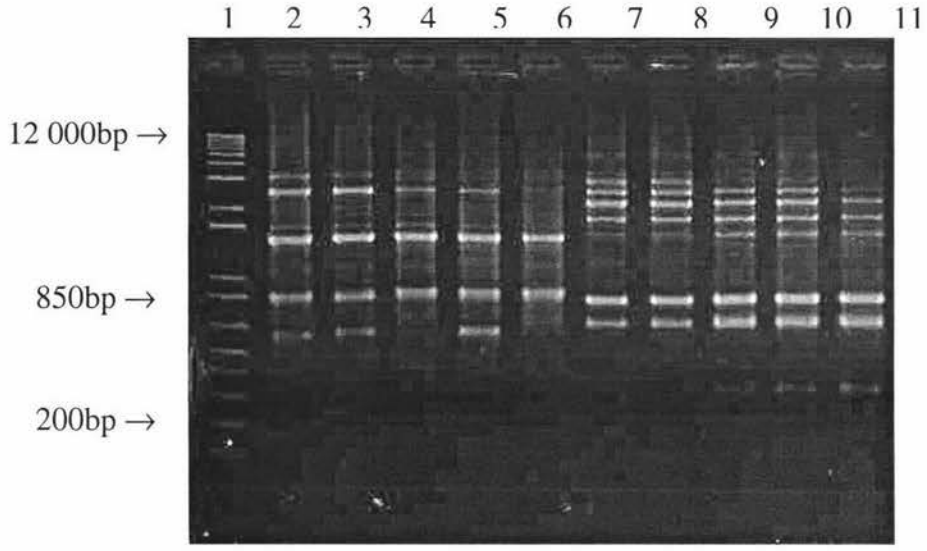



**Figure 3.3:** AFLP analysis of *in vitro* cultures using *Eco*RI digested DNA from *in vitro* cultures of *G. intestinalis* on a 2% agarose gel . Lane 1: 1 Kb plus DNA ladder (Gibco BRL); Lanes 2-6: AFLP analysis using Eco-ACG Primer; Lanes 7-11: AFLP analysis using Eco-ATA primer; Lanes 2 and 7: Beaver isolate (K); Lanes 3 and 8: Human isolate (Iso6); Lanes 4 and 9: Brisbane Human isolate (Bris); Lanes 5 and 10: Human isolate (ATCC); Lanes 6 and 11: Sheep isolate (S2). As each isolate produced an identical banding pattern different primer combinations were tested to detect polymorphisms between the isolates.

AFLP analysis of *Eco*RI restriction fragments, of the same axenic cultures as in figure 3.3, was performed using another set of selective primers to differentiate the isolates. AFLP analysis was performed using Eco-AGC and Eco-ATT selective primers (Figure 3.4) which also produced identical banding patterns between the isolates.

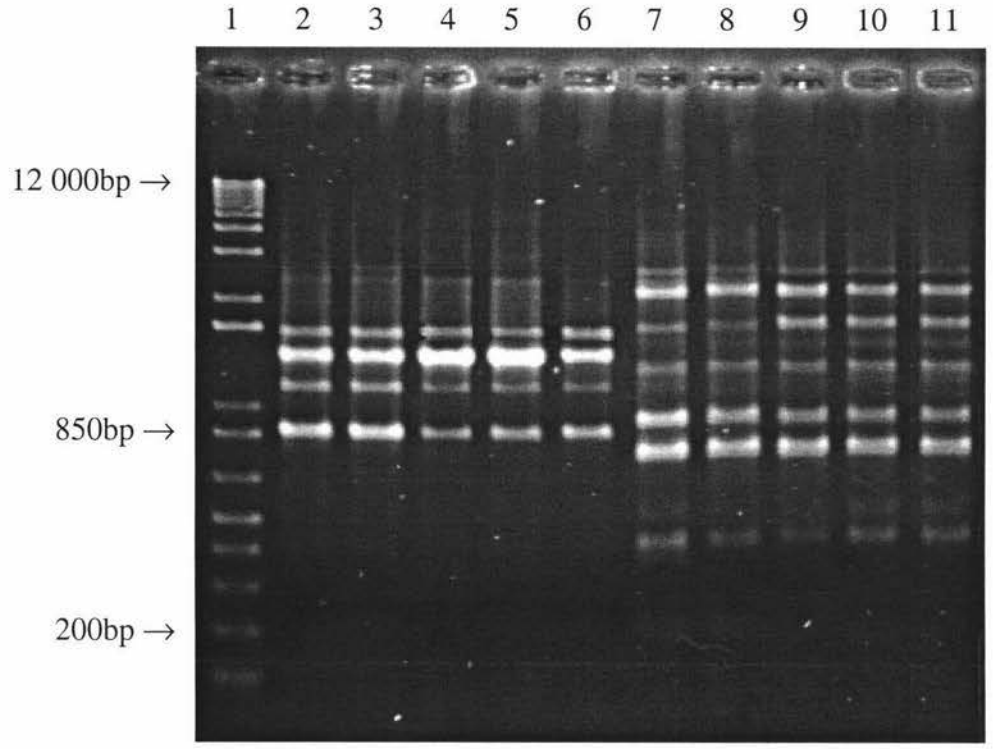



**Figure 3.4:** AFLP analysis using Eco-AGC and Eco-ATT primers of DNA extracted from *in vitro* cultures of *G. intestinalis* on a 2% agarose gel. Lane 1: 1 Kb plus DNA ladder (Gibco BRL); Lanes 2-6: AFLP analysis using Eco-ATT Primer; Lanes 7-11: AFLP analysis using Eco-AGC primer; Lanes 2 and 7: Beaver isolate (K); Lanes 3 and 8: Human isolate (Iso6); Lanes 4 and 9: Brisbane Human isolate (Bris); Lanes 5 and 10: Human isolate (ATCC); Lanes 6 and 11: Sheep isolate (S2). Each isolate produced identical banding patterns so a restriction enzyme recognising a 4 bp sequence (*Mse*I) was used to generate more restriction fragments to increase the likely event of finding polymorphic differences.

*Mse*I was used to produce restriction fragments for AFLP analysis, on the same axenic cultures of *G. intestinalis* as figure 3.3, with different selective primers to detect polymorphisms. AFLP analysis was performed using Mse-CAG and Mse-CTG selective primers (Figure 3.5) which generated identical banding patterns for each isolate.

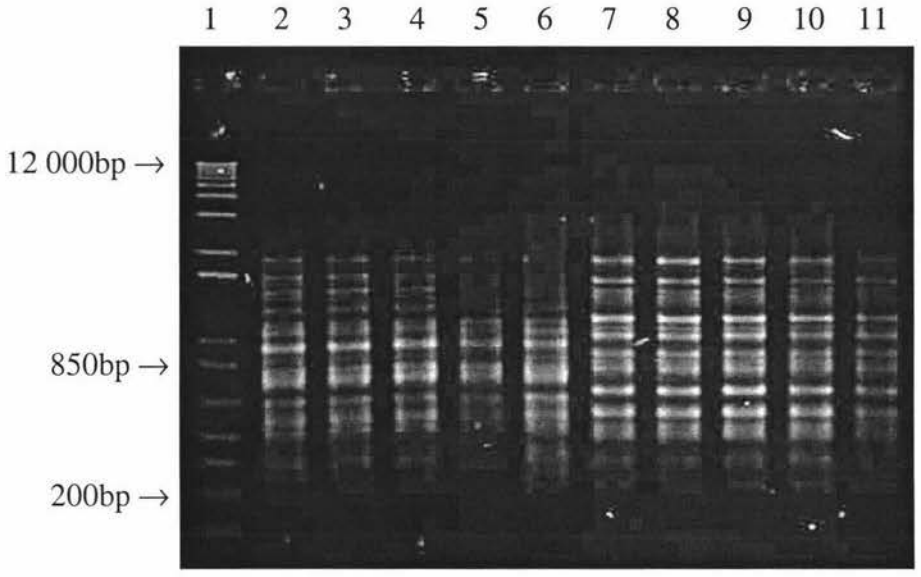



**Figure 3.5:** AFLP analysis using Mse-CAG and Mse-CTG primers of DNA extracted from *in vitro* cultures of *G. intestinalis* visualised on a 2% agarose gel. Lane 1: 1 Kb plus DNA ladder (Gibco BRL); Lanes 2-6: AFLP analysis using Mse-CAG Primer; Lanes 7-11: AFLP analysis using Mse-CTG primer; Lanes 2 and 7: Beaver isolate (K); Lanes 3 and 8: Human isolate (Iso6); Lanes 4 and 9: Brisbane Human isolate (Bris); Lanes 5 and 10: Human isolate (ATCC); Lanes 6 and 11: Sheep isolate (S2). As each isolate produced identical banding patterns different primer combinations were tested to identify polymorphisms.

AFLP analysis was performed on *Mse*I restriction fragments, on the same axenic cultures as in figure 3.3, using Mse-CTA and Mse-CAC selective primers (Figure 3.6) which generated identical banding patterns the isolates.

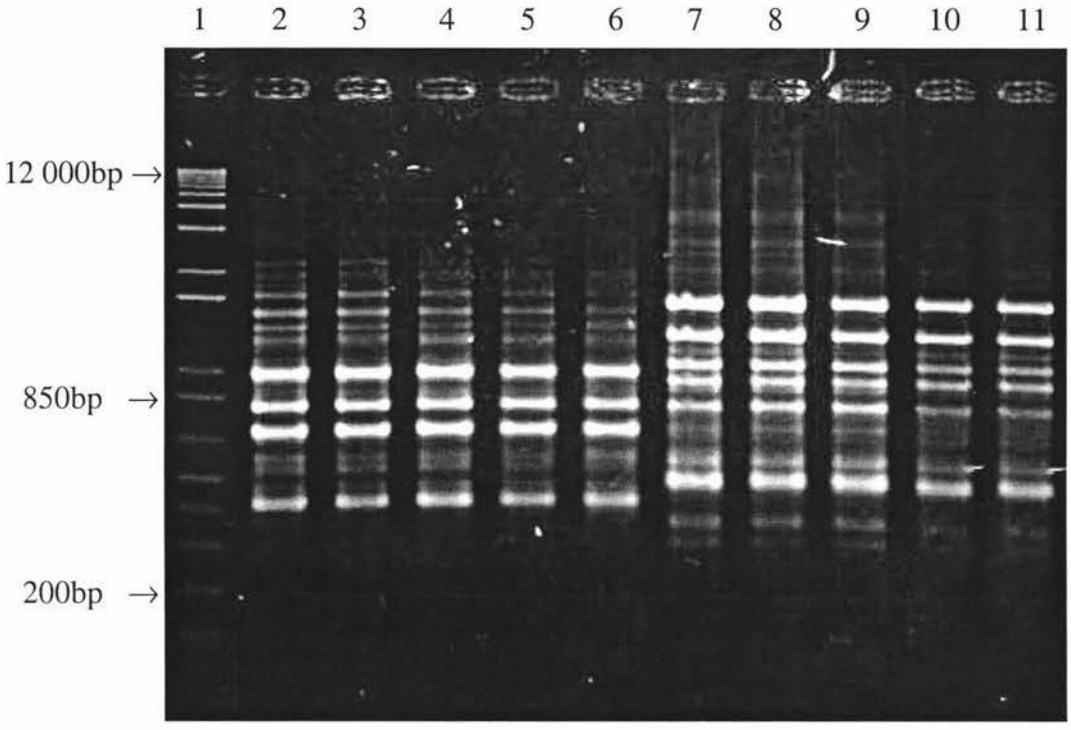



**Figure 3.6:** AFLP analysis using Mse-CTA and Mse-CAC primers of DNA extracted from *in vitro* cultures of *G. intestinalis* on a 2% agarose gel. Lane 1: 1 Kb plus DNA ladder (Gibco BRL); Lanes 2-6: AFLP analysis using Mse-CTA Primer; Lanes 7-11: AFLP analysis using Mse-CAC primer; Lanes 2 and 7: Beaver isolate (K); Lanes 3 and 8: Human isolate (Iso6); Lanes 4 and 9: Brisbane Human isolate (Bris); Lanes 5 and 10: Human isolate (ATCC); Lanes 6 and 11: Sheep isolate (S2). Each isolate produced identical banding patterns so the PCR products were analysed in a 5% polyacrylamide gel to increase the resolution of the banding patterns as all bands may not be detected on the agarose gels with ethidium bromide staining.

Polyacrylamide gel electrophoresis and silver staining were used to increase the resolution of the *Mse*I generated AFLP banding patterns to detect polymorphic differences between two human isolates and one animal isolate. The 5% polyacrylamide gel produced sharp distinct bands but did not reveal any polymorphic differences (Figure 3.7).

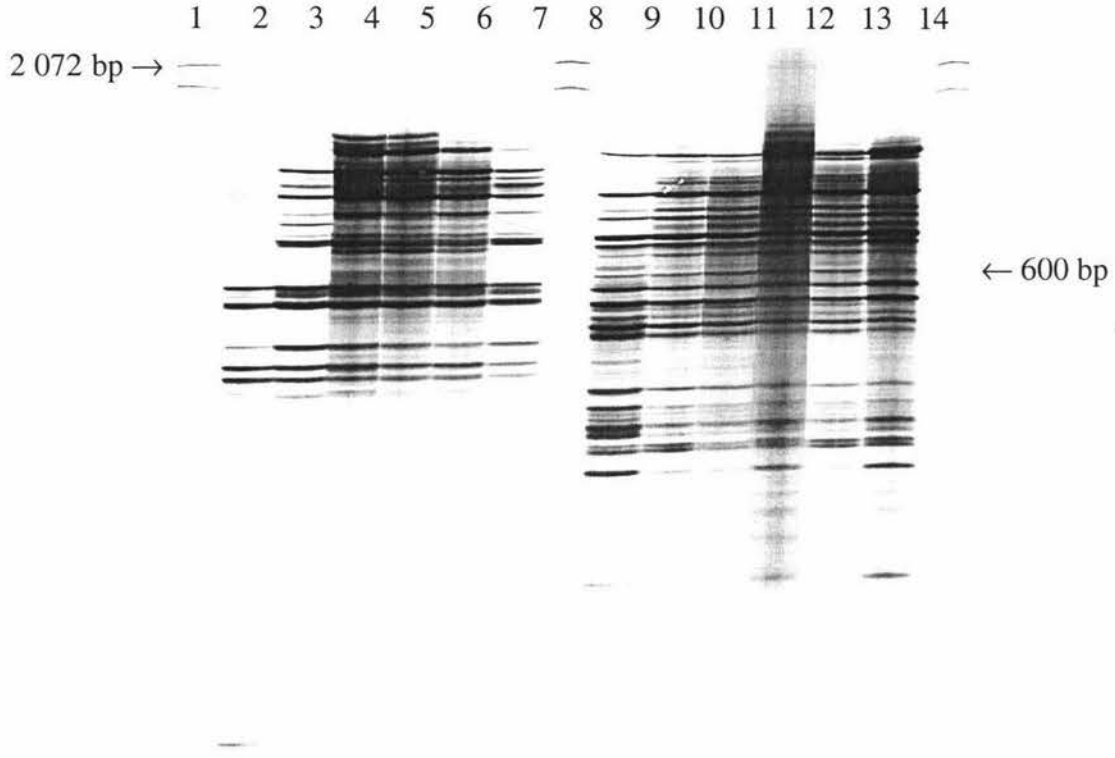



**Figure 3.7:** AFLP analysis of *Mse*I digested trophozoite DNA on a 5% polyacrylamide gel. Lanes 2-7: AFLP analysis using Mse-CAG selective primer; Lanes 9-14: AFLP analysis using Mse-CTG selective primer; Lanes 2, 3, 9, and 10: Sheep isolate (S2) DNA; Lanes 4, 5, 11, and 12: Human isolate (Bris) DNA; Lanes 6, 7, 13, and 14: Human isolate (Iso6) DNA; Lanes 1,8 and 15: 100 bp ladder (Gibco BRL). The polyacrylamide gel did not reveal any polymorphic differences between the isolates using the *Mse*I restriction fragments and the AFLP banding patterns of the *Eco*RI restriction fragments were examined on a 5% polyacrylamide gel.

Polyacrylamide gel electrophoresis and silver staining were used to increase the resolution of the *Eco*RI generated AFLP banding patterns to detect polymorphic differences between axenic cultures of two human isolates and one animal isolate. The 5% polyacrylamide gel produced sharp distinct bands but did not reveal any polymorphic differences (Figure 3.8) indicating the isolates to be identical by AFLP analysis.

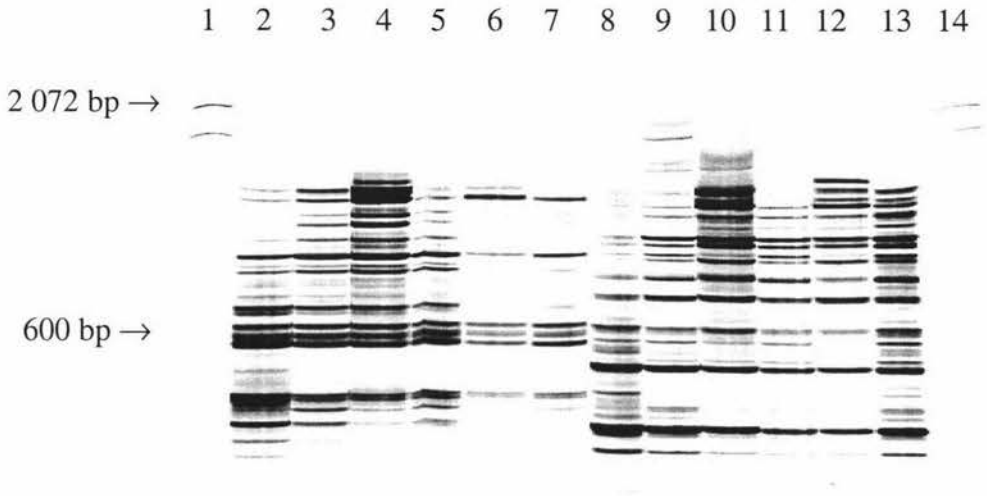



**Figure 3.8:** AFLP analysis of *Eco*RI digested trophozoite DNA on a 5% Polyacrylamide gel. Lanes 2-7: AFLP analysis using Eco-ATT/Eco-CTC selective primer combination; Lanes 8-13: AFLP analysis using Eco-ATT/Eco-CAC selective primer combination; Lanes 2, 3, 8, and 9: Sheep isolate (S2) DNA; Lanes 4, 5, 10, and 11: Human isolate (Bris) DNA; Lanes 6, 7, 12 and 13: Human isolate (Iso6) DNA; Lanes 1 and 14: 100 bp ladder (Gibco BRL). The higher resolution of the 5% polyacrylamide gel and silver staining did not detect polymorphic differences between the isolates so this technique was then tested on DNA extracted directly from the cysts of *G. intestinalis*.

### 3.2.2 AFLP Analysis of G. intestinalis Cysts from Human and Animal Faeces

Due to the identical AFLP patterns obtained from the *in vitro* cultures it was determined to test the analysis procedure directly on DNA extracted from the cysts of *G. intestinalis*. Initially non-reproducible banding patterns were seen (Figures 3.9 and 3.10) but increasing the amount of DNA extracted overcame this problem (Figure 3.11). Only two human and two bovine isolates were used for analysis as the cost of obtaining sufficient amounts of DNA was an expensive process. Analysis of these isolates found each to produce reproducible banding patterns unique for each isolate.

*G. intestinalis* cysts were isolated from human and bovine faeces using a single round of immuno-magnetic separation (IMS) and the extracted DNA used for AFLP analysis using the Mse-CTC selective primer, which produced an non-reproducible banding pattern (Figure 3.9).

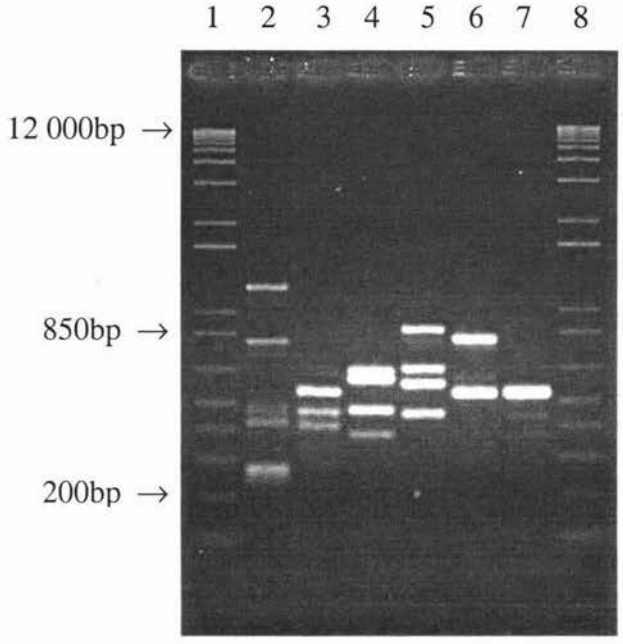



**Figure 3.9:** Non-reproducible AFLP fingerprints of *Mse*I digested *G. intestinalis* cyst DNA of two human isolates and a bovine isolate, amplified using the Mse-CTC primer in duplicate and examined on a 2% ethidium bromide stained agarose gel. Lanes 1 and 8: 1 Kb plus DNA ladder (Gibco BRL); Lanes 2 and 3: AFLP analysis of human isolate (H70) performed in duplicate; Lanes 4 and 5: AFLP analysis of human isolate (H80) performed in duplicate; Lanes 6 and 7: AFLP analysis of calf isolate (C14) in performed in duplicate. AFLP analysis of DNA extracted from *G. intestinalis* cysts produced non-reproducible banding patterns for each duplicate sample and the analysis was repeated using a different selective primer.

*G. intestinalis* cysts were isolated from human and bovine faeces using a single round of IMS and the extracted DNA used for AFLP analysis using the Mse-CAC selective primer, which resulted in an non-reproducible banding pattern (Figure 3.10) thought to be due to the low amount of DNA in the amplification reaction.

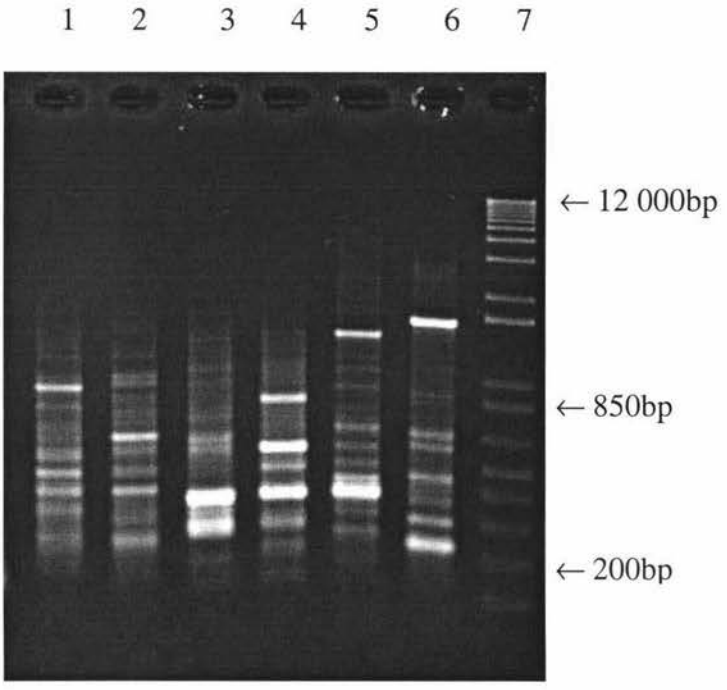



**Figure 3.10:** Non-reproducible AFLP fingerprints of *Mse*I digested DNA from human and bovine *G. intestinalis* isolates, amplified using the Mse-CAC primer in duplicate and examined on a 2% agarose gel. Lane 7: 1 Kb plus DNA ladder (Gibco BRL); Lanes: 1 and 2: Duplicate AFLP analysis of human isolate (H70); Lanes 3 and 4: Duplicate AFLP analysis of human isolate (H80); Lanes 5 and 6: Duplicate AFLP analysis of calf isolate (C14). Each isolate produced non-modified banding patterns that were likely to be due to the low amount of DNA present. The analysis was repeated after increasing the amount of DNA present in the amplification reaction by increasing the amount of cysts in the DNA extraction procedure.

*G. intestinalis* cysts were collected from twelve isolations of IMS of a bovine faecal specimen to increase the amount of DNA in the reaction. AFLP analysis was performed on the DNA extracted using Mse-CTC and Mse-CTA selective primers to amplify *Mse*I restriction fragments, resulting in a stable banding pattern (Figure 3.11).

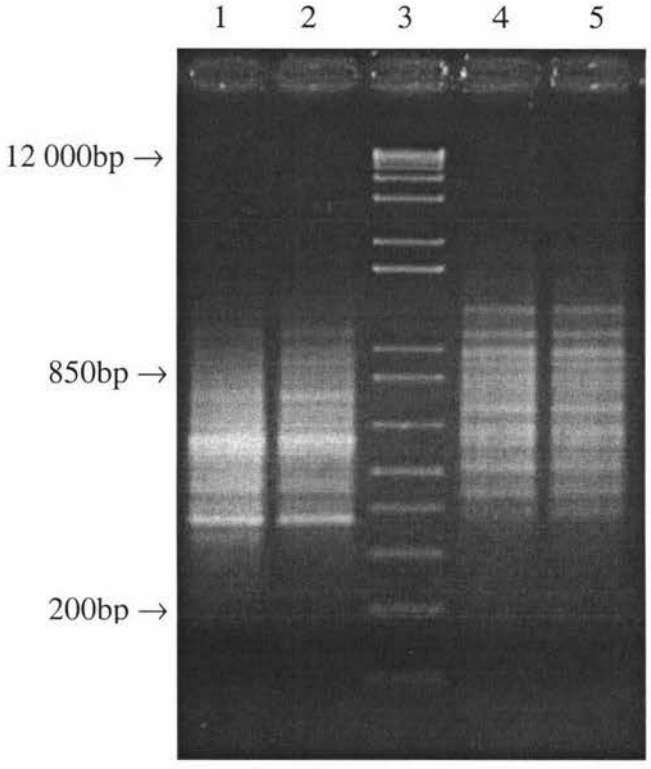



**Figure 3.11:** Reproducible AFLP fingerprints were obtained from the pooling of cysts from twelve IMS isolations from bovine faecal specimen C14 and examined on a 2% agarose gel. Lane 3: 1 Kb plus DNA ladder (Gibco BRL); Lanes 1 and 2: Duplicate AFLP analysis using Mse-CTC primer; Lanes 4 and 5: Duplicate AFLP analysis using Mse-CTA primer. The collection of cysts from twelve IMS isolations resulted in the stability of the AFLP banding pattern due to the increase in the amount of DNA in the amplification reaction. This technique was used on two human and two bovine faecal specimens to determine the ability to perform AFLP analysis on cysts of *G. intestinalis*.

*G. intestinalis* cysts from two human and two bovine faecal specimens were collected using twelve isolations by IMS for each isolate, and the DNA extracted was used for AFLP analysis using Mse-CG and Mse-CAC selective primers which produced stable banding patterns that revealed unique patterns for each isolate (Figure 3.12).

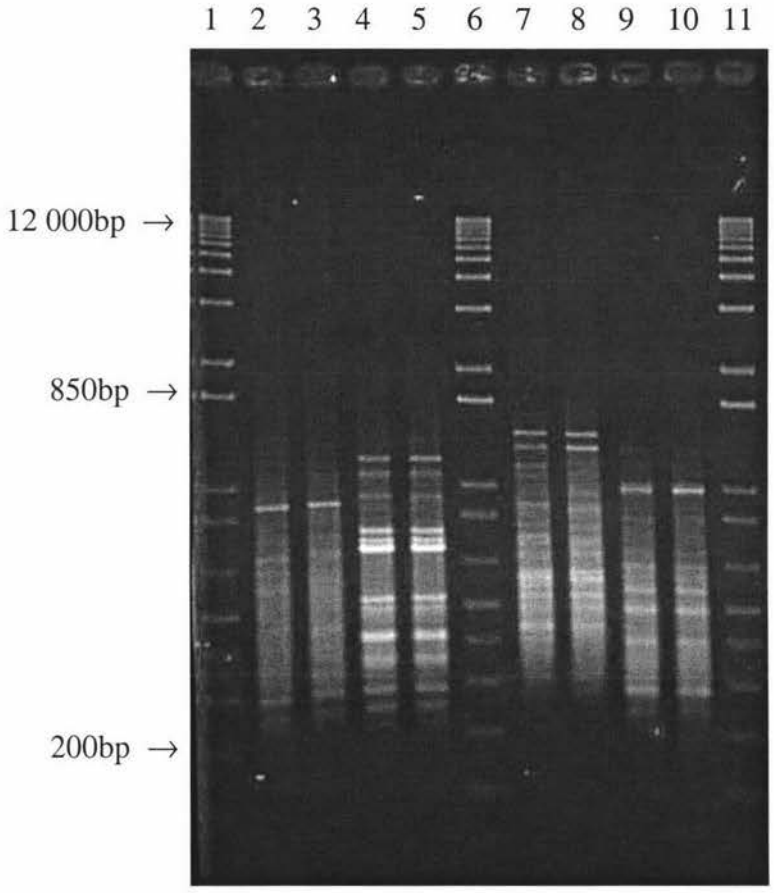



**Figure 3.12:** AFLP analysis from two human and two bovine isolates of *G. intestinalis* using Mse-CG and Mse-CAC primers and examined on a 2% agarose gel. Lanes 1, 6 and 11: 1 Kb plus DNA ladder (Gibco BRL); Lanes 2 and 3: Duplicate AFLP analysis of human isolate (H70); Lanes 4 and 5: Duplicate AFLP analysis of human isolate (H80); Lanes 6 and 7: Duplicate AFLP analysis of calf isolate (C14); Lanes 8 and 9: Duplicate AFLP analysis of calf isolate (C16). Each isolate produced a unique banding pattern revealing polymorphic differences between each isolate and further primer combinations were tested.

*G. intestinalis* cysts from two human and two bovine faecal specimens were collected using twelve isolations by IMS for each isolate, and AFLP analysis was performed on the DNA extracted using the Mse-CT/Mse-CG selective primer combination. The resulting banding patterns revealed unique patterns for each isolate (Figure 3.13).

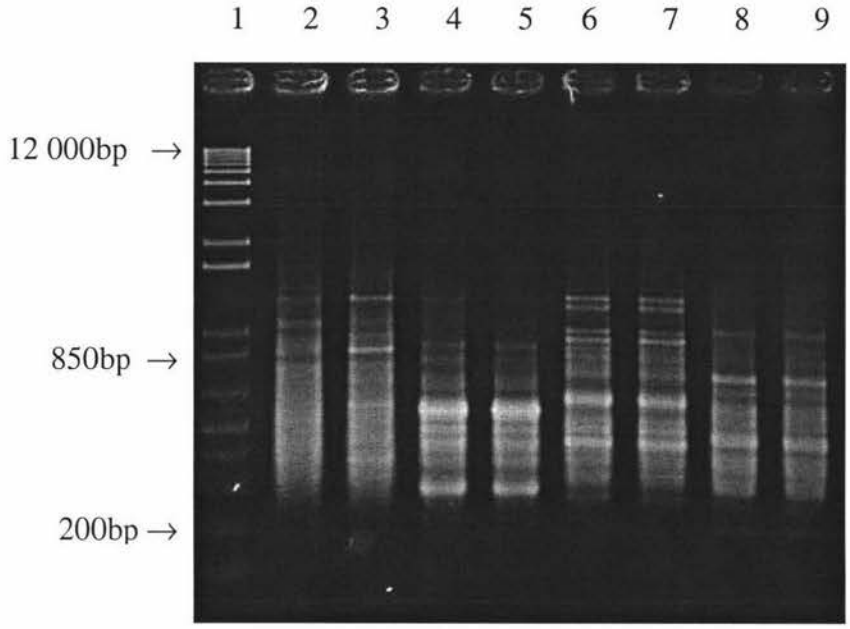



**Figure 3.13:** AFLP analysis using the Mse-CT/Mse-CG selective primer combination, performed on two human and two bovine isolates and examined on a 2% agarose gel. Lane 1: 1 Kb plus DNA ladder (Gibco BRL); Lanes 2 and 3: Duplicate AFLP analysis of human isolate (H77); Lanes 4 and 5: Duplicate AFLP analysis of human isolate (H80); Lanes 6 and 7: Duplicate AFLP analysis of calf isolate (C14); Lanes 8 and 9: Duplicate AFLP analysis of calf isolate (C16). Each isolate produced a unique banding pattern revealing polymorphic differences between the isolates. The reproducibility of the analysis was determined by comparing the AFLP banding patterns of duplicate DNA extractions of *G. intestinalis* cysts.

The reproducibility of AFLP analysis of duplicate DNA extractions from cysts of a bovine faecal specimen using the Mse-AT/Mse-CG selective primer combination to amplify *Mse*I restriction fragments. This produced a reproducible banding pattern from the cysts of *G. intestinalis* isolated from the same faecal specimen (as shown in Figure 3.14).

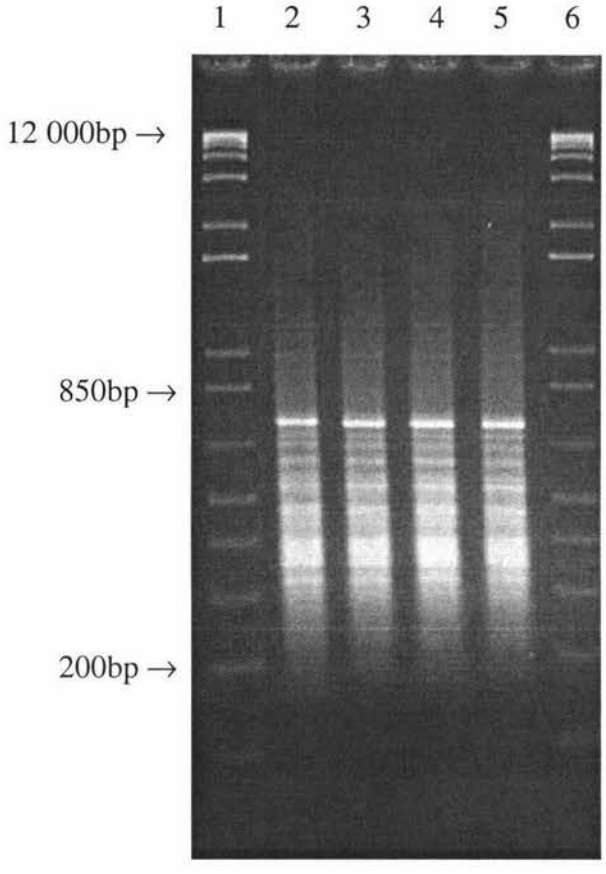



**Figure 3.14:** The reproducibility of AFLP analysis of duplicate DNA extractions from cysts of a bovine faecal specimen using Mse-AT/Mse-CG selective primer combination and examined on a 2% agarose gel. Lanes 1 and 6: 1 Kb plus DNA ladder (Gibco BRL); Lanes 2 and 3: AFLP analysis of calf isolate C16; Lanes 4 and 5: Reproduction of AFLP analysis of calf isolate C16 using duplicate DNA template. The duplicate DNA samples revealed the AFLP analysis technique to be reproducible, indicating that and polymorphic differences between isolates were not due to the procedure itself.

AFLP analysis products, of DNA extracted from the cysts of two human and two bovine isolates of *G. intestinalis*, were separated on a 5% polyacrylamide gel and visualised by silver staining. Polymorphic bands between the human and bovine isolates were excised in duplicate for re-amplification (Figure 3.15).

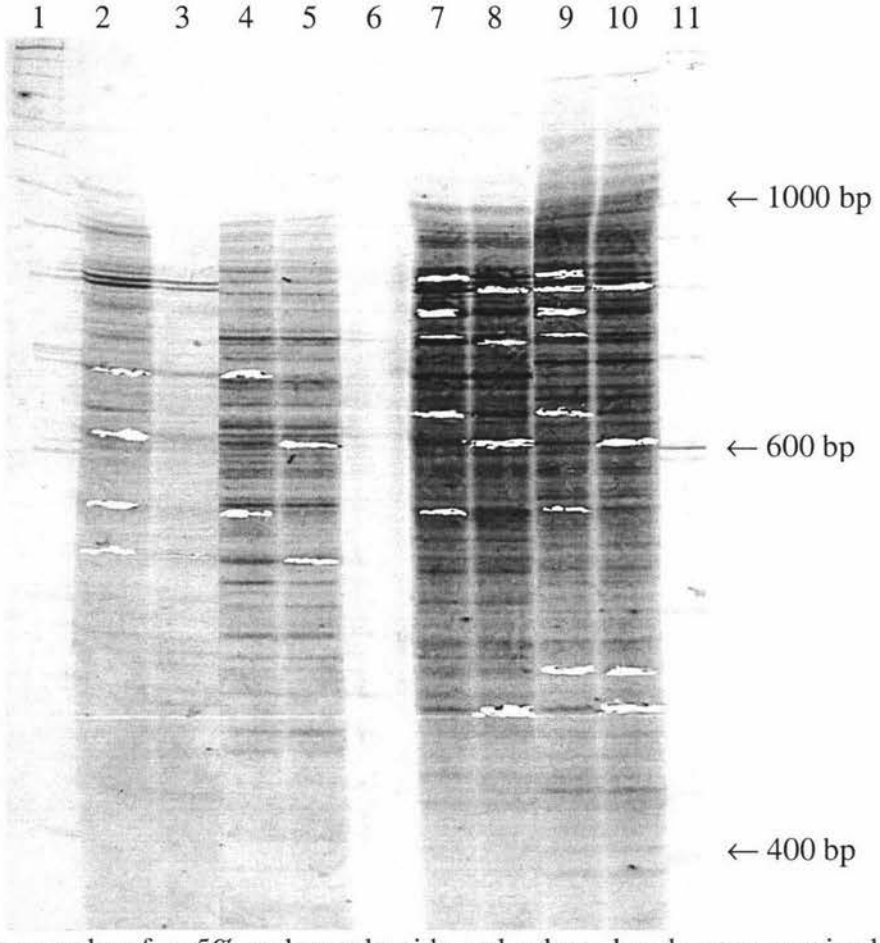



**Figure 3.15:** An example of a 5% polyacrylamide gel where bands were excised in duplicate from human and bovine AFLP banding patterns. These AFLP patterns were generated using the Mse-AT/Mse-CG selective primer combination. Lanes 1 and 11: 100 bp ladder (GibcoBRL); Lanes 2 and 3: AFLP profile of calf isolate (C14); Lanes 4 and 5: AFLP profile of calf isolate (C16); Lanes 7 and 8: AFLP profile of human isolate (H77); Lanes 9 and 10: AFLP profile of human isolate (H80). Only bands excised from the AFLP profile generated by the Mse-AT/Mse-CG selective primer combination are shown as an example.

Polymorphic bands were extracted from the polyacrylamide gels for re-amplification using the selective AFLP primer(s) for use in cloning and sequencing (Figure 3.16). Re-amplification of bands extracted from the polyacrylamide gels was not always successful (Lanes 8-11, Figure 3.16).

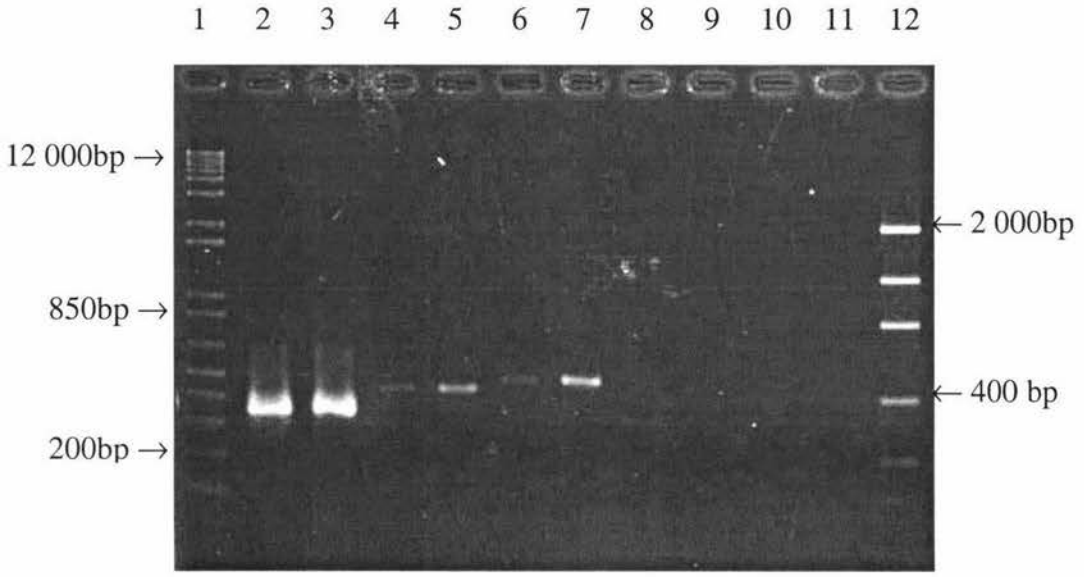



**Figure 3.16:** A 2% agarose gel showing the re-amplification of DNA fragments, in duplicate, extracted from a 5% polyacrylamide gel from the AFLP analysis of the bovine isolates (C14 and C16) generated by the Mse-AT/Mse-CG selective primer combination. Lane 1: 1 Kb plus DNA ladder (Gibco BRL); Lane 12: 50bp DNA ladder (Gibco BRL); Lanes 2 and 3: Re-amplification of a ~380bp fragment; Lanes 4 and 5: Re-amplification of a ~420bp fragment; Lanes 6 and 7: Re-amplification of a ~500bp fragment: Lanes 8 and 9: Unsuccessful re-amplification of a ~610bp fragment; Lanes 10 and 11: Unsuccessful re-amplification of a ~700bp fragment. The excised bands did not always successfully result in re-amplification of the PCR product. Only the re-amplification of five excised fragments is shown as an example.

Re-amplified AFLP fragments excised from the polyacrylamide gels were ligated into vectors to transform *E. coli* and extracted to detect the presence of the vector to ensure the success of the transformation (Figure 3.17). Vectors were detected from each transformation indicating each to be successful.

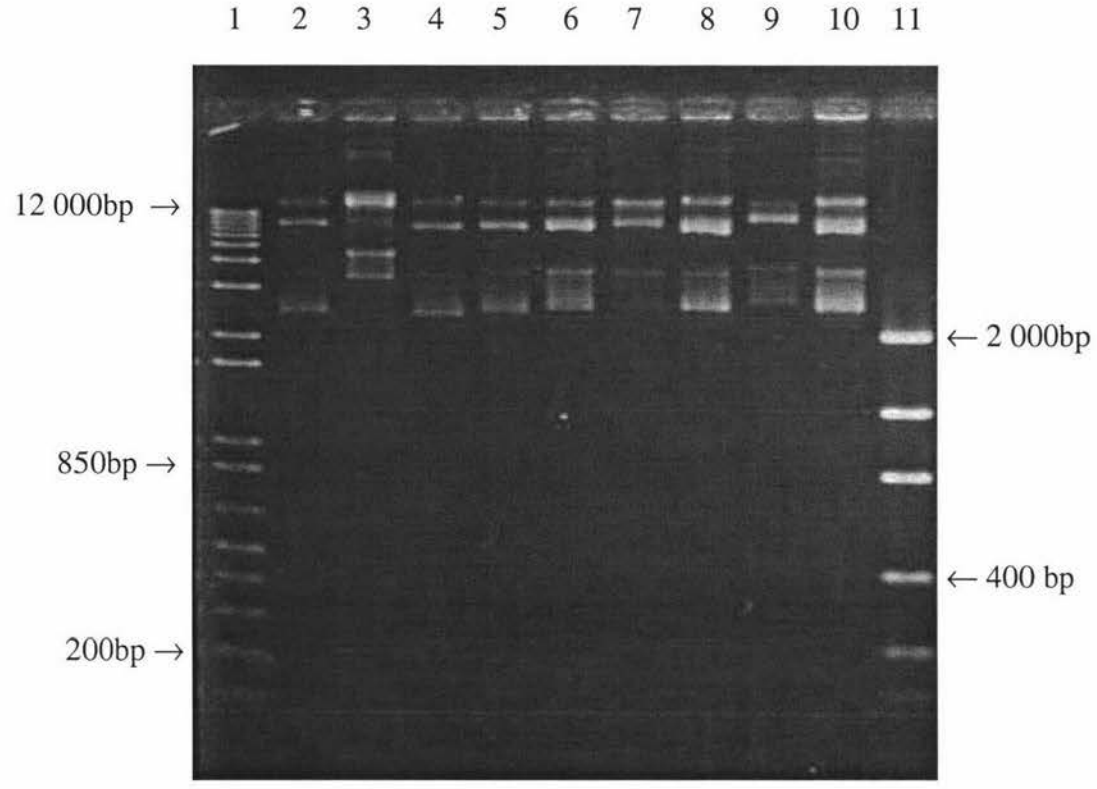



**Figure 3.17:** Vectors containing human and bovine *G. intestinalis* AFLP fragments examined on a 2% agarose gel. Lane 1: 1 Kb plus DNA ladder (Gibco BRL); Lane 11: Low DNA mass ladder (Gibco BRL); Lanes 2-10: Undigested vectors extracted from transformed *E. coli*; Lanes 2-10: Inserts from the Mse-AT/Mse-CG selective primer combination. The agarose gel shows the presence of the vector after extraction from the transformed *E. coli* indicating the successful transformation of the cells.

The vectors extracted from the transformed *E. coli* cells were digested with *Eco*RI to verify the presence of the inserts (Figure 3.18). The enzyme *Eco*RI was chosen, as the target sequence was present either side of the insert site (See Appendix A) and all vectors were shown to contain the presence of an insert by the presence of bands below approximately 3.5 Kb (size of the vector).

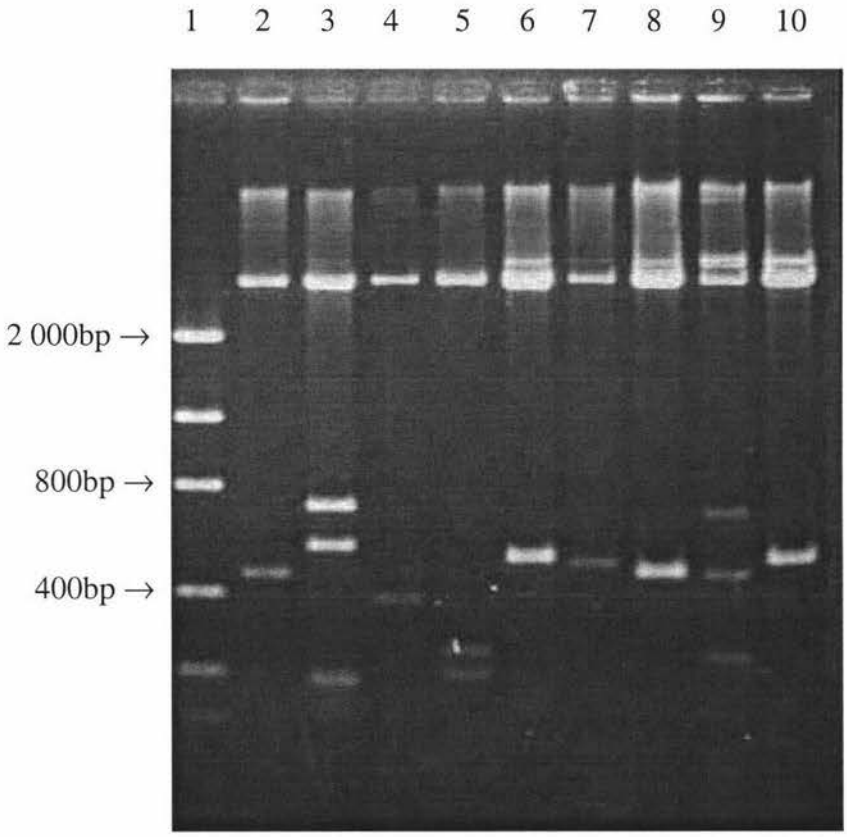
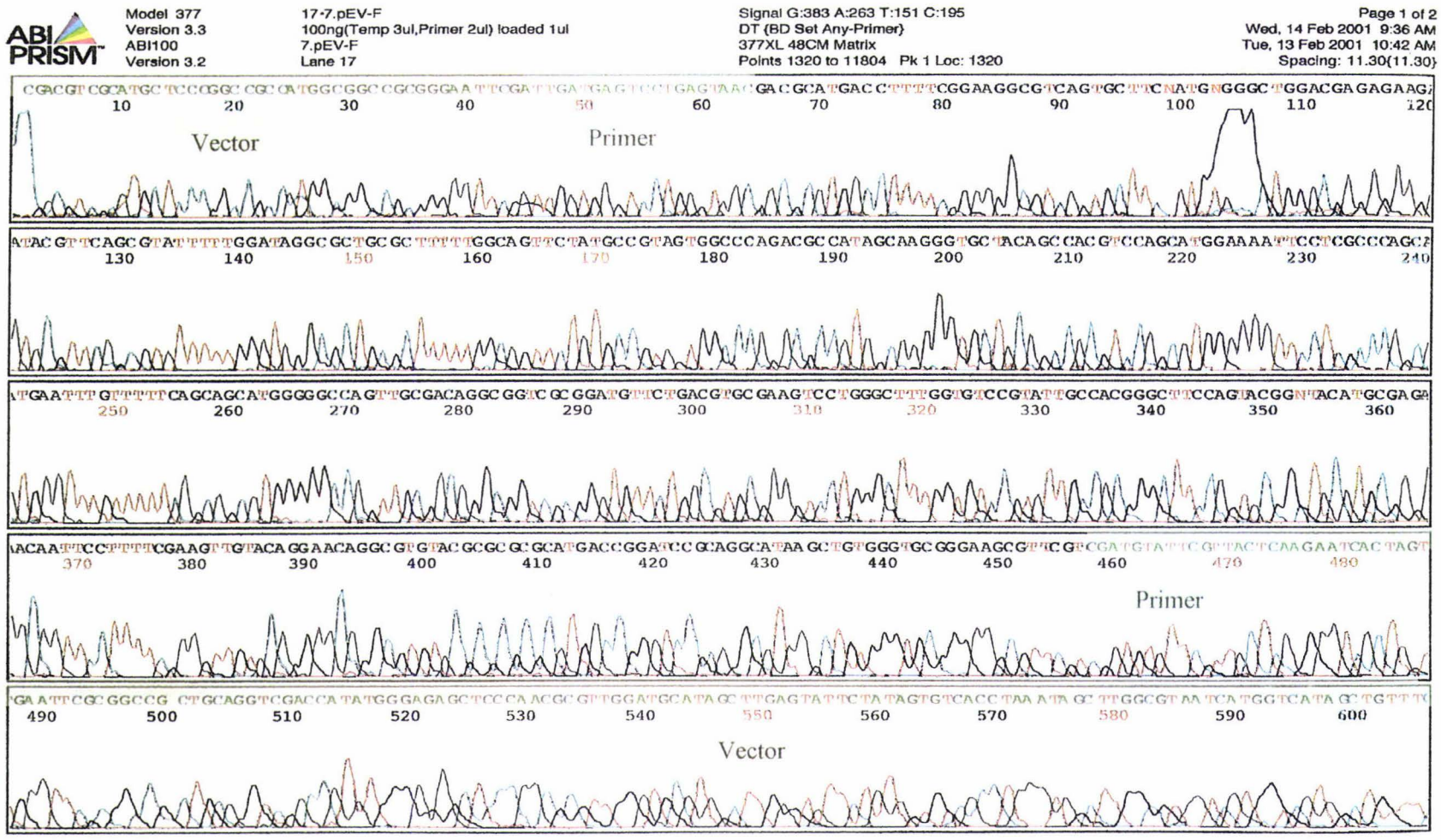



**Figure 3.18:** *Eco*RI digested vectors containing re-amplified AFLP fragments examined on a 2% agarose gel. Lane 1: Low DNA mass ladder (Gibco BRL). Lanes 2-10: Inserts from the Mse-AT/Mse-CG selective primer combination. All digested vectors show the presence of an inserted fragment and the undigested vectors containing an insert were sequenced to determine the insert sequence.

### 3.2.3 DNA Sequencing

The vectors containing the AFLP inserts were sequenced using primers that flanked the insert and were located within the vector. The electrophoretogram in figure 3.19 is an example of the sequences obtained, which shows the vector and inserted fragment including the primers used to amplify the linker-ligated restriction fragment.

**Figure 3.19**: A typical electrophoretogram of a vector containing a fragment excised from a polyacrylamide gel, showing the vector (grey sequence), AFLP primers (blue sequence), and restriction fragment sequences (unlabelled sequence). (OVER PAGE →)


ABI PRISM
Model 377
Version 3.3
ABI100
Version 3.2
17-7.pEV-F
100ng(Temp 3ul,Primer 2ul) loaded 1ul
7.pEV-F
Lane 17
Signal G:383 A:263 T:151 C:195
DT {BD Set Any-Primer}
377XL 48CM Matrix
Points 1320 to 11804 Pk 1 Loc: 1320
Page 1 of 2
Wed, 14 Feb 2001 9:36 AM
Tue, 13 Feb 2001 10:42 AM
Spacing: 11.30(11.30)
Vector
Primer
Primer
Vector

## 3.3 RAPD Analysis of *G. intestinalis*

Due to the large amount of DNA required for AFLP analysis a method relying only on PCR amplification was chosen to reduce the amount of DNA required. For AFLP analysis, the digestion and linker ligation reactions require the presence of a substantial amount of DNA to be extracted from *G. intestinalis* cysts to ensure a sample containing a representation of each restriction fragment is used for the PCR amplification. RAPD analysis was chosen for the use in examining *G. intestinalis* cysts, as this procedure only relies on PCR amplification of genomic DNA with no prior manipulation of the DNA.

RAPD analysis was modified to a similar procedure as AFLP analysis, to include pre-amplification and selective amplification stages. The 10 bp primers used in the unmodified technique were used to design pre-amplification primers where 2 bp were removed from the 3' end of the primers. The selective primer used to amplify the DNA was identical to that used in the pre-amplification stage but had the addition two nucleotides at the 3' end (see Sections 2.5.1 and 2.5.2). The modification to the technique was shown not to alter the banding pattern (Figure 3.22) and was used to examine two human and two bovine isolates of *G. intestinalis*. As with the AFLP technique, RAPD analysis was performed on a duplicate DNA sample to ensure and polymorphic bands were not due to the PCR amplification technique itself but differences in the genome sequence.

### 3.3.1 Optimisation of RAPD Analysis of *in vitro G. intestinalis* Cultures

The sensitivity of the unmodified RAPD analysis technique using the 10bp oligo-nucleotide primer (GC50+GT) was determined using DNA extracted from *G. intestinalis* trophozoites maintained in the laboratory. Ten micrograms of DNA was determined to give the clearest banding pattern (Lane 6, figure 3.20) as increasing the amount did not increase the number of bands detected (Figure 3.20).

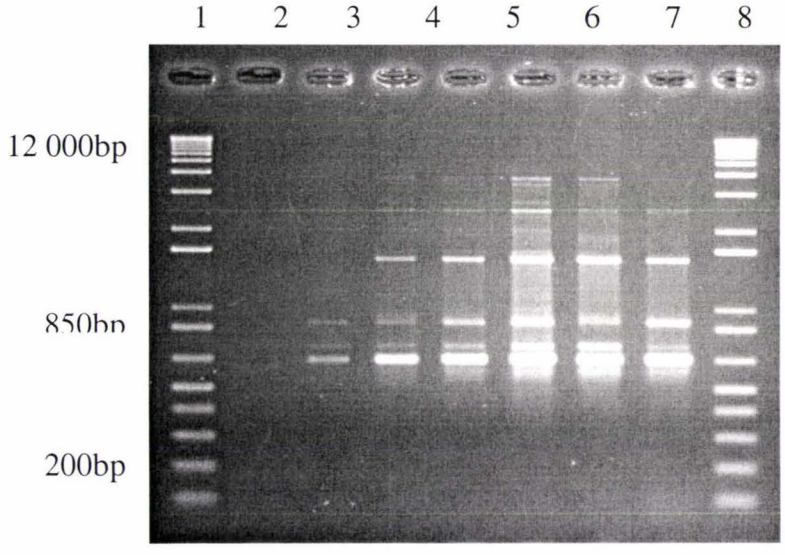



**Figure 3.20:** The sensitivity of RAPD analysis technique was determined using a range of trophozoite (sheep isolate, S2) DNA from 1 x $10^{-9}$ mg to 1 mg, with the GC50+GT primer and examined on a 2% agarose gel. Lanes 1 and 9: 1 Kb plus DNA ladder (Gibco BRL); Lane 2: 1 ng DNA; Lane 3: 10 ng DNA; Lane 4: 100 ng DNA; Lane 5: 1 $\mu$g DNA; Lane 6: 10 $\mu$g DNA; Lane 7: 100 $\mu$g DNA; Lane 8: 1 mg DNA. Ten micrograms of DNA was determined to be the optimal concentration, as more DNA did not increase the number of bands detected.

### 3.3.2 Optimisation of Nested RAPD Analysis of *in vitro G. intestinalis* Cultures

The sensitivity of RAPD analysis using the modified RAPD technique involving the use of nested primers was performed on *G. intestinalis* trophozoite DNA. Ten micrograms of DNA was determined to give the clearest banding pattern (Lane 6, Figure 3.21).

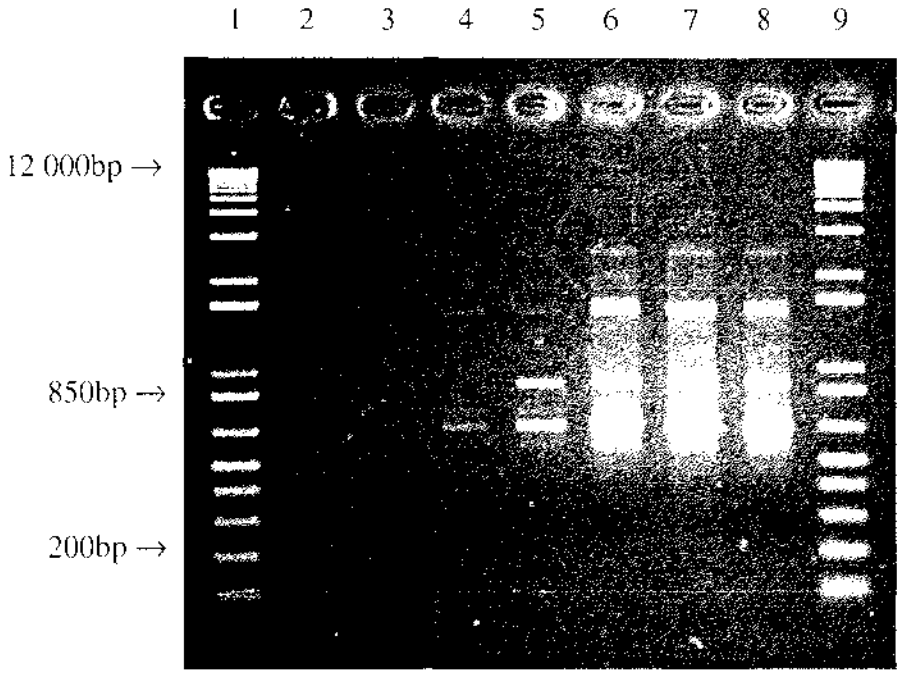



**Figure 3.21:** The sensitivity of nested RAPD technique was determined using a range of trophozoite (sheep isolate, S2) DNA from 1 x $10^{-9}$ mg to 1 mg using the GC50 pre-amplification and GC50+GT selective primers and examined on a 2% agarose gel. Lanes 1 and 9: 1 Kb plus DNA ladder (Gibco BRL); Lane 2: 1 ng DNA; Lane 3: 10 ng DNA; Lane 4: 100 ng DNA; Lane 5: 1 $\mu$g DNA; Lane 6: 10 $\mu$g DNA; Lane 7: 100 $\mu$g DNA; Lane 8: 1 mg DNA. Ten micrograms was determined to be the optimum DNA concentration for the nested RAPD technique making this modification able to detect the same amount of DNA as the unmodified technique. However this modification was able to detect 1 ng of DNA but the banding pattern is unique and not typical of a RAPD fingerprint.

### 3.3.3 Comparison of Nested and Unmodified RAPD Analysis Techniques

The RAPD fingerprints from the modified and unmodified techniques were compared to determine if the nested technique affected the resulting banding pattern. It was determined that the modification to the technique did not affect the banding pattern (as shown in Figure 3.22) as the selective amplification and the unmodified technique used the same primer resulting in the same banding pattern.

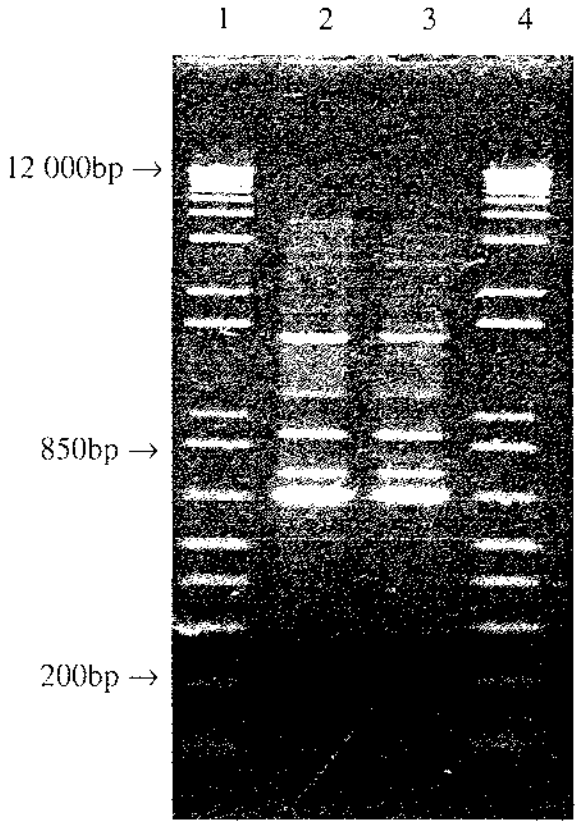



**Figure 3.22:** The RAPD fingerprints using the GC50+GT primer with the modified and unmodified technique were compared using 10 mg of *G. intestinalis* trophozoite DNA and examined on a 2% agarose gel. Lanes 1 and 4: 1 Kb plus DNA ladder (Gibco BRL); Lane 2: RAPD analysis using the GC50+GT primer; Lane 3: Nested RAPD fingerprint using the GC50+GT selective primer. It was possible that the nested modification could produce a different RAPD fingerprint from the unmodified technique, as PCR products from the pre-amplification could remain but the banding patterns were seen to be identical.

### 3.3.4 Nested RAPD Analysis of *G. intestinalis* Cysts from Human and Bovine Faecal Specimens

Two DNA samples were extracted from the cysts of a bovine isolate and each sample was analysed in duplicate to determine if the nested RAPD technique was reproducible. The banding patterns were seen to be identical indicating a reproducible technique (Figure 3.23). A non-reproducible pattern would indicate the presence of DNA from a contaminating organism.

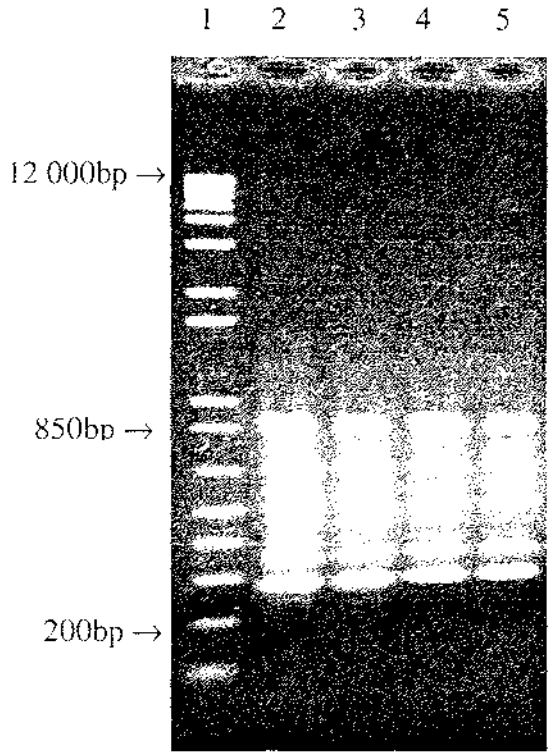



**Figure 3.23:** The reproducibility of RAPD analysis technique was examined using two DNA samples extracted from cysts of a calf isolate (C14) of *G. intestinalis* and analysed in duplicate on a 2% agarose gel. Lane 1: 1 Kb plus DNA ladder (Gibco BRL); Lanes 2 and 3: Duplicate RAPD analysis using the GC50 pre-amplification and GC50+GT selective primer from cysts isolated from calf isolate (C14); Lanes 4 and 5: Repeated duplicate RAPD analysis using the GC50+GT selective primer from cysts of isolate (C14) were re-isolated. The banding patterns were found to be identical. Some bands varied in intensity that may be due to the inability to measure the amount of template used in each reaction. The modified RAPD technique was used to compare human and bovine isolates of *G. intestinalis*.

Analysis of DNA extracted from *G. intestinalis* cysts from two human and two bovine faecal specimens using the nested RAPD technique was performed in duplicate. Using the GC50+GT/GC60+GT selective primer combination reproducible banding patterns was achieved (as shown in Figure 3.24), but were unique for each isolate.

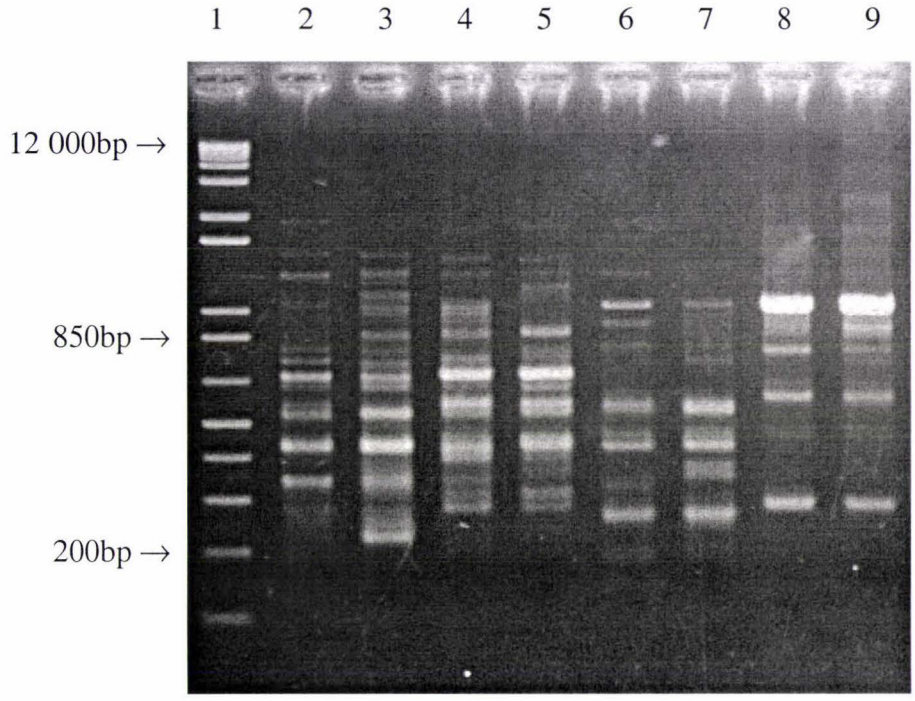



**Figure 3.24:** RAPD analysis of two human and two bovine isolates of DNA from cysts of *G. intestinalis* was performed in duplicate using the GC50/GC60 pre-amplification and GC50+GT/GC60+GT selective primer combination and examined on a 2% agarose gel. Lane 1: 1 Kb plus DNA ladder (Gibco BRL); Lanes 2 and 3: RAPD analysis of human isolate (H77); Lanes 4 and 5: RAPD analysis of human isolate (H80); Lanes 6 and 7: RAPD analysis of calf isolate (C14); Lanes 8 and 9: RAPD analysis of calf isolate (C16). The major banding patterns were stable for each isolate and between them polymorphic differences were seen with each isolate producing a unique banding pattern. Other primers and primer combinations were tested to further examine the resulting DNA fingerprints.

Analysis of DNA extracted from *G. intestinalis* cysts from two human and two bovine faecal specimens using the nested RAPD technique was performed. Using the GC50+GT/GC70+GT selective primer combination stable banding patterns were achieved (as shown in Figure 3.25) for the isolates.

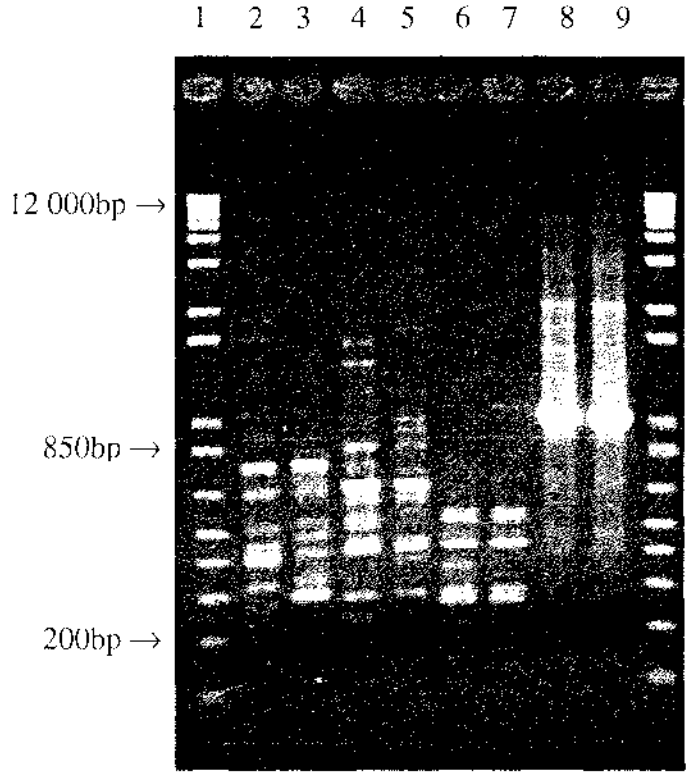



**Figure 3.25:** RAPD analysis of two human and two bovine isolates of DNA extracted from cysts of *G. intestinalis* performed in duplicate using the GC50+GT/GC70+GT selective primer combination and examined on a 2% agarose gel. Lane 1: 1 KB plus DNA ladder (GIBCO BRL); Lanes 2 and 3: RAPD analysis of human isolate (H77); Lanes 4 and 5: RAPD analysis of human isolate (H80); Lanes 6 and 7: RAPD analysis of calf isolate (C14); Lanes 8 and 9: RAPD analysis of calf isolate (C16). The major banding patterns were stable for each isolate and between them polymorphic differences were seen with each isolate producing a unique banding pattern. Only two primer combinations have been shown (Figures 3.24 and 3.25) as an example of the resulting DNA fingerprints.

## 3.4 Selective Pressure on *in vitro* Cultures

The use DNA extracted directly from the cysts of *G. intestinalis* for genotyping experiments removes the tedious process of producing cultures and the selective bias of only examining those isolates capable of being cultured. Due to the fact that the *in vitro* cultures used in this thesis have produced identical DNA fingerprinting patterns it was examined to see if a selective pressure placed on the cultures could alter the AFLP and RAPD banding patterns. This was examined to indicate the possibility that over time the culturing process may have resulted in changes to the DNA fingerprints.

Trophozoites were grown under the selective pressure of metronidazole. As this is the drug used for treating the majority of giardiasis patients it was chosen as a selective pressure as it would be the most likely pressure to result in changes to the DNA fingerprints. Starting at an initial concentration of 0.05 mg/L and slowly increasing to 1 mg/L the DNA was extracted for AFLP and RAPD analysis to compare banding patterns with the wild type to examine the effects of the selective pressure. It was seen that the banding patterns generated by two primers used in the RAPD analysis detected differences between the cultures while the AFLP patterns of two selective primers did not reveal differences.

### 3.4.1 AFLP Analysis

Using the Mse-GA and Mse-CAA selective primers, AFLP analysis was performed to compare the banding patterns of the culture grown under selective pressure with that of the wild type. The analysis with these two primers did not reveal any changes between the two samples (as shown in Figure 3.26).

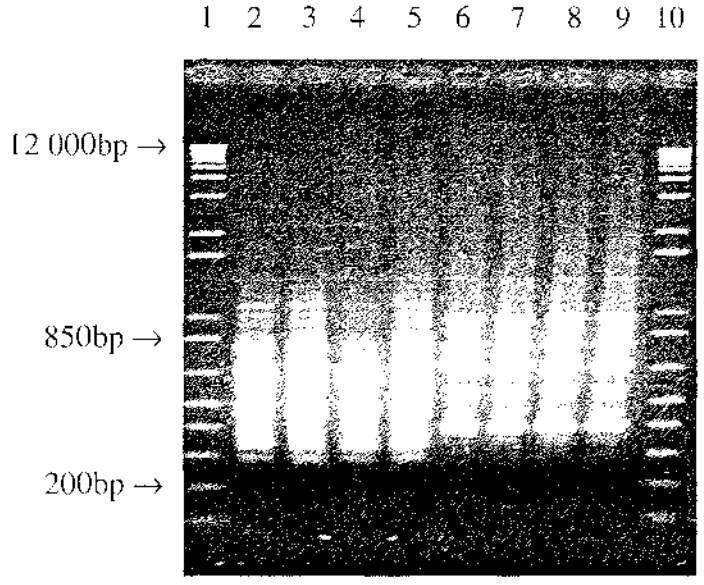



**Figure 3.26:** The banding patterns from the AFLP analysis of *G. intestinalis* grown under selective pressure and wild-type trophozoites using Mse-GA and Mse-CAA selective primers were compared on a 2% agarose gel. Lanes 1 and 10: 1 Kb plus DNA ladder (Gibco BRL); Lanes 2-5: AFLP analysis using the Mse-GA selective primer; Lanes 6-9: AFLP analysis using the Mse-CAA selective primer; Lanes 2, 3, 6, and 7: AFLP analysis of DNA extracted from trophozoites grown under selective pressure; Lanes 4, 5, 8, and 9: AFLP analysis of DNA extracted from wild-type trophozoites. This analysis revealed no changes in the banding patterns between the wild type trophozoites and those grown with metronidazole.

### 3.4.2 RAPD Analysis

Using GC50+GT, GC60+GT, and GC70+GT selective primers, RAPD analysis was performed on wild-type trophozoites and trophozoites grown under selective pressure to compare the resulting banding patterns. Minor differences were seen with GC60+GT selective primer but with GC70+GT selective primer more bands were present in the wild type isolate (Lanes 8, 9, 12, and 13, Figure 3.27).

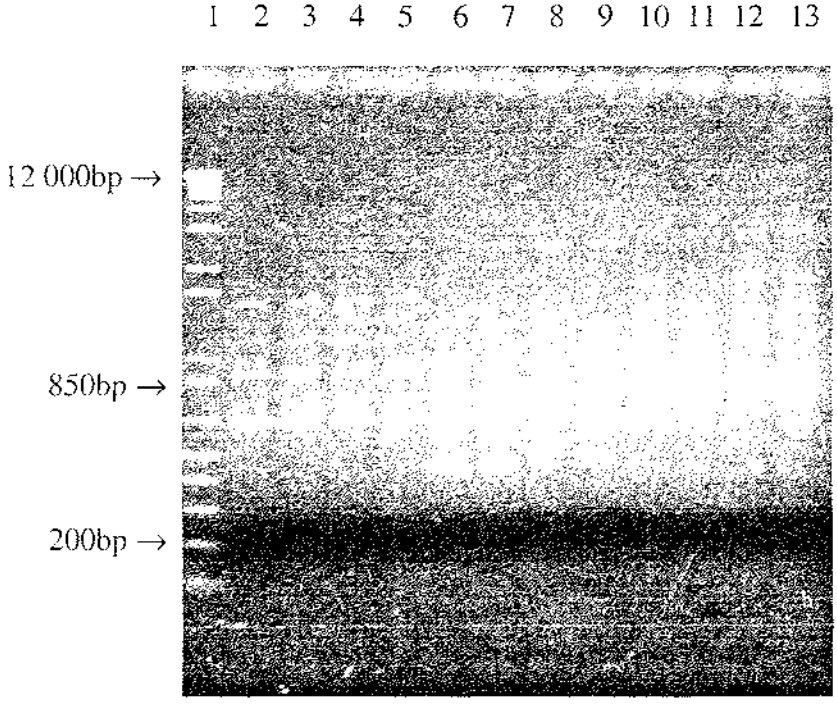



**Figure 3.27:** The banding patterns from the RAPD analysis of wild-type trophozoites of *G. intestinalis* and those grown under selective pressure were compared on a 2% agarose gel. Lane 1: 1 Kb plus DNA ladder (Gibco BRL); Lanes 2–5: RAPD analysis using the GC50+GT primer; Lanes 6–9: RAPD analysis using the GC60+GT primer; Lanes 10–13: RAPD analysis using the GC70+GT primer. Lanes 2, 3, 6, 7, 10 and 11: RAPD analysis of DNA extracted from trophozoites grown under selective pressure; Lanes 4, 5, 8, 9, 12 and 13: RAPD analysis of DNA extracted from wild-type trophozoites. This analysis revealed changes in the RAPD banding patterns, using GC60+GT and GC70+GT primers, between the wild type culture of *G. intestinalis* with that grown with metronidazole. This indicates the possibility that the fingerprints of axenic cultures may change from the process of culturing.

## 3.5 Genotyping of *G. intestinalis* by rDNA Sequence Analysis

The genotyping study by Hunt (1999) using rDNA sequence analysis was shown to be able to separate the isolates of *G. intestinalis* isolates into two distinct groups, and was chosen to analyse human and bovine isolates from the Manawatu and Waikato regions in New Zealand. The rDNA region was chosen for analysis, as it is present in multiple copies within the genome, therefore requiring fewer cysts to extract DNA from than the DNA fingerprinting techniques used in sections 3.2.2 and 3.3.4. The PCR amplified product also contains 2 variable regions and 1 conserved region to analyse for genetic differences.

Amplification of the rDNA was achieved with the Cyn0/Cyn2 primer pair bovine and human isolates. The use of the MAT1 and MAT2 primers as nested primers in conjunction with the Cyn0/Cyn2 primer pair was necessary to obtain PCR products. No amplification product was seen using a single set of primers alone but the use of nested primers gave the expected product. The location of the primers and the expected product sizes are outlined below in Figure 3.28, and the PCR amplified product contains the conserved 5.8S and two variable regions, ITS-1, and ITS-2.

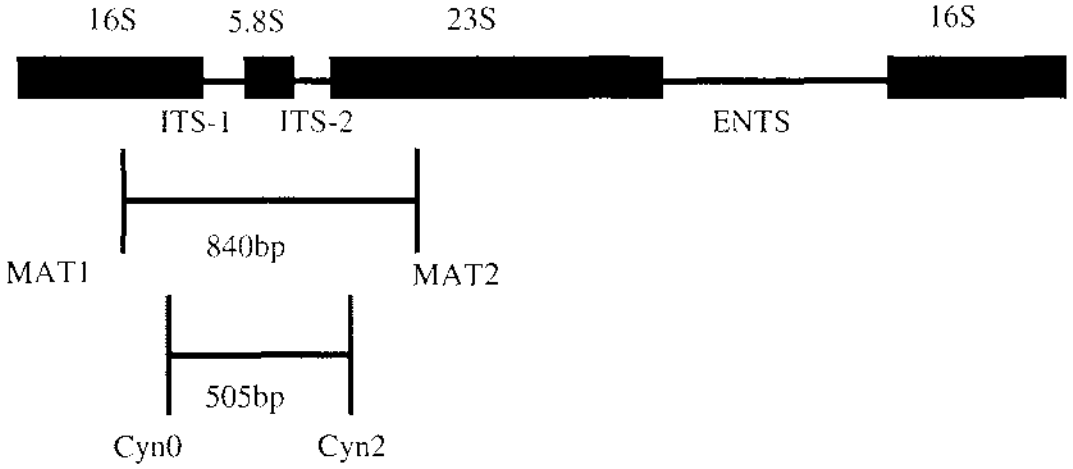



**Figure 3.28:** Structure of the *Giardia* rDNA gene showing the location of MAT1, MAT2, Cyn0 and Cyn2 primers.

### 3.5.1 Collection of Faecal Specimens

Giardia positive faecal specimens were obtained from 53 cows, 59 calves and 11 humans (Table 1). From all of these a total of 53 isolates were genotyped (eight human and 45 bovine).

**Table 1:** Faecal Specimens Collected over the Sampling Period

| | Cow | Calf | Human |
|---|---|---|---|
| Positive | 53 | 59 | 11 |
| Number of Samples | 354 | 304 | 66 |
| Percentage Positive | 15.1% | 19.4% | 16.7% |

**NOTE:** This section has isolates referenced as numbers relating to a laboratory number (See Appendix A) designated by a database held in the laboratory (Protozoa Research Unit, Massey University, Palmerston North, New Zealand).

### 3.5.2 *G. intestinalis* rDNA PCR Amplification

PCR amplification of the rDNA from cysts of *G. intestinalis* was performed using the nested primer pairs MAT1/MAT2 and Cyn0/Cyn2. No PCR product was detected using the MAT1/MAT2 primer pair alone (Figure 3.28, lanes 2-6) but employing the Cyn0/Cyn2 primer pair as nested primers the expected product of 500bp was detected (Figure 3.29, Lanes 8-12).

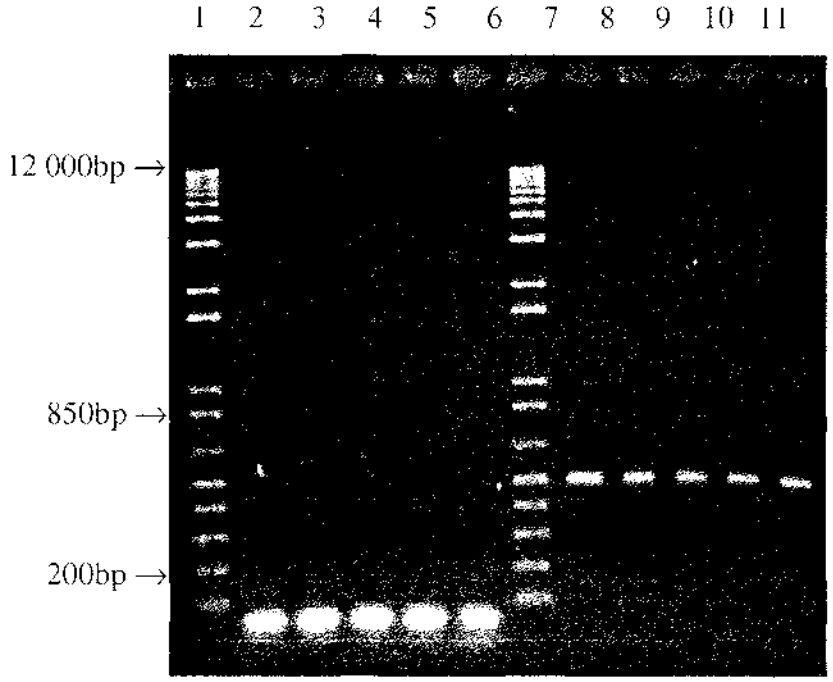



**Figure 3.29:** PCR amplification of *G. intestinalis* rDNA gene showing the requirement of nested primers on bovine *G. intestinalis* cyst DNA on a 1.6% agarose gel. Lane 1 and 7: 1 Kb plus DNA ladder (Gibco BRL); Lanes 2-6: PCR amplification using MAT1/MAT2 primer pair; Lanes 8-12: Nested PCR amplification using Cyn0/Cyn2 primer pair with MAT1/MAT2 PCR product as template. No band is amplified with the use of a single set of primers (Lanes 2-6) but the use of nested PCR amplification gave the expected product size of 505bp (Lanes 8-12).

### 3.5.3 *G. intestinalis* rDNA Automatic Sequencing

The rDNA PCR products of *G. intestinalis* were sequenced using the Cyn0 and Cyn2 primers by the Massey University DNA Analysis Service Sequencing Unit producing a typical electrophoretogram (as shown in Figure 3.30) for sequence analysis.

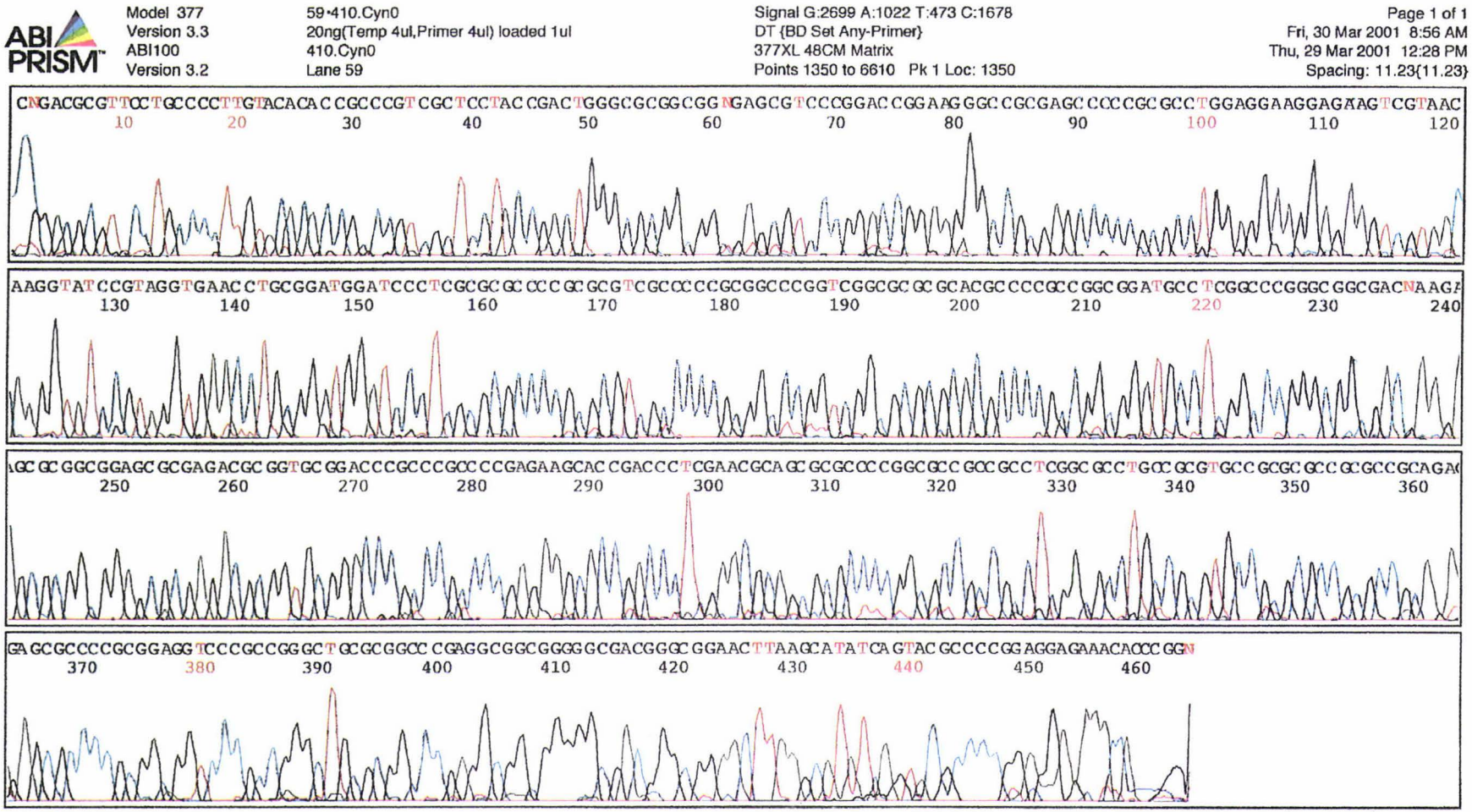



**Figure 3.30:** A typical electrophoretogram of the rDNA sequence of a bovine isolate of *G. intestinalis* using the Cyn0 primer showing 465bp of the 505bp PCR product.

### 3.5.4 *G. intestinalis* rDNA Sequence Analysis

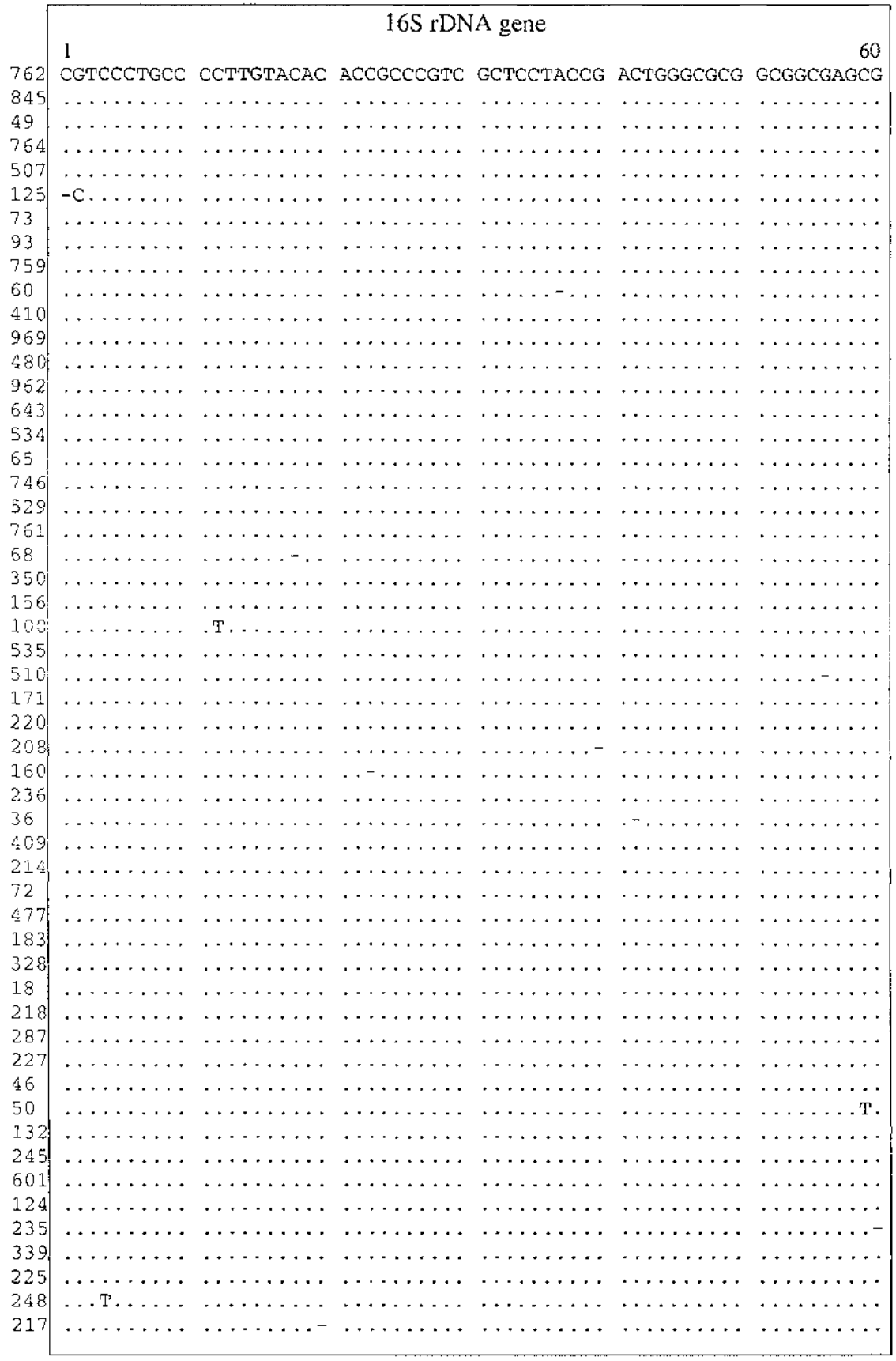
The rDNA sequences generated by the Cyn0 and Cyn2 primers were compared to produce a consensus sequence to remove ambiguous nucleotides in the sequence.

A total of 53 isolates were genotyped using the rDNA and sequences from eight human and 45 bovine isolates were aligned by ClustalX, Version 1.8, and displayed using MEGA, Version 1.02 (Figure 3.31). The information regarding the host and location of each isolate is held in a database in the laboratory (Protozoa Research Unit, Massey University, Palmerston North, New Zealand) (See Appendix A, Table 2).

The rDNA sequence of the isolates of *G. intestinalis* were then compared and the phylogenetic relationships were generated and displayed using SplitsTree, Version 2.4 (as shown in Figure 3.32 and Figure 3.33). The distances of the isolates in the SplitsTree diagram are based upon nucleotide differences between each isolate while the physical orientation of each line is unimportant. Two distinct groups are detected by the base change at position 61, and the GCGCGC sequence at position 190 (shown in Figure 3.32) as described by Hunt (1999), the greater sequence diversity seen in the isolates of Group 2 (Figure 3.33) was also mentioned by Hunt (1999).

**Figure 3.31:** Display of the alignment of the Cyn0 sequences of *G. intestinalis* rDNA PCR products from human and animal isolates generated by MEGA, Version 1.02. The top line displays the complete sequence and identical bases in other isolates are represented by dots, deletions and insertions are displayed as hyphens and base differences are displayed as the base present in the particular sequence. The single nucleotide substitution at position 61 and GCGCGC sequence at position 190, as described by Hunt (1999) were detected.

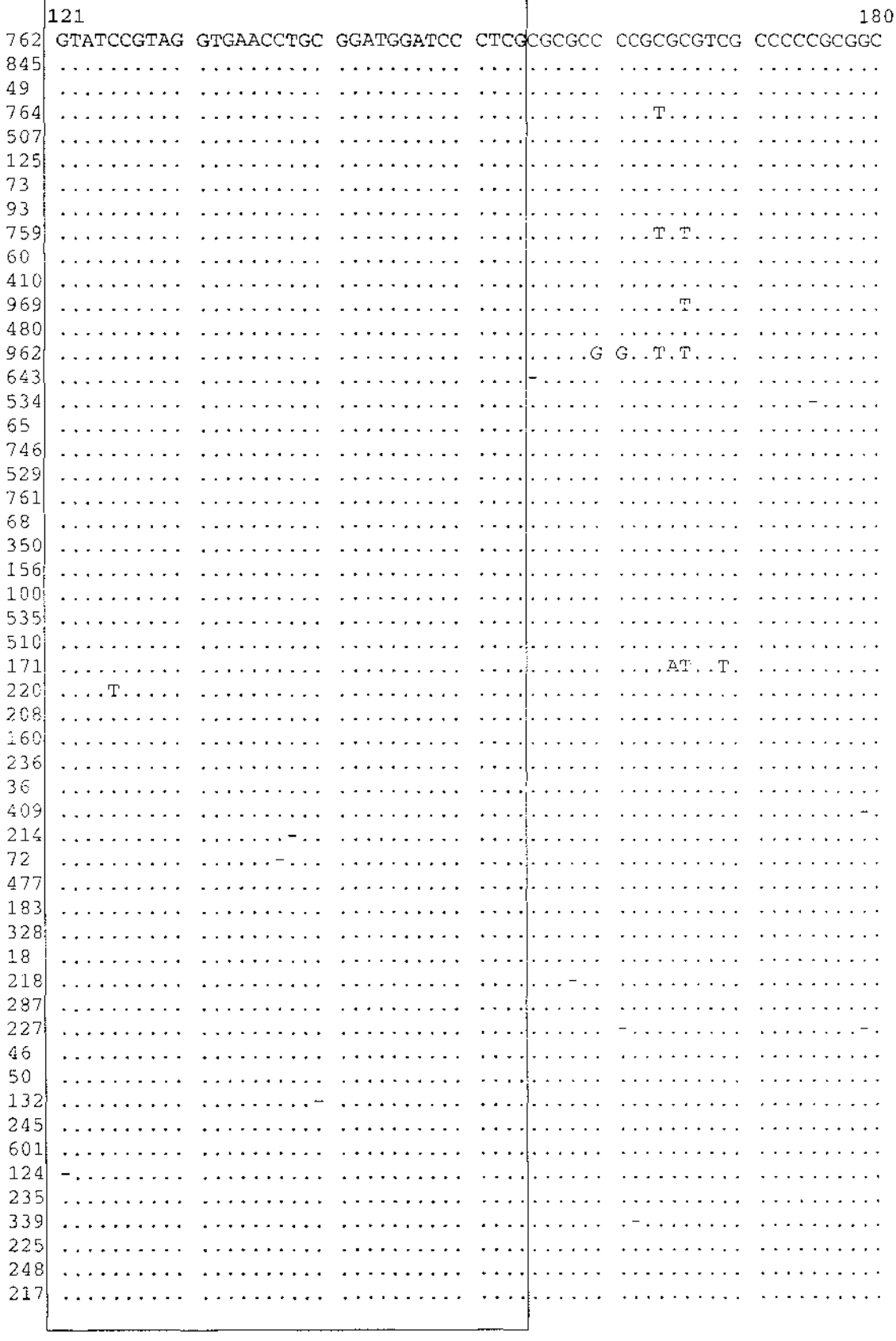
(OVER PAGE →)

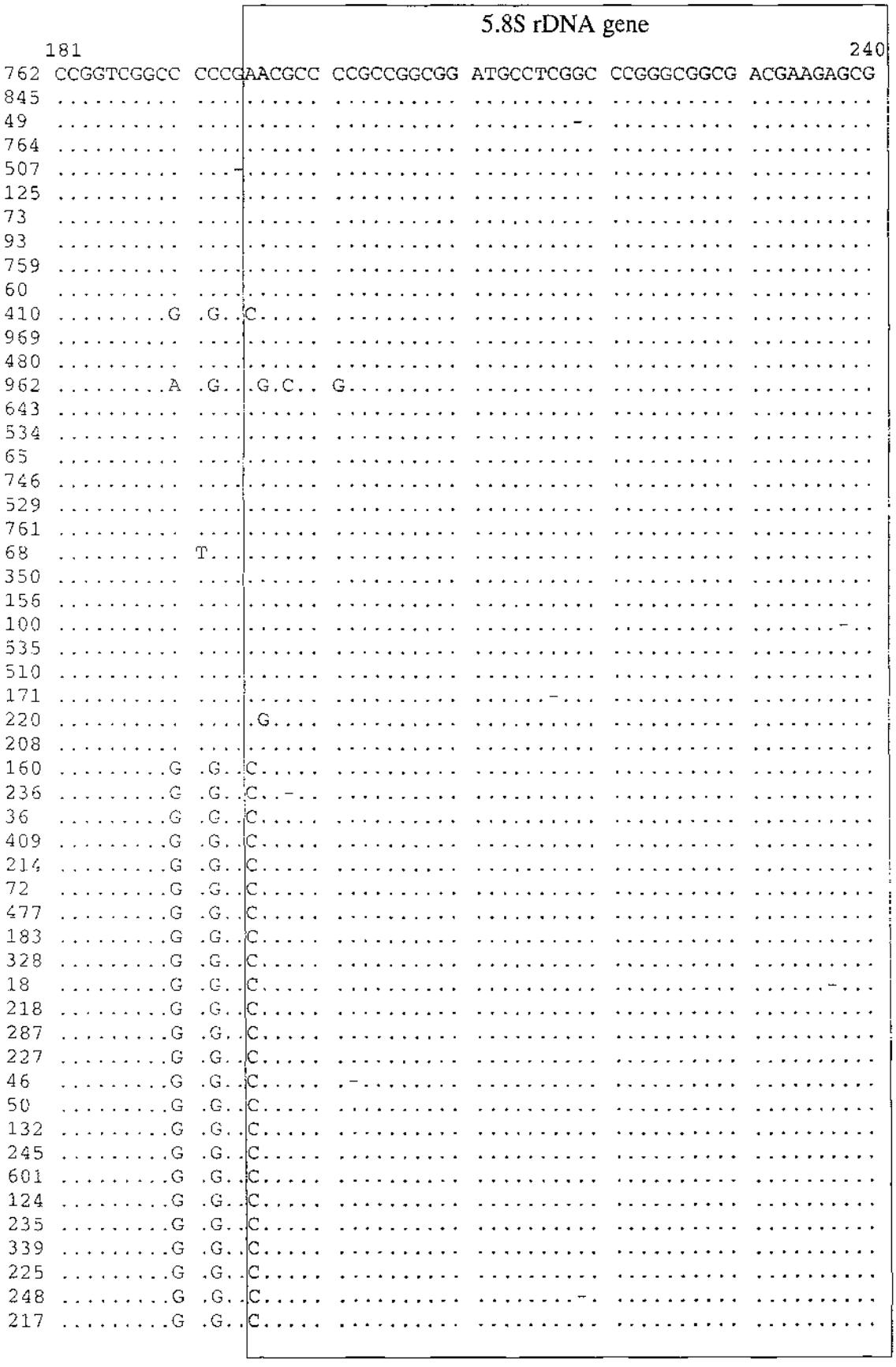

16S rDNA gene
1
60
762 CGTCCCTGCC CCTTGTACAC ACCGCCCGTC GCTCCTACCG ACTGGGCGCG GCGGCGAGCG
845 .......... .......... .......... .......... .......... ..........
49 .......... .......... .......... .......... .......... ..........
764 .......... .......... .......... .......... .......... ..........
507 .......... .......... .......... .......... .......... ..........
125 -C........ .......... .......... .......... .......... ..........
73 .......... .......... .......... .......... .......... ..........
93 .......... .......... .......... .......... .......... ..........
759 .......... .......... .......... .......... .......... ..........
60 .......... .......... .......... ......-... .......... ..........
410 .......... .......... .......... .......... .......... ..........
969 .......... .......... .......... .......... .......... ..........
480 .......... .......... .......... .......... .......... ..........
962 .......... .......... .......... .......... .......... ..........
643 .......... .......... .......... .......... .......... ..........
534 .......... .......... .......... .......... .......... ..........
65 .......... .......... .......... .......... .......... ..........
746 .......... .......... .......... .......... .......... ..........
529 .......... .......... .......... .......... .......... ..........
761 .......... .......... .......... .......... .......... ..........
68 .......... ........-. .......... .......... .......... ..........
350 .......... .......... .......... .......... .......... ..........
156 .......... .......... .......... .......... .......... ..........
100 .......... .T........ .......... .......... .......... ..........
535 .......... .......... .......... .......... .......... ..........
510 .......... .......... .......... .......... .......... .....-....
171 .......... .......... .......... .......... .......... ..........
220 .......... .......... .......... .......... .......... ..........
208 .......... .......... .......... .........- .......... ..........
160 .......... .......... ..-....... .......... .......... ..........
236 .......... .......... .......... .......... .......... ..........
36 .......... .......... .......... .......... .-........ ..........
409 .......... .......... .......... .......... .......... ..........
214 .......... .......... .......... .......... .......... ..........
72 .......... .......... .......... .......... .......... ..........
477 .......... .......... .......... .......... .......... ..........
183 .......... .......... .......... .......... .......... ..........
328 .......... .......... .......... .......... .......... ..........
18 .......... .......... .......... .......... .......... ..........
218 .......... .......... .......... .......... .......... ..........
287 .......... .......... .......... .......... .......... ..........
227 .......... .......... .......... .......... .......... ..........
46 .......... .......... .......... .......... .......... ..........
50 .......... .......... .......... .......... .......... .........T.
132 .......... .......... .......... .......... .......... ..........
245 .......... .......... .......... .......... .......... ..........
601 .......... .......... .......... .......... .......... ..........
124 .......... .......... .......... .......... .......... ..........
235 .......... .......... .......... .......... .......... .........-
339 .......... .......... .......... .......... .......... ..........
225 .......... .......... .......... .......... .......... ..........
248 ...T...... .......... .......... .......... .......... ..........
217 .......... .........- .......... .......... .......... ..........

16S rDNA gene continued

```
     61                                                                120
762  CCCCGGACGC GCGAAGGGCC GCGAGCCCCC GCGCCTGGAG GAAGGAGAAG TCGTAACAAG
845  .......... .......... .......... .......... .......... ..........
49   .......... .......... .......... .......... .......... ..........
764  .......... .......... .......... .......... .......... ..........
507  .......... .......... .......... .......... .......... ..........
125  .......... .......... .......... .......... .......... ..........
73   .......... .......... .......... .......... .......... ..........
93   .......... .......... .......... .........- .......... ..........
759  .......... .......... .......... .......... .......... .........-
60   .......... .......... .......... .......... .......... ..........
410  ...-....-. .-........ .......... .......... .......... ..........
969  .......... .......... .......... .......... .......... ..........
480  .......... .........- .......... .......... .......... ..........
962  .......... .......... .......... .......... .......... ..........
643  .......... .......... .......... .......... .......... ..........
534  .......... .......... .......... .......... .......... ..........
65   .......... .......... .......... .......... .......... ..........
746  .......... .......... .......... .......... .......... ..........
529  .......... .......... .......... .......... .......... ..........
761  .......... .......... .......... .......... .......... ..........
68   .......... .......... .......... .......... .......... ..........
350  .......... .......... .......... .......... .......... ..........
156  .......... .......... .......... .......... .......... ..........
100  .......... .......... .......... .......... .......... ..........
535  .......... .......... .......... .......... .......... ..........
510  .......... .......... .......... .......... .......... ..........
171  .......... .......... .......... .......... .......... ..........
220  .......... .......... .......... .......... .......... ..........
208  .......... .......... .......... .......... .......... ..........
160  T.......-. .-........ .......... .......... .......... ..........
236  T.......-. .-........ .......... .......... .......... ..........
36   T.......-. .-........ .......... .......... .......... ..........
409  T.......-. .-........ .......... .......... .......... ..........
214  T.......-. .-........ .......... .......... .......... ..........
72   T.......-. .-........ .......... .......... .......... ..........
477  T.......-. .-........ .......... .......... .......... ..........
183  T.......-. .-........ .......... .......... .......... .........-
328  T.......-. .-........ .......... .......... .......... ..........
18   T.......-. .-........ .......... .......... .......... ..........
218  T.......-. .-........ .......... .......... .......... ..........
287  T.......-. .-........ .......... .......... -......... ..........
227  T.......-. .-........ .......... .......... .......... ..........
46   T.......-. .-........ .......... .......... .......... ..........
50   T.......-. .-.....-.. .......... .......... .......... ..........
132  T.......-. .-........ .......... .......... .......... ..........
245  T.......-. .-.......- .......... .......... .......... ..........
601  T......T-. A-........ .......... .......... .......... ..........
124  T.......-. .-........ .......... .......... .......... ..........
235  T.......-. .-........ .......... .......... .......... ..........
339  T.......-. .-........ .......... .......... .......... ..........
225  T.......-. .-........ .......... .........-. .......... ..........
248  T.......-. .-........ .......... .......... .......... ..........
217  T.......-. .-........ .......... .......... .......... ..........
```

16S rDNA gene continued




5.8S rDNA gene
181
240
762 CCGGTCGGCC CCCGAACGCC CCGCCGGCGG ATGCCTCGGC CCGGGCGGCG ACGAAGAGCG
845 .....
49 .....
764 .....
507 .....
125 .....
73 .....
93 .....
759 .....
60 .....
410 .....
969 .....
480 .....
962 .....
643 .....
534 .....
65 .....
746 .....
529 .....
761 .....
68 .....
350 .....
156 .....
100 .....
535 .....
510 .....
171 .....
220 .....
208 .....
160 .....
236 .....
36 .....
409 .....
214 .....
72 .....
477 .....
183 .....
328 .....
18 .....
218 .....
287 .....
227 .....
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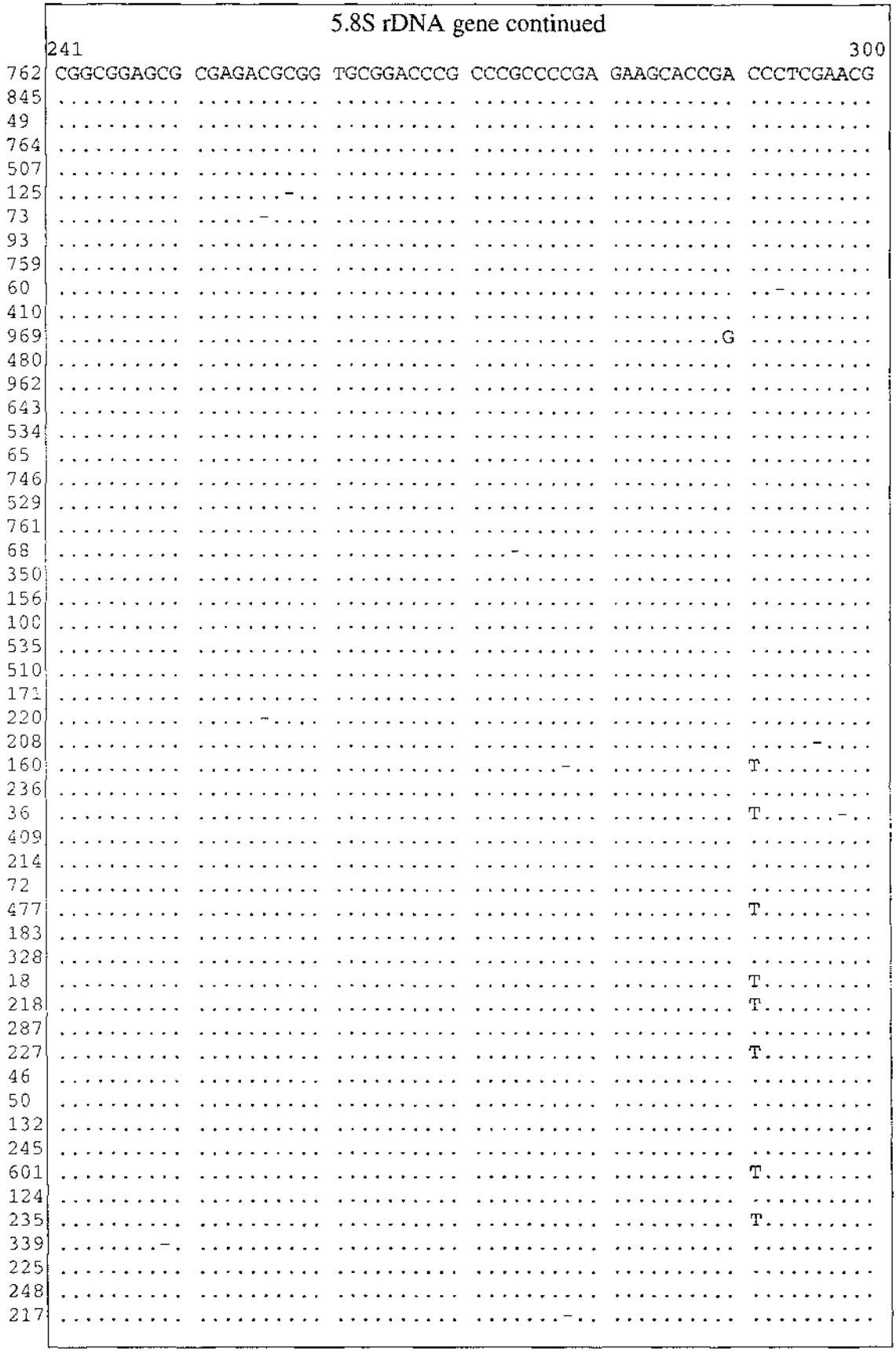
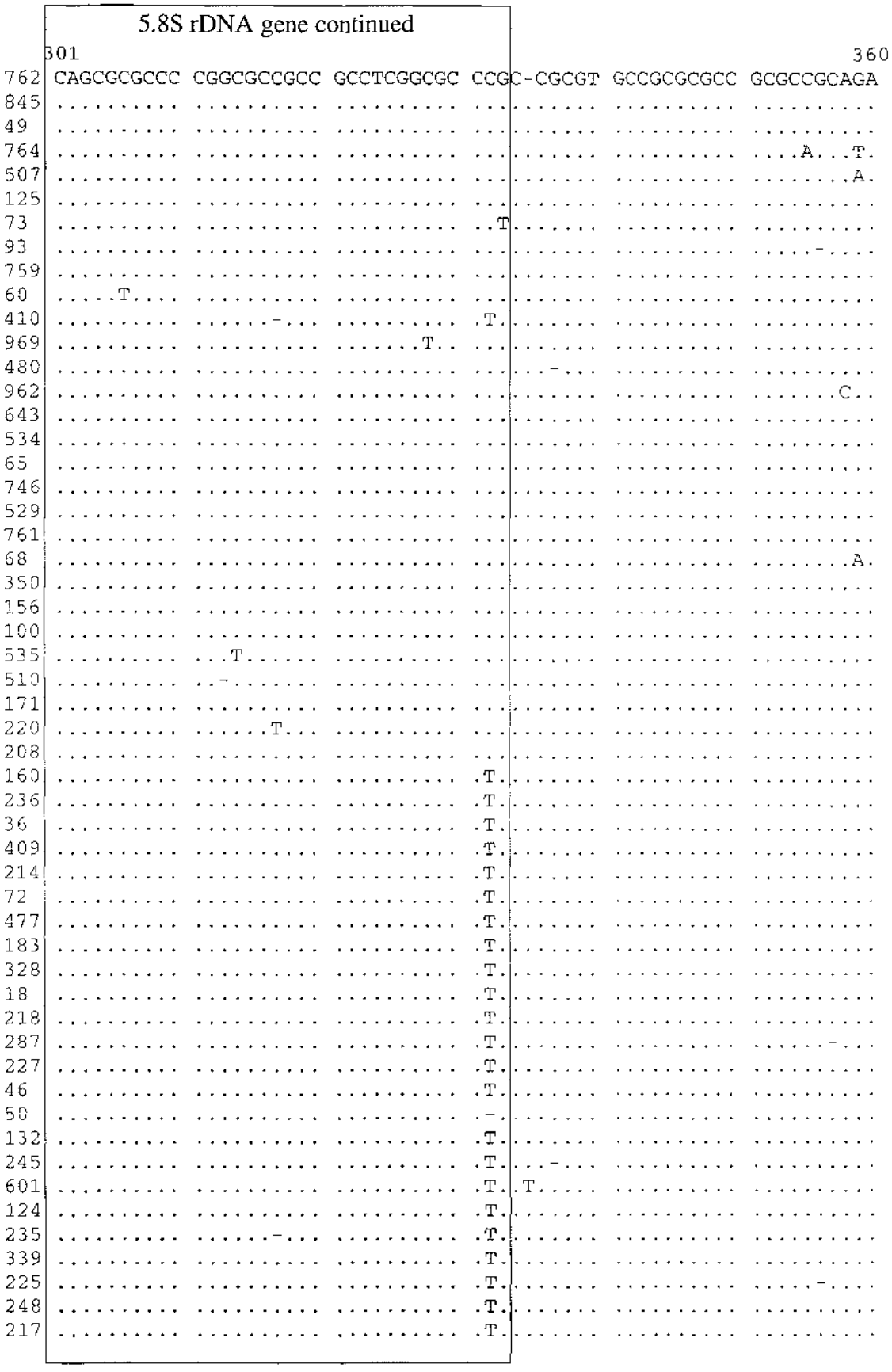
245 .....
601 .....
124 .....
235 .....
339 .....
225 .....
248 .....
217 .....


5.8S rDNA gene continued
241
300
762 CGGCGGAGCG CGAGACGCGG TGCGGACCCG CCCGCCCCGA GAAGCACCGA CCCTCGAACG
845 .......... .......... .......... .......... .......... ..........
49 .......... .......... .......... .......... .......... ..........
764 .......... .......... .......... .......... .......... ..........
507 .......... .......... .......... .......... .......... ..........
125 .......... ........-. .......... .......... .......... ..........
73 .......... .....-.... .......... .......... .......... ..........
93 .......... .......... .......... .......... .......... ..........
759 .......... .......... .......... .......... .......... ..........
60 .......... .......... .......... .......... .......... ..-.......
410 .......... .......... .......... .......... .......... ..........
969 .......... .......... .......... .......... .........G ..........
480 .......... .......... .......... .......... .......... ..........
962 .......... .......... .......... .......... .......... ..........
643 .......... .......... .......... .......... .......... ..........
534 .......... .......... .......... .......... .......... ..........
65 .......... .......... .......... .......... .......... ..........
746 .......... .......... .......... .......... .......... ..........
529 .......... .......... .......... .......... .......... ..........
761 .......... .......... .......... .......... .......... ..........
68 .......... .......... .......... ...-...... .......... ..........
350 .......... .......... .......... .......... .......... ..........
156 .......... .......... .......... .......... .......... ..........
100 .......... .......... .......... .......... .......... ..........
535 .......... .......... .......... .......... .......... ..........
510 .......... .......... .......... .......... .......... ..........
171 .......... .......... .......... .......... .......... ..........
220 .......... .....-.... .......... .......... .......... ..........
208 .......... .......... .......... .......... .......... ......-...
160 .......... .......... .......... ........-. .......... T.........
236 .......... .......... .......... .......... .......... ..........
36 .......... .......... .......... .......... .......... T.......-..
409 .......... .......... .......... .......... .......... ..........
214 .......... .......... .......... .......... .......... ..........
72 .......... .......... .......... .......... .......... ..........
477 .......... .......... .......... .......... .......... T.........
183 .......... .......... .......... .......... .......... ..........
328 .......... .......... .......... .......... .......... ..........
18 .......... .......... .......... .......... .......... T.........
218 .......... .......... .......... .......... .......... T.........
287 .......... .......... .......... .......... .......... ..........
227 .......... .......... .......... .......... .......... T.........
46 .......... .......... .......... .......... .......... ..........
50 .......... .......... .......... .......... .......... ..........
132 .......... .......... .......... .......... .......... ..........
245 .......... .......... .......... .......... .......... ..........
601 .......... .......... .......... .......... .......... T.........
124 .......... .......... .......... .......... .......... ..........
235 .......... .......... .......... .......... .......... T.........
339 .........-. .......... .......... .......... .......... ..........
225 .......... .......... .......... .......... .......... ..........
248 .......... .......... .......... .......... .......... ..........
217 .......... .......... .......... ........-. .......... ..........


5.8S rDNA gene continued
301
360
762 CAGCGCGCCC CGGCGCCGCC GCCTCGGCGC CCGC-CGCGT GCCGCGCGCC GCGCCGCAGA
845 .......... .......... .......... ...........
49 .......... .......... .......... ...........
764 .......... .......... .......... ....A..T.
507 .......... .......... .......... .......A.
125 .......... .......... .......... ...........
73 .......... .......... .......... ..T.......
93 .......... .......... .......... ......-....
759 .......... .......... .......... ...........
60 .....T.... .......... .......... ...........
410 .......... .......-.. .......... .T.........
969 .......... .......... .......T.. ...........
480 .......... .......... .......... ....-......
962 .......... .......... .......... .......C..
643 .......... .......... .......... ...........
534 .......... .......... .......... ...........
65 .......... .......... .......... ...........
746 .......... .......... .......... ...........
529 .......... .......... .......... ...........
761 .......... .......... .......... ...........
68 .......... .......... .......... .......A.
350 .......... .......... .......... ...........
156 .......... .......... .......... ...........
100 .......... .......... .......... ...........
535 .......... ...T...... .......... ...........
510 .......... ..-....... .......... ...........
171 .......... .......... .......... ...........
220 .......... ......T... .......... ...........
208 .......... .......... .......... ...........
160 .......... .......... .......... .T.........
236 .......... .......... .......... .T.........
36 .......... .......... .......... .T.........
409 .......... .......... .......... .T.........
214 .......... .......... .......... .T.........
72 .......... .......... .......... .T.........
477 .......... .......... .......... .T.........
183 .......... .......... .......... .T.........
328 .......... .......... .......... .T.........
18 .......... .......... .......... .T.........
218 .......... .......... .......... .T.........
287 .......... .......... .......... .T.......-...
227 .......... .......... .......... .T.........
46 .......... .......... .......... .T.........
50 .......... .......... .......... .-.........
132 .......... .......... .......... .T.........
245 .......... .......... .......... .T.....-....
601 .......... .......... .......... .T..T.......
124 .......... .......... .......... .T.........
235 .......... .......-.. .......... .T.........
339 .......... .......... .......... .T.........
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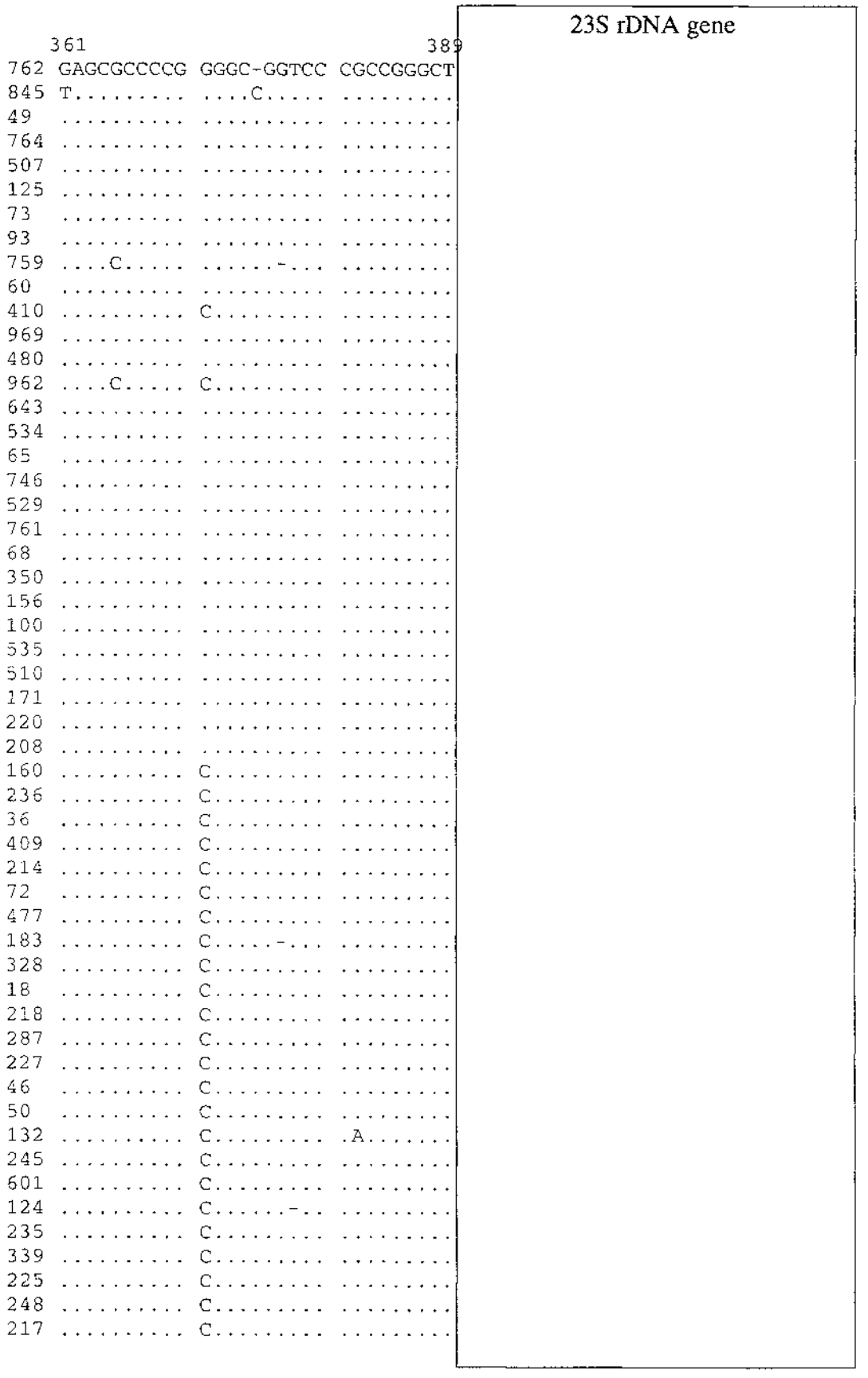
248 .......... .......... .......... .T.........
217 .......... .......... .......... .T.........


23S rDNA gene
361
389
762 GAGCGCCCCG GGGC-GGTCC CGCCGGGCT
845 T..... ....C..... .....
49 ..... ..... .....
764 ..... ..... .....
507 ..... ..... .....
125 ..... ..... .....
73 ..... ..... .....
93 ..... ..... .....
759 ....C..... .....-... .....
60 ..... ..... .....
410 ..... C..... .....
969 ..... ..... .....
480 ..... ..... .....
962 ....C..... C..... .....
643 ..... ..... .....
534 ..... ..... .....
65 ..... ..... .....
746 ..... ..... .....
529 ..... ..... .....
761 ..... ..... .....
68 ..... ..... .....
350 ..... ..... .....
156 ..... ..... .....
100 ..... ..... .....
535 ..... ..... .....
510 ..... ..... .....
171 ..... ..... .....
220 ..... ..... .....
208 ..... ..... .....
160 ..... C..... .....
236 ..... C..... .....
36 ..... C..... .....
409 ..... C..... .....
214 ..... C..... .....
72 ..... C..... .....
477 ..... C..... .....
183 ..... C.....-... .....
328 ..... C..... .....
18 ..... C..... .....
218 ..... C..... .....
287 ..... C..... .....
227 ..... C..... .....
46 ..... C..... .....
50 ..... C..... .....
132 ..... C..... .A.....
245 ..... C..... .....
601 ..... C..... .....
124 ..... C.....-.. .....
235 ..... C..... .....
339 ..... C..... .....
225 ..... C..... .....
248 ..... C..... .....
217 ..... C..... .....

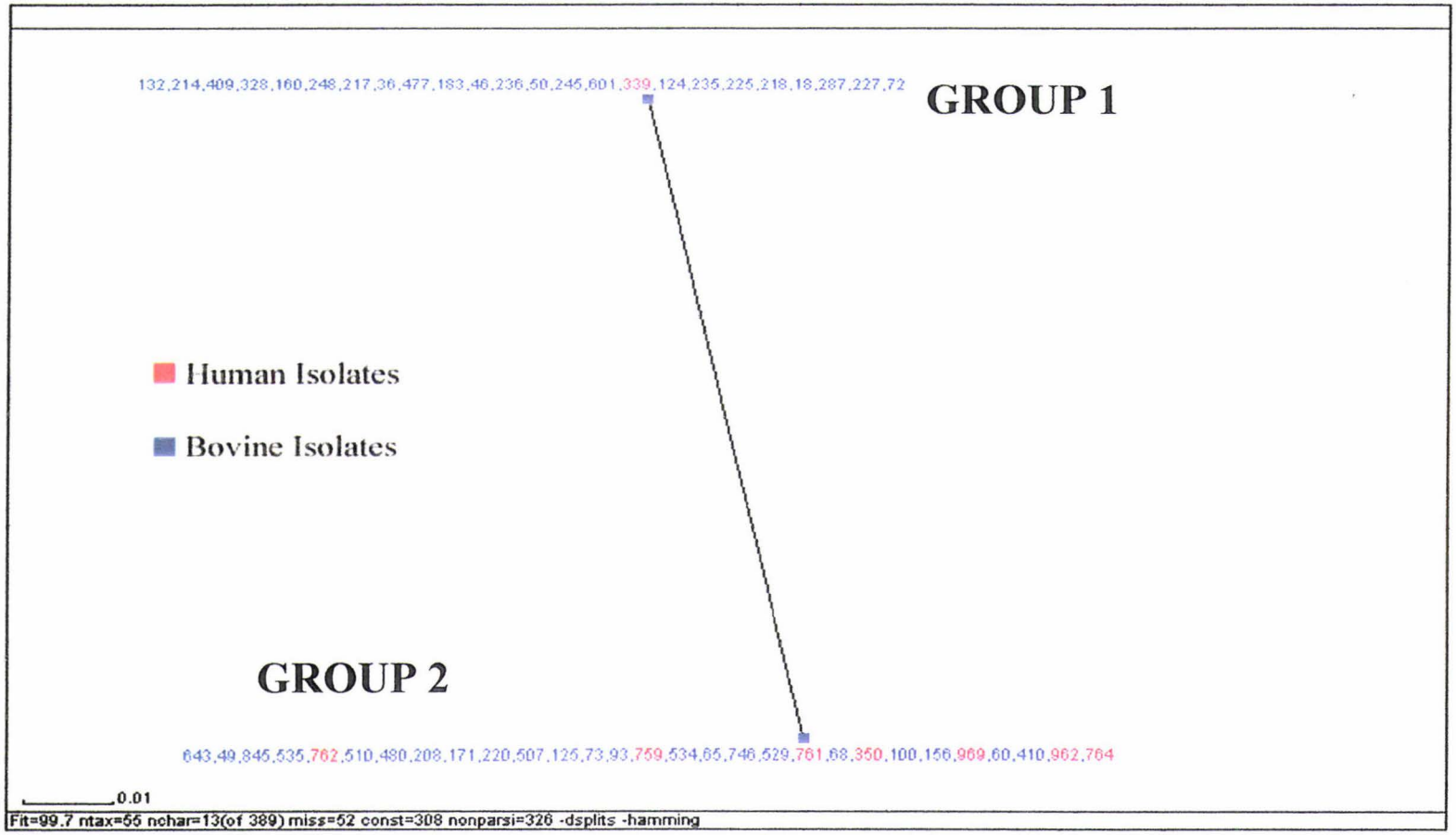



**Figure 3.32:** Splitstree diagram showing the phylogenetic relationships of human and bovine *G. intestinalis* isolates using a 13bp region covering the single nucleotide substitution at position 61. This separated the isolates into two distinct groups with a 99.7% fit, the distances between the groups represents the sequence diversity between them.

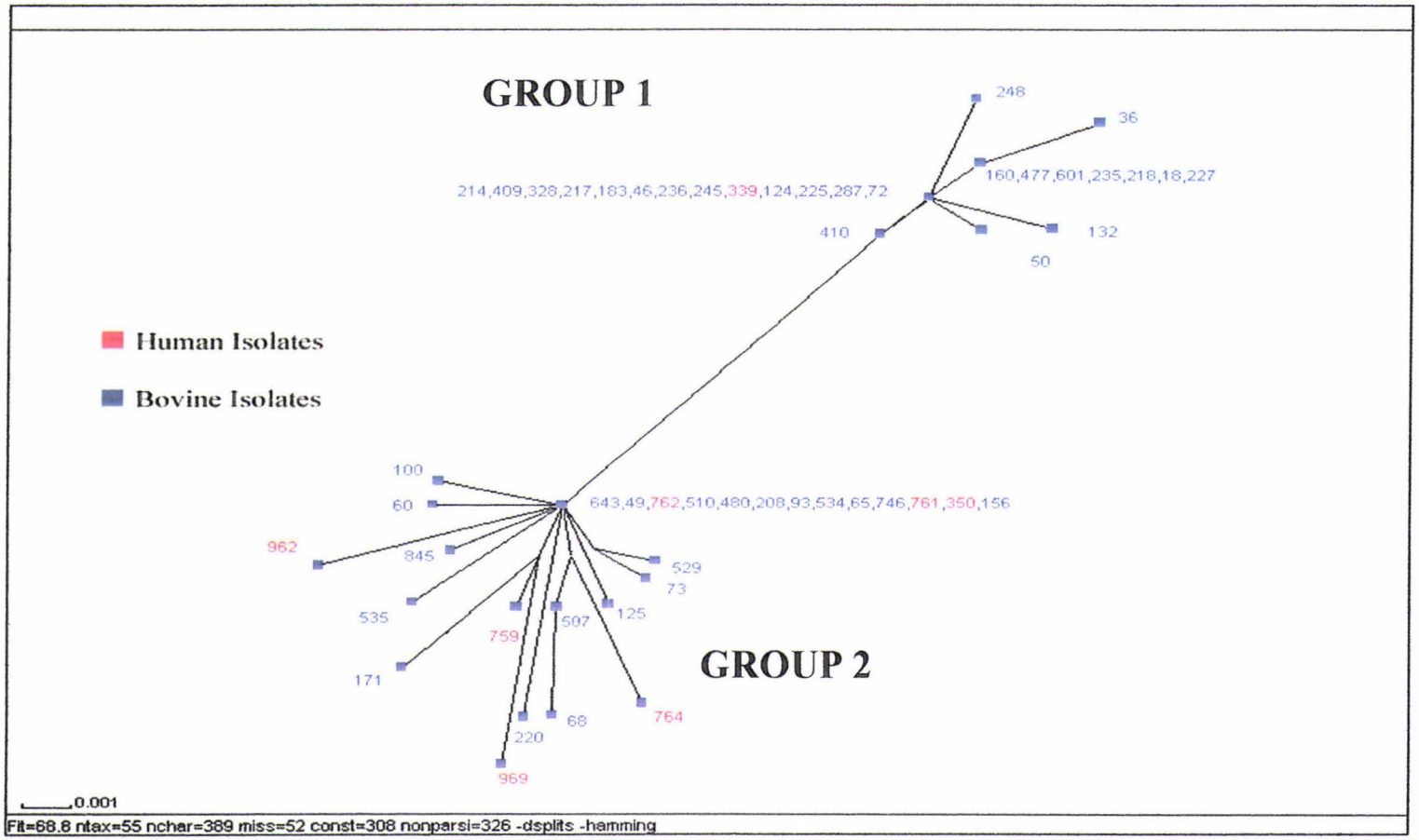



**Figure 3.33:** Splitstree diagram showing the phylogenetic relationship of human and bovine *G. intestinalis* isolates using the 389 nucleotides of the rDNA sequence, showing greater diversity amongst the isolates in group 2. The orientation of the branches is unimportant, and the distances represent the sequence diversity.

# CHAPTER 4: DISCUSSION

This thesis had three major goals. Firstly, to determine the feasibility of AFLP and RAPD DNA fingerprinting techniques for direct genotyping of cysts of *G. intestinalis*. Secondly, to examine the stability of AFLP and RAPD analysis of *in vitro* cultures of *G. intestinalis* placed under selective pressure and finally, to genotype a large population of human and bovine isolates of *G. intestinalis* using rDNA sequence analysis.

## 4.1 AFLP analysis

### 4.1.1 AFLP Analysis of Trophozoite DNA

The use of AFLP analysis on DNA extracted from *in vitro* cultures of two human and three animal isolates of *G. intestinalis* revealed identical banding patterns when visualised on 2% agarose gels (Figure 3.3, Figure 3.4 and Figure 3.5, not all primer combinations shown). The higher resolution of a 5% polyacrylamide gel improved the separation of the PCR products and the greater sensitivity of silver staining, over agarose gels and ethidium bromide staining, also produced identical AFLP fingerprints in the isolates tested (Figure 3.7 and Figure 3.8). A previous DNA fingerprinting study, by RAPD analysis using the same *in vitro* cultures had also shown the cultures to have identical patterns (Farrant, 1995). This information lead to two possibilities:

a) That the cultures had been contaminated at the point of their establishment or over time during their maintenance, or

b) That the selective pressure of axenic culture and the flexibility of the *Giardia* genome had lead to the cultures to become genotypically identical.

Due to the fact that the *in vitro* cultures were displaying identical banding patterns, it was decided to trial AFLP and RAPD analysis directly on DNA extracted from *Giardia* cysts isolated from the faeces of an infected host. The use of DNA extracted directly from *Giardia* cysts would also remove the selection process culturing creates, where only those isolates that can be grown *in vitro*, or passaged *in vivo* through rodents are examined.

### 4.1.2 *G. intestinalis* Cyst DNA Extraction

The filamentous layer of the cyst wall has been shown to be disrupted by repeated cycles of freezing and thawing (Erlandsen, 1990b), and this information was used to develop a method to extract intact genomic DNA from *G. intestinalis* cysts for AFLP and RAPD analysis. DNA extraction procedures that would break the DNA would result in false polymorphic bands as they would result in no PCR amplification.

The extraction of the DNA was detected by the presence of a high molecular weight band on a 1.6% gel, with no smearing being present in the lane, which would indicate damage to the DNA. The repeated cycles of freezing and thawing was not seen to release DNA, so the addition of a detergent to aid in the lysis of the cysts was determined to be sufficient for this release. The addition of proteinase K to digest proteins in the lysate was found not to be necessary, but the addition of RNase to remove the RNA was incorporated into the final method that was used to extract DNA for both AFLP and RAPD analysis (Figure 3.1).

The DNA was purified to remove inhibitors from the faecal material that were not removed during the DNA extraction procedure. This was achieved using a 0.025 µm MF-Millipore$^{TM}$ Membrane Filter (Millipore) to dialyse the DNA against sterile TE buffer. The extracted DNA was PCR amplified using *Giardia* genus specific (Gsp) and *Giardia intestinalis* specific (GI) primers to verify that the DNA extracted was that of *Giardia* were employed, and both sets of primers gave the expected band size (Figure 3.2) verifying the DNA to be from *G. intestinalis*.

### 4.1.3 AFLP Analysis of DNA from *G. intestinalis* Cysts

AFLP analysis amplifies restriction fragments of genomic DNA to detect polymorphic bands between samples. False polymorphic bands can be produced from the breakage of the DNA during DNA extraction, insufficient template DNA, or the generation of different restriction fragments by the star activity of some enzymes, for example *Eco*RI. All of the restriction fragments from DNA extracted from *G. intestinalis* cysts were generated using *Mse*I. Since the extract volume was to small to determine the amount of DNA, star activity of *Eco*RI could not be avoided, which would have produced false polymorphic bands.

Initially, results were displaying non-reproducible patterns (Figure 3.9 and Figure 3.10) and this was determined to be due to the low amount of DNA in the PCR reaction. Repeating the isolation from the faeces and pooling the cysts together for each isolate increased the number of cysts present in the DNA extraction procedure. This increased the amount of DNA and resulted in the patterns becoming reproducible (Figure 3.11, Figure 3.12 and Figure 3.13). This process was very expensive due to the large volume of immuno-magnetic beads required to obtain a reproducible banding pattern, so only four isolates could be used in the study. The technique was shown to be reproducible when the cysts isolated in duplicate from the same faecal specimen were fingerprinted by AFLP analysis (Figure 3.14). This reproducibility indicates the presence of DNA from contaminating organisms to not be present, as the same organisms would have to be present in the same proportion to maintain the reproducibility. The likely event that during each isolation procedure the same contaminating organisms were present in the same proportion does not seem highly plausible.

Although AFLP analysis of DNA extracted from the cysts of *G. intestinalis* has been achieved, the amount of DNA required creates a significant cost for the analysis due to the large amount of immuno-magnetic beads required to obtain sufficient numbers of cysts. So in order to understand whether human and animal isolates represent a single population or several populations of host-specific stains requires the analysis

of a large population of *G. intestinalis* but the cost involved would not make this practical

The incorporation of the restriction digestion for AFLP analysis requires a significant amount of DNA to be present to ensure that the sample taken for PCR amplification contains a representation of each restriction fragment. The high cost associated with this technique does not make this a practical genotyping method which is why another genotyping method that did not rely on restriction fragments but only PCR was examined as this would require less DNA to produce reproducible banding patterns. For this reason RAPD analysis was chosen as this method to analyse genomic DNA by PCR alone.

## 4.2 RAPD Analysis

Results obtained from the rDNA sequence analysis study indicated the requirement for nested primers to obtain PCR products (Figure 3.29) and it was decided to modify the RAPD technique to include pre-amplification and selective amplification stages as with the AFLP analysis technique. Due to the modification of the technique, it was examined to see whether this would alter the resulting banding patterns using DNA extracted from an axenic trophozoite culture. The technique was then used to examine use with DNA extracted from the cysts of *G. intestinalis* isolated from infected hosts.

### 4.2.1 RAPD Analysis of Trophozoite DNA

The use of the modified (with nested primers) RAPD technique was shown to be capable of producing DNA banding patterns (Figure 3.19 and Figure 3.20). The comparison of the banding patterns from the modified and unmodified techniques showed that when sufficient DNA was available to produce stable patterns there was no variation between the banding patterns of the two techniques (Figure 3.21).

### 4.2.2 RAPD Analysis of DNA from *G. intestinalis* Cysts

RAPD analysis from *G. intestinalis* cysts was initially shown to be non-reproducible but could be stabilised by increasing the amount of cysts in the DNA extraction procedure (data not shown). Compared to AFLP analysis, the amount of cysts required in the extraction procedure is far less, only requiring the cysts from two IMS cyst isolations as opposed to twelve, making this a cost effective alternative to AFLP analysis.

When the cyst numbers were increased in the DNA extraction procedure, the isolates were shown to have reproducible banding patterns (Figure 3.22), although the four isolates showed four different banding patterns (Figure 3.23 and Figure 3.24). Due to the low number of isolates studied, whether the four isolates show a great diversity in the species requires a study of a greater sample size but this initial study shows the possibility for direct genotyping of *G. intestinalis* cysts examining the entire genome. Although the banding patterns were the same between duplicates there were variations in the intensities of some of the bands. These variations in the band intensities are likely to be due to the inconsistent amount of DNA used in the each PCR reaction as the volume obtained from the DNA extraction process does not allow the concentration of the DNA to be measured.

The possibility of directly genotyping the cysts of *G. intestinalis* would allow a greater number of isolates to be tested giving a better representation of the genetic diversity of the species and also remove the tedious method of producing axenic and *in vivo* cultures.

## 4.3 Selective Pressure on *in vitro* Cultures

A study using 25 *in vitro* cultures established from a single infected host has detected different genotypes (Upcroft, 1994) possibly resulting from the process of producing axenic cultures which may be a selective pressure. The possibility that selective pressure can result in the change of the genotypes of *in vitro* cultures was briefly examined in this thesis to determine the genotype of the cultures may be an due to the culturing procedure itself. By comparison of the DNA fingerprints of a culture placed under selective pressure to a wild type culture, both AFLP and RAPD analysis could detect changes in the genome.

An initial concentration of 0.05 mg/L metronidazole was initially selected, as this concentration was enough to kill the majority of the trophozoites. The removal of the selective pressure after two days allowed the trophozoites to recover without killing the remaining cells with continual antibiotic treatment. Each time the metronidazole was placed back into the growth media, the concentration was increased by 0.025 mg/L to aid the cells to develop a higher tolerance for the antibiotic, to a maximum of 1 mg/L. Using RAPD analysis, the banding pattern was seen to change from the original cells that had not been placed under selective pressure (Figure 3.26). This supports the idea of the flexibility of the *Giardia* genome and the idea that producing axenic cultures to genotype *G. intestinalis* introduces a bias due to this, along with the bias of selecting only those isolates capable of being grown. AFLP analysis did not detect any polymorphic differences (Figure 3.25), which may indicate this method is less sensitive than RAPD analysis, but only two primers were examined for AFLP analysis and other primers and/or primer combinations may have detected differences.

This initial study shows changes in some of the banding patterns of the RAPD analysis that would indicate the selective pressure can result in genotyping results and would be beneficial for further study. If it were found that the process of culturing *G. intestinalis* results in the alterations to the genome, it would raise the

question as to the validity of genotyping results of studies that rely on using cultures. Further selective pressure studies of *in vitro* cultures to examine the genome stability of *G. intestinalis* cultures would be beneficial to understanding the genotyping results when using cultures.

## 4.4 rDNA Sequence Analysis

The study by Hunt C. L. (1999) using rDNA sequence analysis of 9 human and 15 bovine isolates from the Manawatu and Waikato region had identified sequence differences at position 61 and 189. These differences in the rDNA locus separated the isolates into two groups and this was also seen in this study.

Sufficient template was not obtained to allow PCR amplification as in the original study so a new set of outer PCR primers were used in conjunction with the original inner primer set to increase the sensitivity of the method. The new primer pair (MAT1/MAT2) amplifies an 840bp PCR product size and a 505bp product with the Cyn0/Cyn2 primer pair. No PCR product is seen when using DNA isolated from *G. intestinalis* cysts using the outer primer pair (MAT1/MAT2). The lack of amplification by a single set of primers may be due to either:

1. A very low amount of DNA in the reaction or
2. The presence of inhibitors that are removed during the incubation stages leading to a very low amount of amplification that is undetectable by ethidium bromide staining.

In the second PCR reaction using the Cyn0/Cyn2 primer pair and the MAT1/MAT2 PCR product as template, the expected 505bp fragment was detected (Figure 3.28). The increase in the sensitivity of this technique by the inclusion of a second primer pair to use as nested primers may make it possible to genotype isolates of *G. intestinalis* from waterways were numbers of cysts found at approximately 10 cysts/100L at the high point of water contamination.

For sequence analysis a 389bp fragment was analysed for sequence alignment of the isolates. This 389bp sequence started 154bp upstream of the 3' end of the 16S rDNA gene and extended to the beginning of the 23S rDNA including the internal non-transcribe spacer 1 (ITS-1) and ITS-2 regions and the 5.8S rDNA gene.

The results from this current study separated the isolates into two groups based on the base substitution at position 61 and the GCGCGC sequence at position 189. The two groups were the same detected by Hunt (1999). As with the study by Hunt (1999) there was also greater sequence diversity among the isolates in Group 2 (Figure 3.33). Unlike the initial study there was no correlation between the groups and the host species that would support the theory of *G. intestinalis* being a zoonotic parasite. This is of importance in farming communities where environmental waterways can become contaminated with *G. intestinalis* cysts from infected cattle from the local farms, exposing humans to the potential of acquiring giardiasis. For the interaction between the human and bovine isolates to be more robust, additional human isolates are required.

## 4.5 Summary and Future Directions

This thesis has shown that the use of genomic DNA extracted directly from the cysts of *G. intestinalis*, isolated from infected hosts, can be fingerprinted using AFLP and RAPD analysis techniques. Although use of both of these two techniques have been achieved the use of more samples would have been more beneficial. However if these techniques are to be applied to study *G. intestinalis* isolates, it would be more practical to perform RAPD analysis, as the cost required to isolate the cysts by IMS is one sixth that required for AFLP analysis.

If RAPD analysis is to be used there are two possible uses for the DNA fingerprinting technique:

1 The genotyping of isolates of *G. intestinalis* can be performed directly on cysts by comparison of the DNA fingerprints generated.

2 A large number of samples can be fingerprinted to identify the different genotypes. Bands representing each of these genotypes can be extracted and sequenced to produce specific primers for each genotype. Genotyping can then be done by specific PCR.

RAPD analysis requires cysts free from contaminating organisms that could produce polymorphic bands not originating from *G. intestinalis*. This has been achieved using IMS and the cost of this procedure is significant. Future work with RAPD analysis would be best if a more cost effective isolation technique could be developed. The current method of immuno-magnetic separation is effective in isolating the cysts but the cost involved makes it impractical for regular use.

The use of genotyping techniques that can be applied directly to DNA extracted from cysts of *G. intestinalis* is of importance as this thesis has shown the ability to alter the RAPD fingerprints when *G. intestinalis* trophozoites are grown under selective pressure. The change in the growth conditions from the host to an axenic culture or *in vivo* culture could also place selective pressures on the organism that could result in changes to the genotyping results. A selection process is also placed on the

current genotyping methods due to the inability to genotype those isolates that fail to convert to *in vitro* culture or *in vivo* passage.

The rDNA analysis results found the same separation of the isolates into two major groups as described in the study by Hunt (1999). Unlike the original study these results support the idea of a zoonotic parasite and have implications where cattle excrete *G. intestinalis* cysts into natural waterways *via* farm runoff, but analysis of more human isolates are required to supplement this data for definitive conclusions.

Ultimately it is hoped that this thesis will instigate the use of RAPD or rDNA sequence analysis to study the cysts of *G. intestinalis*, eliminating the use of cultures, to enable a better understanding of the zoonotic potential of this organism.

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# APPENDICES

## APPENDIX A: REAGENTS

**TY1-S-33 Growth Media (pH 7.0)**

| | |
|---|---|
| Trypticase Soy Broth (DifcoLaboratories) | 20.0 g |
| Yeast extract powder (DifcoLaboratories) | 10.0 g |
| Glucose (BDH) | 10.0 g |
| Sodium Chloride (BDH) | 2.0 g |
| Di-potassium hydrogen phosphate (BDH) | 1.0 g |
| Potassium dihydrogen phosphate (BDH) | 0.60 g |
| L-Cysteine Monohydrochloride (Sigma) | 1.50 g |
| Ferric Ammonium Citrate (BDH) | 0.0023 g |
| L-Ascorbic Acid (BDH) | 0.20 g |
| NCTC 135 (Sigma) | 0.94 g |
| Bile Bacteriological (Sigma) | 0.80 g |
| Benzyl Penicillin (Biochemie) | 0.060 g |
| Gentamycin Sulphate (Sigma) | 0.050 g |
| Vancomycin (Sigma) | 0.020 g |
| Bovine Serum | 100.0 mL |
| Distilled water to | 1000.0 mL |

All dry ingredients were dissolved in 500 mL of distilled water, bovine serum added and adjusted to pH 7.0 with 1 M NaOH and made up to 1000 mL with sterile distilled water.

The solution was clarified by positive pressure filtration through five sheets of Whatman 1 filter paper, followed by a 0.45 $\mu$m pore membrane filter and then a 0.2 $\mu$m pore membrane filter (Advantec MFS, Inc). The media was sterilised by positive pressure filtration through a Supor$^{TM}$ 0.2 $\mu$m filter (Gelman Sciences) that had previously been sterilised by autoclaving at 121$^{o}$C and 103 kilopascals (kPa) for

15 minutes. Aliquots of 500 mL of the media were placed into sterile Schott® bottles and stored at 4°C for up to two weeks.

**Saline Tris-EDTA (STE) Buffer 10x**

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

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

**0.2 M EDTA (pH 7.2)**

| | |
|---|---|
| EDTA (Disodium salt, BDH) | 74.45 g |
| Distilled Water to | 1000 mL |

The solution was adjusted, using 5.0 M NaOH, to pH 7.2 and stored at room temperature.

**1.0 M Tris HCl (pH 7.5)**

| | |
|---|---|
| Tris (GibcoBRL) | 121.14 g |
| Distilled Water to | 1000 mL |

The solution was adjusted with 5.0 M hydrochloric acid to pH 7.5 and stored at room temperature.

**PBS**

| | |
|---|---|
| Sodium Chloride | 8.50 g |
| di-Sodium Hydrogen Orthophosphate (BDH) | 2.70 g |
| Sodium di-Hydrogen Orthophosphate (BDH) | 0.39 g |
| Distilled Water to | 1000 mL |

The solution was sterilised by autoclaving at 121°C and 103 kPa for 15 minutes and stored at room temperature.

**Tris EDTA (TE) Buffer**

| | |
|---|---|
| 1.0 M Tris-HCl (pH 7.5) | 10.0 mL |
| 0.2 M EDTA (pH 7.2) | 5.0 mL |
| Distilled Water to | 1000.0 mL |

The solution was sterilised by autoclaving at 121°C and 103 kPa for 15 minutes and stored at room temperature.

**Bromophenol Blue Loading Dye**

| | |
|---|---|
| 0.1% w/v Bromophenol Blue | 1.0 g |
| 80% v/v Glycerol | 800 mL |
| 1x E Buffer up to | 1000 mL |

This solution was stored at room temperature.

**1.0 M Sucrose**

| | |
|---|---|
| Sucrose | 171.15 g |
| Distilled Water to | 500 mL |

**1 Kb Plus DNA Ladder™**

| | |
|---|---|
| Bromophenol Blue Loading Dye | 100 μl |
| 1 Kb Plus DNA Ladder | 40 μl |
| 1x TE Buffer | 860 μl |

This solution was stored at room temperature.

**1 Kb Plus DNA Ladder**

The 1 Kb Plus DNA Ladder is suitable for sizing linear, double-stranded DNA fragments from 100 bp to 12 kb. The ladder consists of 12 bands ranging in size from 1,000 to 12,000 bp in exact 1,000-bp increments, as well as 7 bands from 100 to 850 bp. For easy reference, there is a doublet of orientation bands at 1,650 and 2,000 bp. This ladder can be stained with ethidium bromide, SYBR® Green or radiolabeled using T4 polynucleotide kinase, T4 DNA polymerase, DNA polymerase I, or the large fragment of DNA polymerase I (Klenow fragment). Fill-in labeling can use any radiolabeled dNTP.

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

**Recommended storage condition:** –20°C.

**Related Products:**
DNA Polymerase I
DNA Polymerase I, Large (Klenow) Fragment
T4 Polynucleotide Kinase
T4 DNA Polymerase
10X BLUE-JUICE™ Gel Loading Buffer



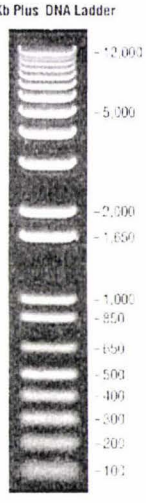


**Fig 3.34** Description of the 1Kb Plus DNA Ladder™ taken from the Gibco BRL 1999-2000 Product Catalogue.

**Low DNA Mass™ Ladder**

| | |
|---|---|
| Bromophenol Blue Loading Dye | 1 μl |
| Low DNA Mass™ Ladder | 2 μl |
| 1x TE Buffer | 7 μl |

This solution was make directly prior to use.

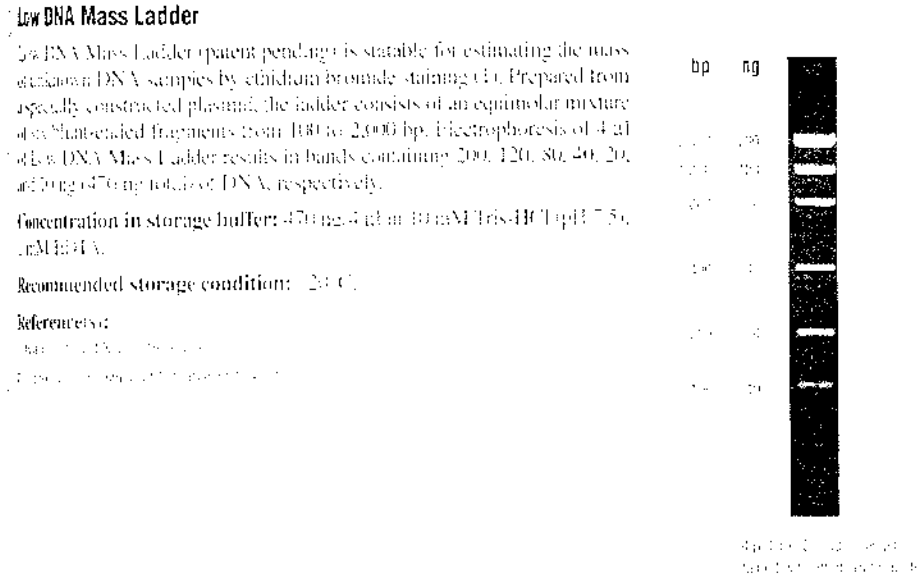


**Fig 3.35** Description of the Low DNA Mass™ Ladder taken from the Gibco BRL 1999-2000 Product Catalogue.

**pGEM®-T Easy Vector Map**

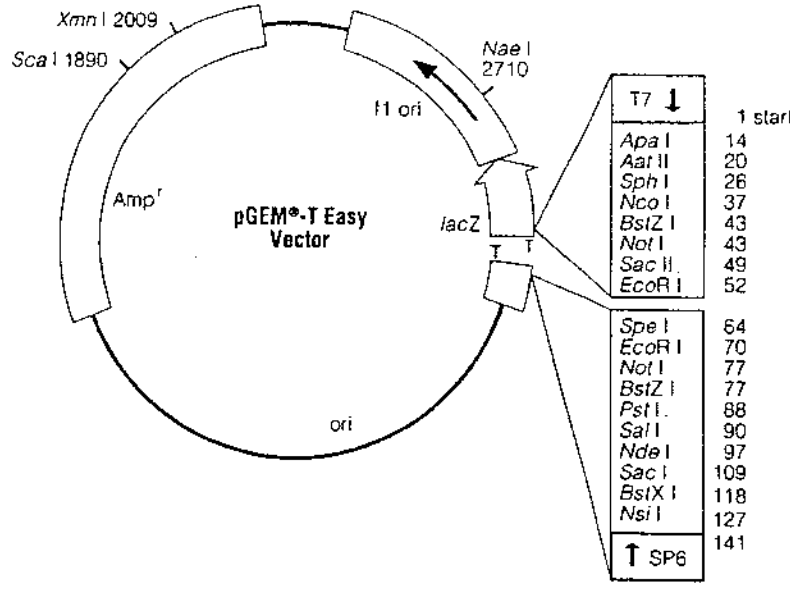


**Figure 3.36:** Map of pGEM®-T Easy Vector

**Table 2:** Table of 53 isolates used in rDNA sequence analysis study.

| Laboratory Reference Number | Isolate Information |
|---|---|
| 762 | Waikato human |
| 845 | Manawatu cow |
| 49 | Waikato calf |
| 764 | Waikato human |
| 507 | Waikato cow |
| 125 | Waikato cow |
| 73 | Waikato calf |
| 93 | Waikato calf |
| 759 | Waikato human |
| 60 | Waikato calf |
| 410 | Waikato calf |
| 969 | Waikato human |
| 480 | Waikato calf |
| 962 | Waikato human |
| 643 | Waikato cow |
| 534 | Waikato cow |
| 65 | Waikato cow |
| 746 | Waikato cow |
| 529 | Waikato cow |
| 761 | Waikato human |
| 68 | Waikato cow |
| 350 | Waikato human |
| 156 | Waikato cow |
| 100 | Waikato calf |
| 535 | Waikato cow |
| 510 | Waikato cow |
| 171 | Waikato calf |
| 220 | Waikato cow |
| 208 | Waikato calf |
| 160 | Waikato calf |
| 236 | Waikato calf |

| | |
|---|---|
| 36 | Waikato calf |
| 409 | Waikato calf |
| 214 | Waikato calf |
| 72 | Waikato calf |
| 477 | Waikato calf |
| 183 | Waikato calf |
| 328 | Waikato calf |
| 18 | Waikato calf |
| 218 | Waikato calf |
| 287 | Waikato calf |
| 227 | Waikato calf |
| 46 | Waikato calf |
| 50 | Waikato calf |
| 132 | Waikato calf |
| 245 | Waikato calf |
| 601 | Waikato calf |
| 124 | Waikato cow |
| 235 | Waikato calf |
| 339 | Waikato human |
| 225 | Waikato calf |
| 248 | Waikato calf |
| 217 | Waikato calf |

# APPENDIX B: METHODOLOGIES

## 1 Primer Concentrations

All primers were obtained from Invitrogen and were resuspended to a concentration of 1 nmol/$\mu$l in sterile TE buffer for a stock solution. For PCR the stock solution was diluted 50 fold, and for sequencing the stock solution was diluted 1000 fold for working solutions.

## 2 Agarose Gel Electrophoresis

**Materials:**

- High Strength Analytical Grade Agarose (Ultra Pure DNA Grade, Biorad)
- 1x E Buffer
- Bromophenol Blue Loading Dye
- 1 Kb Plus DNA Ladder$^{TM}$ (Gibco BRL)

**10x E Buffer**

| | |
|---|---|
| Tris HCl | 48.44 g |
| EDTA (Disodium Salt) | 3.72 g |
| Anhydrous Sodium Acetate | 4.10 g |
| Distilled Water to | 1000 mL |

The solution was prepared by dissolving the Tris HCl, EDTA, and sodium acetate in 500 mL distilled water, then adjusting the pH to 7.8 with glacial acetic acid, and the volume adjusted to 1 L. The solution was stored at room temperature and diluted 10 fold for a working strength solution.

**Method:**

A 5 μl aliquot of the PCR product was mixed with 1 μl of bromophenol blue loading dye and loaded on to the gel, along with 5 μl of the 1 Kb plus DNA ladder™. A 1.6% agarose gel was run at 80V for 1.5 hours then stained in a solution of ethidium bromide (5 μg/mL) for 10 minutes, destained in 1x E buffer for 5 minutes and visualised by UV illumination. A 2% gel was run at the same voltage for 2 hours, stained for 15 minutes, destained for 10 minutes and visualised by UV illumination.

## 3 PCR Product Quantification

**Materials:**

- High Strength Analytical Grade Agarose (Ultra Pure DNA Grade, Biorad)
- 1x E Buffer
- Bromophenol Blue Loading Dye
- Low DNA Mass™ Ladder (Gibco BRL)

**Method:**

For quantification of the PCR product, a 2 μl aliquot was mixed with 1 μl of bromophenol blue loading dye and 7 μl 1x TE buffer, loaded on a 1.6% agarose gel, along with the Low DNA mass™ ladder. The gel was run for 1.5 hours at 80V, stained in a solution of ethidium bromide (5 μg/mL) for 10 minutes, destained in 1x E buffer for 5 minutes and visualised by UV illumination. The quantity of PCR product was determined by the comparison of the band to those seen in the ladder.

## 4 DNA Concentration for Sequencing

For DNA sequencing the concentration of DNA required was dependent of the size of the product. For the plasmids containing the inserts from AFLP analysis, at least 2μl of a 100-200 ng/μl solution was required at all of the plasmids remained <5 Kb in size. The rDNA PCR product was 505bp in size and required at least 2 μl of a 10-20 ng/μl solution

## 5 AFLP Flow Diagram

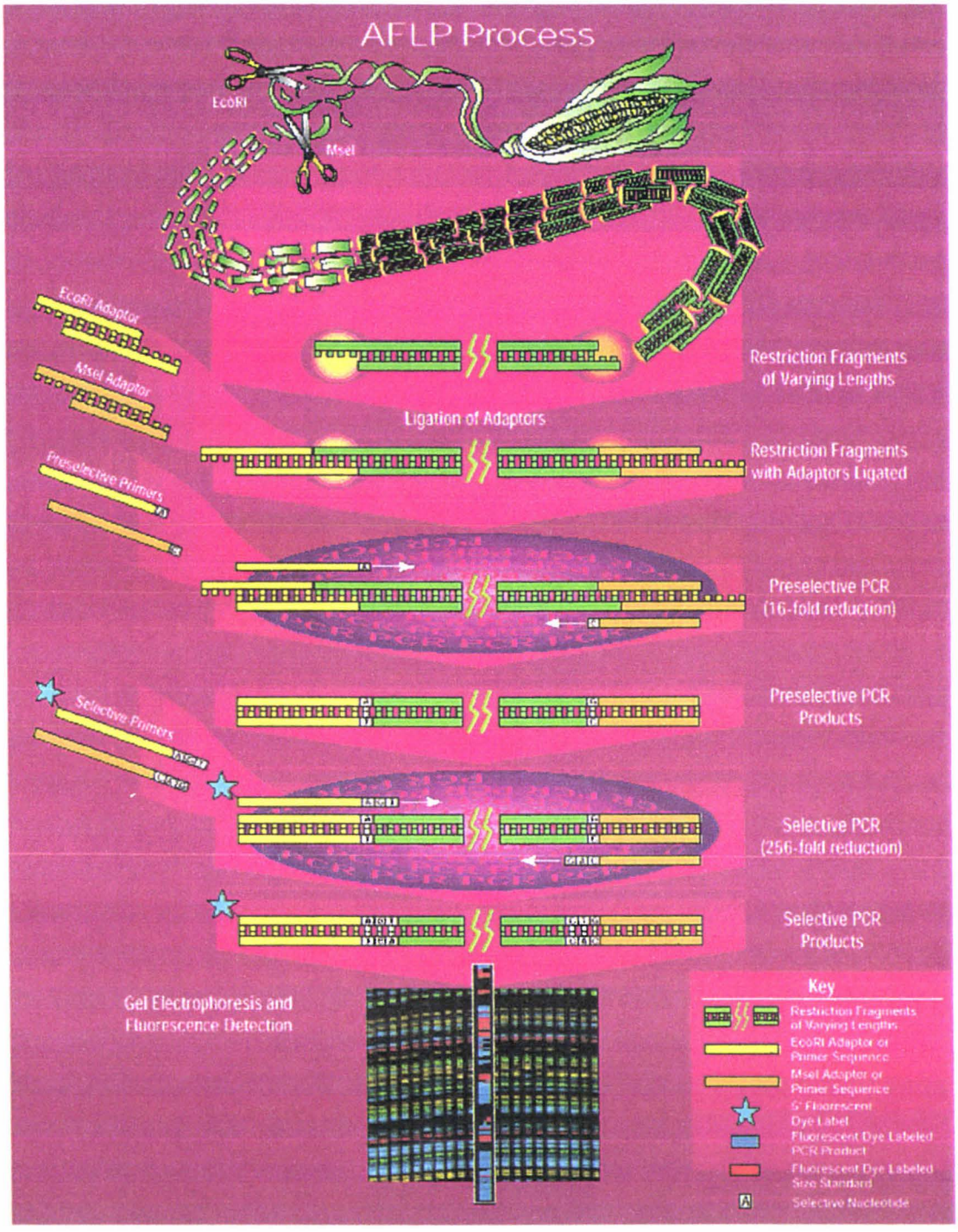


**Fig 3.37** Flow diagram describing the AFLP methodology.

(http://www.pebio.com/ag/775601/flowchart/figureNoFrames.html)