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**The Development of
Techniques to Distinguish
Species and Strains of
Giardia.**

A Thesis Presented in Partial Fulfilment of the
Requirements for the Degree of
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Kirsty Jane Farrant
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Abstract

Water supplies, in some rural areas of New Zealand, contain *Giardia* cysts. This is assumed to make the water unsuitable for human consumption. *G. intestinalis* and/or *G. muris* cysts may be present but are not distinguished by the standard test. *G. muris* infects rodents only so it is not infectious for humans. However *G. intestinalis* infects humans and a wide range of animals, but it is unclear if the strains which infect animals also infect humans. If *G. intestinalis* strains are host-specific, then since water in rural areas may contain cysts derived only from animal species it would follow that the water (even if *G. muris* and/or *G. intestinalis* cysts were found) may not be infectious for humans.

Investigation of host-specificity of *G. intestinalis* would be facilitated by a reliable test to distinguish strains of the organism and this thesis investigates the use of PCR for this purpose. A series of random primers were investigated for their ability to distinguish strains of *G. intestinalis* when used with a variety of PCR protocols. We found that several of these primers (especially GC50 at an annealing temperature of 35°C, and GC80 at an annealing temperature of 35°C) had the potential to distinguish strains. The differences seen were not large but this may be because some of the isolates were clonally related. Consequently we concluded that further modifications and extensions of PCR when applied to human and animal strains should distinguish strains and may have the potential to address the question of host-specificity.

The major aim of the thesis however was to produce primers which when used in the PCR are capable of distinguishing *G. muris* from *G. intestinalis*. The same approach, ie the use of a random primer, was used to distinguish *G. muris* from *G. intestinalis*. Clear differences were seen but the non-specificity of the random primer would allow the organisms to be reliably distinguished only in the absence of other organisms. To avoid this lack of specificity an amplified band produced with *G. muris* DNA but not with *G. intestinalis* DNA was sequenced and a primer pair was selected. These primers were, in principle, long enough (21-mer and 23-mer) to be specific for the target DNA and were chosen so as to have matched melting temperatures. The selected primer pair amplified a sequence 307bp long, and the primer sequences were specific for the target species, namely *G. muris*. Thus in our hands using PCR this primer pair amplified DNA from the available strains of *G. muris* but failed to amplify DNA from any of seven *G. intestinalis* strains.

Further work is required to establish both an optimal method for lysing cysts and to estimate the minimum number of cysts required to ensure that DNA is available for amplification. However, the availability of the *G. muris* -specific primers, along with the recently developed genus and *G. intestinalis* -specific primers should allow us to undertake investigations of water supplies to see if *G. muris*, *G. intestinalis* or both species are present. In the case of a small rural supply it would seem reasonable to accept the potability of water supplies containing *G. muris* only, as long as assurance could be given that *G. intestinalis* was not present.

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Chapter 1

Historical Review of *Giardia* and Giardiasis

1.1 Discovery

Giardia is the agent of an intestinal disease known as giardiasis. It was first described over 300 years ago, in 1681, by Antony van Leeuwenhock (1632-1723), an amateur lens maker from Delft, Holland, from his own stools (Adam, 1991; Erlandson and Meyer, 1984; Meyer, 1990). The description included notes on the size, movement and morphology of the organism, now thought to be the trophozoite of *Giardia*. He associated it with the diarrhoeic nature of his stools and his dietary habits (Adam, 1991):

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

However, it is a Czech physician, Vilem Lambl, who is credited with the discovery of the organism, which he named *Lamblia*. The organism has since been named *Giardia* after the French biologist Giard, who died in 1903 (Ilson *et al*, 1984). However, neither he, nor van Leeuwenhock, recognised the relationship between the trophozoite and the cyst form of the parasite. Grassi (1888) (reported in Adam, 1991) first noted *Giardia* cysts, thinking that they may be coccidia, but concluded that they represented the flagellated form of the organism.

1.2 Biology

1.2.1 Morphology

Giardia is found in two morphologically distinct forms: in fresh faeces as the resistant, infective cyst, and in the intestine of the infected host as the reproductive trophozoite. The trophozoite may sometimes be seen in faeces, if the stool is very diarrhoeic.

The trophozoite is responsible for the pathogenesis of the disease, and it is found free-living in the duodenum. They measure approximately 12-15 μm in length and 5-9 μm wide. Trophozoites are bilaterally symmetrical organisms, with a pear-shaped appearance. Each trophozoite has four pairs of flagella, and the anterior half of the organism contains a pair of nuclei. The posterior half of the organism contains the median body. In *G. intestinalis* it is described as 'claw-hammer' shaped, and as 'short and round' for *G. muris*. This is of use in taxonomy (Filice, 1952). No mitochondria-like structures have been detected in *Giardia* trophozoites (Adam, 1991).

The trophozoite is dorsally convex and ventrally concave. The anterior of the ventral surface is occupied by an ovoid 'sucking' disc. It is by means of this sucking disc that the organism attaches to the microvilli of the duodenum and jejunum. This feature, along with dorso-ventral flattening, differentiates *Giardia* from other Diplomonads (Feely, *et al*, 1984).

The cyst form of *Giardia* is oval in shape and ranges in size from 8-12 μm long and 7-10 μm wide. The cyst wall is hyaline and varies in thickness from 0.3-0.5 μm (Filice, 1952). They consist of 2-4 nuclei, depending on whether cell division has taken place, intracytoplasmic axenomes and median bodies. The cyst forms during the passage through the jejunum (Burke, 1977; Lambert, 1970), and passes out in the faeces. The cyst is a resistant survival mechanism, and, as the means of transmission and survival outside the host, predominates in the faeces.

1.2.2 Life Cycle

Giardia requires no intermediate host. It is transmitted from host to host, via the faecal-oral route. It has been found that only 10-100 cysts need to be ingested to cause infection (Rendtorff, 1954)

Giardia cysts are ingested, and pass through the stomach to the duodenum. Here they excyst, forming two trophozoites. These then attach firmly to the microvilli of the duodenum and jejunum by means of their sucking discs. The trophozoites then replicate by binary division.

Encystment occurs as the trophozoites pass further down the intestinal tract towards the colon. These cysts are then excreted with the faeces. The cysts are transmitted to a new host either directly, by the faecal-oral route, or indirectly, by water contamination (Adam, 1991).

1.3 Taxonomy

Two different generic names have been applied to this organism. In 1882 *Giardia* was isolated from the tadpole by Kunstler, and in 1888 Blanchard isolated *Lamblia* from mammals. It was demonstrated that both these organisms were of the same genus by Hegner (1922). The name *Giardia* is now given to all representatives of this group and the name giardiasis to the disease they cause.

Species of *Giardia* have been differentiated principally on the basis of three criteria (Filice, 1952):

1. host specificity.
2. various body dimensions.
3. variations in structure among the forms.

Using these criteria, more than 40 species of *Giardia* have been differentiated (Filice, 1952; Kulda and Nohynkova, 1978). The statistical validity of morphometric comparisons has been questioned, and host specificity of trophozoites has been disputed after observation of some degree of cross-infectivity (Grant and Woo, 1978b; Davies and Hibler, 1979).

Filice (1952) can probably be credited with the most significant work on taxonomy. He proposed the division of the genus *Giardia* into three species, based upon morphometric characterisation of the median bodies only, which he states as the only reliable characters for differentiation. The three species are:

1. *G. intestinalis* : -median bodies lie approximately across the body of the trophozoite.
-body outline is pyriform.
-found in man and other mammals, some birds and reptiles.
2. *G. muris* : -rounded median bodies parallel to the long axis of the trophozoite.
-body is short and broad.
-found in rodents and birds.
3. *G. agilis* : -median bodies fused into a single, club-shaped rod, parallel to the long axis of the trophozoite.
-narrow elongate body.
-found in amphibians, typically disappears after metamorphosis of the host.

The classification of *Giardia* is as follows (Levine, 1979):

Phylum: **Protozoa**.

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

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

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

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

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

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

1.4 Disease

1.4.1 Introduction

It was not until the 1960's and 1970's that *G. intestinalis* became acknowledged as the cause of illness in humans. This was due to the large numbers of people who became ill at this time after drinking sewage contaminated or improperly filtered water in several United States communities, and after travellers to foreign countries, especially the then USSR, returned ill from endemic areas (Logsdon, 1988).

Giardia intestinalis is one of the most common enteric protozoan parasites of man, especially prevalent in children in developing countries, and has been identified as the etiologic agent in numerous common-source outbreaks (Erlandson and Meyer, 1984). It is the intestinal parasite most frequently identified in public health laboratories in the United States (Ellmer, 1984).

1.4.2 Pathogenesis

Giardiasis has a prepatent period of between 9 and 20 days (Rendtorff, 1954). This is followed by the acute phase of infection, which usually lasts for 3-4 days. During this phase there is dense colonisation of the jejunal epithelium (Owen, *et al*, 1979). From a mouse model, it has been established that during infection the mucosa of the jejunum undergoes morphological changes. These include a reduction in villus:crypt ratio, microvillus deformation or blunting and an increase of inflammatory cells in the lamina propria. In some cases, however, changes in the mucosa may be minimal (Roberts-Thomson, *et al*, 1976).

Trophozoites are commonly detected in the duodenum of infected patients. Although they attach to the intestinal epithelium, they do not invade the cells unless there is necrosis or has been mechanical trauma of the mucosa (Owen *et al*, 1979).

The trophozoite adheres, by means of its ventral sucking disc, to the epithelial cell surface, and cysts are shed in the faeces in large numbers (approximately 10^8 /g faeces/day). The acute phase usually resolves spontaneously, and it is followed by either a sub-acute or chronic stage. Cysts may disappear from faeces and the host becomes an asymptomatic cyst-passer. Others may have brief periods of recurring acute symptoms.

It is thought that the sheer number of trophozoites colonising the intestine cause a problem in itself, providing a physical barrier to absorption (Speghelyi, 1940). Kirkpatrick (1986) noted that malabsorption is partly due to the blunting of villi and a decrease in the villus:crypt ratio, which combine to produce a marked reduction in the surface area available for absorption.

1.4.3 Symptoms

The symptoms seen in patients with giardiasis vary widely, from asymptomatic carriers, who shed cysts but show no other symptoms of infection, through to acute patients. The onset of giardiasis is characterised by a variety of symptoms. These include nausea, anorexia, a feeling of queasiness in the upper intestine, malaise, and sometimes a low grade fever and chills. During the acute infection there is a sudden onset of explosive watery profuse foul-smelling diarrhoea with foul flatulence and regular abdominal cramps. "Purple burps" (foul belching) may also occur. Mucus or blood are rarely found in the stool. Blood, if present, is indicative of anal irritation from diarrhoea. If acute infection is prolonged, as in the case of immuno-compromised persons and small

children, then the patient may suffer from malabsorption, steatorrhoea, debility and weight loss (Meyer, 1990).

Subacute infection is characterised by mild or moderate symptoms, often without a history of any acute infection and may last many months. Chronic infection is characterised by periodic episodes of soft foul-smelling stools with increased flatulence and abdominal discomfort. Anorexia is sometimes noted. Chronic infections may follow acute or subacute infection. Asymptomatic infections are least likely to be diagnosed and treated, and are therefore important in the transmission of the disease. Excretion of cysts by carriers may continue for months or years (Logsdon, 1988).

1.4.4 Diagnosis

Diagnosis of infection is by identification of *Giardia* cysts in the microscopic examination of a faecal specimen. Cysts are shed intermittently so two or three samples should be taken over one to three days (Logsdon, 1988). A negative stool sample does not rule out infection because of the intermittent nature of shedding. Small bowel biopsies and duodenal fluid examinations are used if faecal smears are negative, but symptoms are indicative of a *Giardia* infection (Meyer, 1990).

1.4.5 Treatment

A number of effective agents are available for the treatment of patients with giardiasis, including quinacrine, the nitroimidazoles, metronidazole and tinidazole, and furazolidone (Adam, 1991; Meyer, 1990).

Quinacrine, because of its high effectiveness when given over 5-10 day period, is considered by some to be the agent of choice. However, gastro-intestinal side effects are common. Other side effects include toxic psychosis and haemolysis in glucose-6-phosphate dehydrogenase-deficient patients, although these are rare. It is thought that the mechanism of action of quinacrine may be due to its effect on the flavoprotein and quinone components of respiration (Adam, 1991).

The nitroimidazoles (metronidazole and tinidazole) are also highly effective in the treatment of giardiasis. They have a broad spectrum of activity against the anaerobic bacteria and protozoa.

Metronidazole (FlagylTM) is more than 90% effective when given for a 5-day course. It is widely used in the United States of America and New Zealand for the treatment of giardiasis. Nausea and general malaise are common during therapy, but serious side effects are rare. High dosage of metronidazole for prolonged periods has been shown to be carcinogenic in mice. However, two case-control studies have shown no increased frequency of cancer in people who have taken the drug (Beard, *et al*, 1988; Beard, *et al*, 1979).

Tinidazole (FasigynTM) is well tolerated and effective when given in a single dose (Adam, 1991). Its use in New Zealand is now common place.

Furazolidone is less effective than quinacrine and metronidazole. It is, however, used in small children instead of quinacrine. The bitter taste and gastro-intestinal side effect of quinacrine make it less than suitable for small children. It has also been known to be carcinogenic in animals (rats), but has not been shown to be carcinogenic in humans (Adam, 1991).

A second course of treatment with the same drug or a different treatment is used for patients who fail to respond to treatment. Treatment of asymptomatic carriers is often carried out for public health reasons. Treatment of asymptomatic carriers is futile in endemic areas, where the rate of reinfection is high. However, symptomatic giardiasis can result after exposure to persons with asymptomatic infection. Therefore people with asymptomatic infections should be treated to prevent transmission to others in areas where the rate of reinfection is lower.

1.4.7 Transmission

Giardia can be transmitted either directly, by the faecal-oral route, or indirectly, by waterborne contamination.

1.4.7.1 Direct Transmission

It has been well documented that *Giardia* can be transmitted by the faecal-oral route. This is seen particularly in situations where people are living or working in close confines, such as institutions and day care centres. The prevalence of giardiasis in children attending day care may be as high as 35% (Pickering *et al*, 1986; Polis *et al*, 1986; White *et al*, 1989). The infection in these children is usually asymptomatic, but

transmission of the infection may result in symptomatic infection of their family members and others (Polis *et al*, 1986).

Veneral transmission can also occur. Giardiasis is more prevalent in homosexual men, most probably as a result of faecal-oral transmission (Schermerin *et al*, 1978). Animals may also serve as a reservoir of infection, as will be discussed later.

1.4.7.2 Indirect Transmission

Waterborne giardiasis accounts for a much lower proportion of giardiasis cases than faecal-oral transmission. Although it is the least common mode of transmission, this mode receives the most notoriety (Hibler and Hancock, 1990). However, the listings of waterborne outbreaks are numerous, occurring worldwide, and in the United States of America between 1972 and 1981, *Giardia* was the most commonly identified pathogen in waterborne outbreaks (Craun, 1984). There were 53 reported waterborne outbreaks of giardiasis in the United States of America between 1965 and 1981. In most cases there had been a defect in water treatment or contamination post treatment.

Data from waterborne outbreaks in the United States of America have indicated the following (Craun, 1984):

1. Most outbreaks have occurred in water systems using surface water sources.
2. Animals have been implicated as the source of contamination in outbreaks where no obvious human source of contamination can be found.
3. Surface water systems where chlorination is the only form of water treatment of the water source have been implicated.
4. In filtered water supplies, outbreaks occur only where there have been structural defects, inadequate pretreatment, or operational problems.
5. Outbreaks have occurred in systems meeting the current EPA Drinking Water Standards for coliforms and turbidity.

There has been one reported waterborne outbreak of *Giardia* in New Zealand. The 1990 outbreak in the coastal North Otago settlement of Kakanui has been described as minor, involving up to 35 laboratory confirmed cases (Cochrane, 1991) Distribution of *Giardia* in New Zealand is fairly widespread (Brown *et al*, 1992).

1.5 Cultivation

G. intestinalis is the only trophozoite that has been successfully cultured *in vitro* ; neither *G. muris* or *G. agilis* have been cultured (Meyer and Radulescu, 1979).

The axenic culture of *G. intestinalis* was achieved in 1976, from a patient in Portland, Oregon. The most commonly used medium for culture is modified TYI-S-33, which includes bovine serum, bile salts, casein digest, yeast extract, cysteine, dextrose, ferric ammonium, citrate, and ascorbic acid. Especially notable are the absolute requirements for cysteine and low oxygen concentration (Adam, 1991).

For *in vivo* culturing, the following animal models have been established:

Mongolian gerbil (*Meriones unguiculatus*) for human *Giardia* strains (Belosevic *et al* , 1989).

mice for *G. muris* (Roberts-Thomson *et al* , 1976).

1.6 Cryopreservation

In the presence of glycerol or dimethyl sulphoxide (DMSO) *Giardia* trophozoites are able to survive storage at temperatures ranging from 8°C to -70°C (Bemrick, 1961; Meyer and Chadd, 1967; Warhurst and Wright, 1979). The trophozoites are placed in a solution of 7.5% DMSO, and cooled under controlled conditions. They can then be stored in liquid nitrogen. When needed, rapid thawing at 37°C gives optimal recovery (Phillips *et al* , 1982).

1.7 Animal Reservoirs and Host Specificity

Besides humans, *Giardia* has been isolated from many animal sources. It is therefore important to be able to identify if these animal reservoirs can act as an infection source for humans.

Giardia has been isolated from many different animals, both domestic and feral. At the Poznan Zoological Gardens, Poland, *Giardia* was isolated from primates (slow loris, lesser slow loris and siamang) and from rodents (Gambian giant pouched rat and cuis) (Majewska and Kasprzak, 1990). Other researchers have isolated *Giardia* from cats (Davies and Hibler, 1979; Kirkpatrick, 1986; Swan and Thompson, 1986; Tonks *et al* , 1991), dogs (Davies and Hibler, 1979; Barlough, 1979; Swan and Thompson, 1986; Tonks *et al* , 1991), budgerigars (Box, 1981; Hirai *et al* , 1980), cattle (Buret *et al* , 1990;

Craighead, 1991; Davies and Hibler, 1979; Deshpande and Shastri, 1981), sheep (Buret *et al* , 1990; Craighead, 1991), beaver (Davies and Hibler, 1979), coyote (Davies and Hibler, 1979), deer mice (Grant and Woo, 1978a), voles (Grant and Woo, 1978a), wild rats (Grant and Woo, 1978a; Grant and Woo, 1979), hamsters (Grant and Woo, 1978b), amphibians (Callow, 1984), goats (Callow, 1984) and many other animals. It has also been suggested that animals and insects may carry the cysts of *Giardia* on their bodies (Grant and Woo, 1978b). Animals may therefore act as an important reservoir of infection. Meyer (1979) showed that transmission to animals was possible by infecting dogs and cats with a human strain of *Giardia* . The occurrence of giardiasis in backpackers who drank in regions with no human inhabitation suggests that wild animals may be acting as reservoirs (Davies and Hibler, 1979).

A New Zealand study by Marino (1993) tested the faeces of 24 different species of animals, as shown below:

<u>Origin of faeces tested</u>	<u>Number tested</u>	<u>Number positive</u>	<u>% positive</u>
Blackbird	8	3	37.5
Cattle	32	7	21.9
Chaffinch	3	1	33.3
Cockatiel	1	0	0.0
Deer	2	1	50.0
Dogs	10	6	60.0
Ducks	5	0	0.0
Ferret	1	0	0.0
Goats	3	1	33.3
Hedgehog	3	1	33.3
Hedge sparrow	2	0	0.0
Hens	16	3	18.8
Horse	1	1	100.0
Magpie	1	1	100.0
Mice	263	60	22.8
Possums	177	28	15.8
Pukeko	1	0	0.0
Rabbits	1	0	0.0
Rats	94	59	62.8
Sheep	80	24	30.0
Sparrow	17	1	5.9
Swan	4	0	0.0

Thrush	3	3	100.0
Turkey	6	0	0.0

Grant and Woo (1978a, 1978b, 1979) concluded, after very careful cross-species transmission studies, that some species are highly host specific, while others are not. For example, *G. simoni* in rats, *G. muris* in mice and *G. peromysci* in deer mice are highly host specific. However, *G. microti* in voles and *G. mesocricetus* in hamsters are not as host specific.

In a survey done over 1975-77, Davies and Hibler (1979) concluded that the beaver acted as an important reservoir of *Giardia* in Colorado, USA. They concluded that beavers were an important reservoir because they carried the infection for at least three months, and defecated into the water where *Giardia* cysts survive very well. While out tramping and backpacking people often drink water from beaver dams.

Cysts from beavers were given to three human volunteers. Two of the three began passing cysts in their faeces within 11 days of infection. The third was later found to be on tetracycline treatment, which may have affected his/her susceptibility to infection (Davies and Hibler, 1978). From these results, it could be concluded that people drinking from water that infected beavers have defecated in could pick up *Giardia* infections. Therefore, beavers are acting as an important reservoir.

Cattle, which congregate around the water because their grazing range is dry, also become important reservoirs. They drink the water, become infected, and then spread the infection to other sources by defecating in or near water sources.

Domestic animals are also important as a reservoir, due to their close association with man. A Perth study found 21% of dogs and 14% of cats infected with *Giardia* (Swan and Thompson, 1986). Similarly, a New Zealand study, of two cities, tested for the prevalence of *Giardia* cysts in faeces of cats and dogs. In Palmerston North 6.7% of cats and 7.7% of dogs proved positive for *Giardia* cysts, while in Hamilton 2.8% of cats and 24.7% of dogs were positive (Tonks *et al* , 1991). In his review article, Barlough (1979) cites positive infections in dogs all over the United States, in Brazil, Montreal, Milan, Netherlands, Romania, Austria and Italy. Canine infection is therefore very wide spread. Kirkpatrick, in his review article (1986), reports feline giardiasis to be wide spread, with infection ranging from 1-11%.

Dogs accompany humans to many places, including recreational trips, such as tramping in the bush. Dogs not only drink from infected waters, but they also have a tendency to roll in faeces. This increases the animals chance of becoming infected, and also increase the animal's chances of bringing cysts back to its owner. Dogs will also defecate near the water, and if they are infected, the cysts shed in the faeces will get into the water.

Although cats tend not to go on recreational trips with their owners, they may still pick up an infection near home, and bring it back to its owners. Two cases in the United States are referred to by Davies and Hibler (1979): in the first case, a family of five had giardiasis, and the family cat was found to be shedding large numbers of *Giardia* cysts in normal appearing stools; in the second case, a resident in Colorado and her boyfriend both had severe giardiasis, and the cat was passing large numbers of *Giardia* cysts in her stools. However, the question that must be asked is this. Was the cat infected first, or have the humans passed the infection on to the cat?

Humans also act as their own reservoir by virtue of their sometimes improper hygiene habits. Many trampers regard the bush as "nature's toilet", and treat it as such. If infected people defecate in other's drinking water, infection will spread.

Cross transmission between primates in a zoo and their keepers have been shown (Erlandson and Meyer, 1984). It is presumed that transmission stopped after the keeper's hygiene was altered and improved.

A study by Issac-Renton *et al* (1992) was the first report of water source and epidemic associated *Giardia* isolates being adapted to *in vitro* propagation. The aim of the study was to epidemiologically relate isolates from patients in an outbreak, with samples collected from animals and water.

The source of the outbreak was found to be the community water supply, which was found to have a beaver living approximately 1km above the water intake. The beaver was found to be shedding a large number of *Giardia* cysts, and water sample tests became negative once the beaver was removed.

Isoenzyme analysis placed the *Giardia* isolate from the beaver in the same zymodeme as those of the water isolates and isolates collected from patients. These results tend to indicate that the beaver was acting as a reservoir of infection.

1.8 Differentiation of Isolates *Giardia intestinalis* .

Isolates of *G. intestinalis* have been examined by nonmorphological criteria, and significant differences have been demonstrated. *G. intestinalis* infections are quite common in dogs. In New Zealand, studies by Tonks *et al* (1991) have indicated that 24.7% of dogs tested in Hamilton, and 7.7% of dogs tested in Palmerston North were shedding *Giardia* cysts in their faeces. Despite this, well-documented cases of transmission from dogs to humans, or humans to dogs are rare (Castor and Lindqvist, 1990).

Meloni and Thompson (1987) attempted to axenize *G. intestinalis* isolates of canine and human origin. Of the human origin isolates, 44% were successfully axenized, whereas none of the canine origin isolates were. This suggests a biological difference between *G. intestinalis* isolates of human and canine origin. A similar study (Meloni *et al* , 1988) using isolates of human and feline origin found that *G. intestinalis* isolates obtained from cats were more easily axenized, than those from dogs, and that they are similar to human isolates. Like human isolates, feline isolates could be transmitted to gerbils, but not to mice (Kirkpatrick and Farrel, 1984). In another study, however, it was found that human *G. intestinalis* isolates were not easily transmitted to cats (Kirkpatrick and Green IV, 1985).

Research into the molecular characterisation of *G. intestinalis* has been extensive. Various techniques have been employed, as shown below. This list is not exhaustive.

1.8.1 Restriction-Endonuclease Analysis

A study by Nash *et al* (1985) used restriction-endonuclease analysis as a method to compare 15 *Giardia* isolates. Of these isolates, 11 were of human origin and 4 were of animal origin.

DNA was extracted from the isolates and subjected to restriction enzyme digestion. The banding patterns were compared by two techniques. The digests were compared by agarose gel electrophoresis and ethidium bromide staining, and after Southern blot analysis using recombinant plasmids containing *Giardia* DNA as probes.

Two major groups could be distinguished with ethidium bromide staining of eight isolates. The similarity of the ethidium bromide staining pattern of many of the isolates

indicated a general lack of DNA-sequence divergence although the geographical location and the host of the *Giardia* were diverse.

Southern blot analysis again resulted in two major groups. However, another seven of the fifteen isolates showed unique banding patterns, resulting in a total of nine different banding patterns. The banding patterns showed no relationship to geographical location and host, except for two identical isolates from sisters.

A 1989 study by Meloni *et al* compared 47 isolates of *G. intestinalis* by restriction-endonuclease analysis of DNA with hybridisation to a non radiolabelled probe. The probe distinguished seven schizodemes. This compares to the 17 zymodemes that were found in the same isolates when compared by isoenzyme analysis. The extensive heterogeneity shown by these results suggests that the group that is morphologically defined as *G. intestinalis* encompasses a wide spectrum of genetic variation.

1.8.2 Ribosomal RNA Gene Sequence.

Weiss *et al* (1992) used a polymerase chain reaction (PCR) based assay to detect and analyse polymorphism in the 18S ribosomal RNA (rRNA) gene of *G. intestinalis*. Thirty five isolates of *G. intestinalis* were obtained from a variety of hosts and geographical locations, and analysed by this method.

It was found that the 35 isolates could be divided into 3 groups on the basis of the defined nucleotide substitutions within the 183 base pair (bp) fragment of amplified DNA of the 18S rRNA gene. The 3 groups matched identically the groupings assigned to these isolates previously according to both phenotypic and genotypic criteria. These criteria include patterns of monoclonal antibody reactivity with surface antigens, surface antigen labelling, banding following restriction-endonuclease analysis and electrophoretic analysis of intact chromosomes.

1.8.3 Isoenzyme Analysis

Isoenzyme analysis has also revealed the heterogenous nature of *G. intestinalis*. A total of 13 zymodemes were found among the 47 isolates tested by isoenzyme analysis (Homan *et al*, 1992). When the same isolates were examined by Southern blot analysis, 17 schizodemes were found. These correlated with the previously defined zymodemes, 4 of the zymodemes being split into 2 schizodemes each.

1.8.4 Pulse-Field Gel Electrophoresis.

Pulse-field gel electrophoresis (PFGE) has allowed the karyotypic characterisation of protozoa at the species and subspecies level. Sarafis and Issac-Renton (1993) used PFGE as a method of biotyping of *G. intestinalis*. Ethidium bromide staining of chromosomes of twenty different isolates of *G. intestinalis* showed discriminating patterns, and six biotypes were formed, on the basis of size and number of chromosome bands as well as the overall organisation of the karyotype. All but one of the twelve isolates from a single waterborne outbreak fell into one biotype, which also included one non outbreak isolate. The remaining isolates showed no correlation between geographical location and biotype.

1.8.5 Antigenic Analysis.

An early study by Smith *et al* (1982) examined the protein and antigen characteristics of *G. intestinalis*. Sensitive immunochemical and electrophoretic separation techniques were used on four isolates from individuals who acquired their infections in Afghanistan, Oregon, Ecuador and Puerto Rico. Results of sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoelectrophoresis (IEP) analysis showed an overall similarity in the trophozoite proteins and antigens. However, comparison by crossed immunoelectrophoresis (CIE) more clearly revealed qualitative and quantitative antigenic differences. An enzyme-linked immunosorbent assay (ELISA) also showed subtle antigenic differences between the four isolates.

It was concluded that although the PAGE and IEP showed overall similarity between the *G. intestinalis* isolates from widely differing locations, subtle differences detected by CIE and ELISA suggest the existence of potentially important antigenic differences among these isolates.

Further studies by the same laboratory (Nash and Keister, 1985), aimed to confirm and extend these findings, by examining the surface proteins from 19 isolates of *Giardia*. These proteins were compared and analysed by acrylamide gel electrophoresis, and the reactivity of excretory-secretory products with antisera to homologous and heterologous isolates was determined. These results were compared with previously established DNA banding patterns.

The isolates could be divided into three broad groups, correlating to previous DNA banding pattern studies. It was found that surface-antigen differences were common among isolates of *Giardia*.

1.8.6 Drug Sensitivity and Experimental Human Infection.

G. intestinalis infections in humans are highly variable in their clinical manifestation (cf asymptomatic and symptomatic infections) and response to chemotherapy. This variability could be due to factors within the patient. However, a study by McIntyre *et al* (1985) has indicated variation in *G. intestinalis* isolates may also be involved. *G. intestinalis* isolates from 13 patients were cultured and tested *in vitro* for their susceptibility to drugs, and their doubling times. The isolates examined varied by a factor of up to 20 in their susceptibility to metronidazole, tinidazole, furazolidine and quinacrine, the drugs used to treat *Giardia* infections. The population doubling time varied widely also, ranging from 12 to 44 hours.

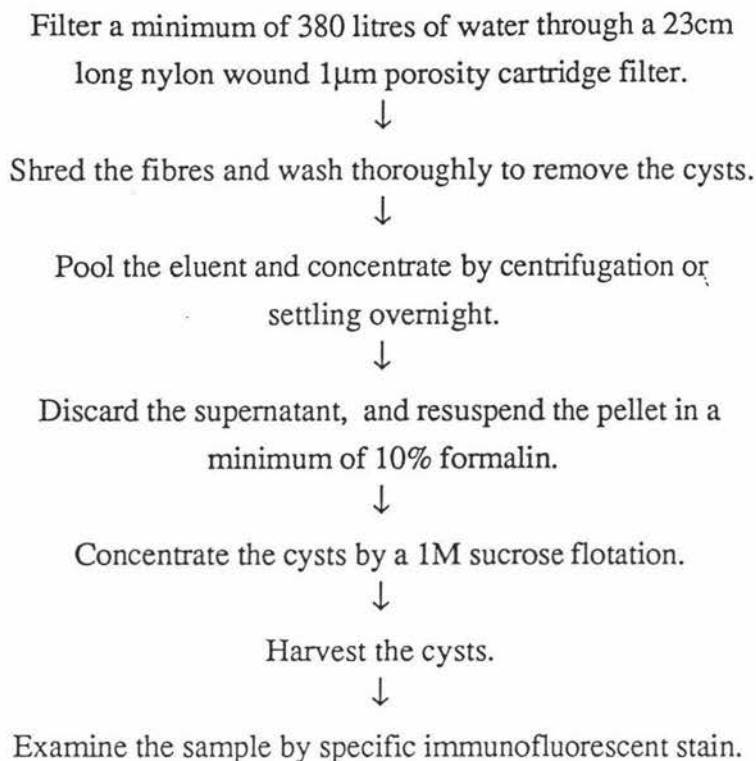
In one study, volunteers were inoculated enterally with the trophozoites of two different human isolates of *G. intestinalis*, GS/M or Isr. All of the volunteers inoculated with GS/M became infected, whereas none of those inoculated with Isr did. Although host factors may have been involved, it appears that there is some intrinsic difference in the infectivity of these two isolates.

1.8.7 DNA Fingerprinting.

DNA fingerprinting of *Giardia* using M13 DNA as a probe was used to compare 5 *Giardia* isolates of human and animal origin (Archibald *et al*, 1991). All 5 isolates had been shown to be identical by restriction-endonuclease cleavage of genomic DNA. It was found that although many of the bands generated were common to all of the isolates, differences in the DNA banding patterns were seen, each isolate having a unique DNA fingerprint pattern. This result demonstrated that the isolates were not genetically identical.

1.9 Detection of *Giardia* in Water.

Guidelines set down by the United States Environmental Protection Agency (USEPA) in 1978 are used in the detection of *Giardia* cysts in fresh-water sources. These were modified by Jakubowski in 1979. The test is conducted as follows:



A positive sample contains a cyst which exhibits the typical apple-green fluorescence when examined under UV light. The test is specific for *Giardia* genus, and is unable to distinguish at the species level, between *G. intestinalis*, *G. muris* or *G. agilis*.

1.10 Significance of *Giardia* in Water

Potable water in urban areas is treated in such a way as to exclude *Giardia* cysts, so the presence of any such cysts in the water implies a defect in processing. Nevertheless it does not follow that the presence of *Giardia* cysts in water in itself is a risk to human health. Apart from the possibility that the cysts may not be viable there are two other situations where cysts may not be infectious for the human population. This is if:

1. The cysts are *G. muris* only.
2. The cysts, although *G. intestinalis*, come from an animal and are host-specific and thus may not infect humans.

The first point is generally accepted, ie *G. muris* does not infect humans, whereas the second point remains to be established, ie it is not yet known if all or indeed any (apart from beavers) animals carry *Giardia* strains which infect humans. The question of host-specificity could in theory be established by transmission experiments but these would probably not be ethically acceptable. Consequently another approach could be taken, ie

to examine many strains of *G. intestinalis* from humans and animals and find if they fall into one or several populations. This requires a reliable technique to distinguish *G. intestinalis* strains but no such method is yet available.

With this in mind, this thesis has two major aims:

1. To investigate the use of random amplification of polymorphic DNA (RAPD) analysis as a means to distinguish strains of *G. intestinalis*. This could be used in subsequent studies to investigate host specificity.
2. To develop a method by which *G. muris* and *G. intestinalis* in water can be reliably distinguished. This could be used at least in the context of small rural supplies to avoid condemnation of supplies containing just *G. muris*.

Chapter 2

The investigation of the use of Random Amplification of Polymorphic DNA (RAPD) to distinguish strains of *Giardia intestinalis*.

2.1 Introduction

G. intestinalis is the only species of *Giardia* which is known to infect humans. However, it is also found in a number of other animals, such as cats and dogs (Adam, 1991). It is therefore desirable to know if the same strains of *G. intestinalis* infect both humans and animals, or if the strains which infect humans are different from those which infect animals.

Different strains of *G. intestinalis* are known to exist, eg, restriction-endonuclease analysis of total genomic DNA of *G. intestinalis* DNA shows heterogeneity. However, this method gives such a large number of bands, that they can not be resolved, so it is not a suitable technique for identifying strains. Consequently there is no easy and reliable method of identifying individual strains of this organism. Such a method, if available, would be useful in at least two contexts: tracing the source of epidemics and answering the question - Are the strains infecting humans and animals identical? The latter is an important question because it has significant epidemiological implications.

We are concerned here with the application of the polymerase chain reaction (PCR) in an attempt to identify individual strains of *G. intestinalis*. A PCR-based technique, called random amplification of polymorphic DNA (RAPD) was described by Williams *et al* (1990), and called arbitrary primed PCR (AP-PCR) fingerprinting by Welsh and McClelland (1990). With this technique, single arbitrary primers are used to amplify random sequences of DNA.

By using a PCR cycle with low stringency (low annealing temperature) at the primer annealing step, a single 10-mer primer with no known homology to the DNA being examined will anneal to a number of sites. Providing the sites occur less than several kilobases (kb) apart and in the correct orientation, the sequence between two such sites are amplified. The products of this amplification are then examined by agarose gel electrophoresis. This method is applied here to examine differences in eleven isolates of *G. intestinalis* of human origin. One of these isolates was obtained from Brisbane. The

other ten were obtained by a graduate student in this department, from human faecal material, sourced from various parts of New Zealand.

Since any one arbitrary primer may be unsuitable for the purpose, we used a series of 10-mers (see Materials and Methods) starting with an AT rich sequence which was progressively modified by a single base change to give a GC rich sequence. This allows the relative merits of a range of arbitrary primers to be compared.

2.2 Materials

2.2.1 For Cryopreservation of Isolates.

1) Dimethyl Sulphoxide (DMSO) 15% (w/v) in TY1-S-33

2.2.2 For Propagation and Preparation of *G. intestinalis* Isolates for DNA extraction.

1) TY1-S-33 Medium for the culture of *Giardia*

Trypticase Soy Broth (BBL. No. 11768)	60.00g
yeast extract powder (Difco No. 0127-01)	30.00g
glucose	30.00g
sodium chloride	6.00g
dipotassium hydrogen phosphate	3.00g
potassium dihydrogen phosphate	0.60g
L-cysteine Monohydrochloride (Sigma No. C-7880)	4.50g
ferric ammonium citrate (Brown Pearls)	0.069g
L-Ascorbic acid	0.60g
NCTC 109 (Difco No. 5927-23)	
or NCTC 135 (Gibco No. 440-1100)	2.82g
bile bacteriological (Sigma No. B-8381)	2.40g
benzyl penicillin	0.18g
gentamycin sulphate	0.15g
vancomycin	0.06g
bovine serum	300.0ml
distilled water to	3000.0ml

The pH was adjusted to pH 7.0-7.2 with 1M NaOH. The media was clarified using positive pressure membrane filtration through non sterile 5.0 μ m and 0.45 μ m pore membranes.

Finally, the media was filter sterilised by positive pressure membrane filtration using a sterile Supor 0.2 μ m pore filter membrane. Sterilisation of the filter was achieved by autoclaving the filter within the filter unit at 121°C for 15 minutes. Sterile media can then be stored until use for up to 10 days at 4°C.

2) Phosphate Buffered Saline (PBS) pH 7.2

NaCl	8.5g
Na ₂ HPO ₄ .12H ₂ O	2.7g
NaH ₂ HPO ₄ .2H ₂ O	0.39g
distilled water to	1000.0ml

Autoclave for 15 minutes at 121°C, and store at room temperature.

3) Tris-EDTA Solution

- 0.1M Tris-HCl (pH 7.5)
- 0.1M EDTA (disodium salt) (pH 7.5)

2.2.3. For DNA Extraction.

1) Sodium Dodecyl Sulphate (SDS) 10% (w/v)

Stored at room temperature without autoclaving.

2)Pronase Type XIV 10mg/ml

This solution was preincubated at 37°C for three hours to self-digest contaminants, especially DNase activity. This was then stored at -20°C.

3) RNase (Ribonuclease 1) 2mg/ml

This solution was preincubated at 90°C for 10 minutes to destroy DNase activity. It was then stored at -20°C.

4) 5.0M NaCl

5) **Saline Tris-EDTA (STE) Buffer (x10)**

1.0M NaCl

0.5M Tris-HCl (ph 7.5)

10mM EDTA (disodium salt) (pH 7.2)

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

A 25:24:1 solution, respectively, was prepared.

7) **0.001M EDTA**

8) **Absolute Ethanol**

9) **5M Sodium Perchlorate**

2.2.4 For Spectrophotometric Determination of the Purity and Concentration of the DNA.

1) **TE Buffer (Tris-EDTA)**

10mM Tris-HCl (pH 7.5)

1mM disodium salt EDTA (pH 7.2)

2.2.5 For RAPD Analysis

1) **Sterile Distilled Water**

2) **dNTP's 1.25mM**

3) **DNA Taq Polymerase Buffer (x10)**

As supplied by manufacturer (Gibco).

4) **DNA 1µg/µl**

Isolate Code	Species	Source	Location
Hast 76	<i>G. intestinalis</i>	Human	Hastings
Bris/83/Hepu 106	<i>G. intestinalis</i>	Human	Australia
Hast 86	<i>G. intestinalis</i>	Human	Hastings
Whang 48	<i>G. intestinalis</i>	Human	Whangarei
Ham 7	<i>G. intestinalis</i>	Human	Hamilton
Whang 8	<i>G. intestinalis</i>	Human	Whangarei

Wang 3	<i>G. intestinalis</i>	Human	Wanganui
Hast 11	<i>G. intestinalis</i>	Human	Hastings
N.Ply 77	<i>G. intestinalis</i>	Human	New Plymouth
Ham 84	<i>G. intestinalis</i>	Human	Hamilton
Hast 68	<i>G. intestinalis</i>	Human	Hastings

5) MgCl₂ 50mM

As supplied by manufacturer (Gibco).

6) Primer 1nmol/μl

Supplied as dry pellet by Oligos etc, and diluted with sterile distilled water to a concentration of 1nmol/μl.

The primer sequences are as follows:

GC30	5' TAATCACTGT 3'
GC40	5' TAGTCACTGT 3'
GC50	5' TGGTCACTGT 3'
GC60	5' CGGTCCTGT 3'
GC70	5' CGGCCACTGT 3'
GC80	5' CGGCCCTGT 3'
GC90	5' CGGCCCGGT 3'
GC100	5' CGGCCCGGC 3'

7) Taq Polymerase 5units/μl

As supplied by manufacturer (Gibco).

2.2.6 For Gel Electrophoresis

See appendix

2.3 Methods

2.3.1 Cryopreservation of Isolates

To preserve the cultures in liquid nitrogen, trophozoites were harvested at the late log phase of growth by putting tubes of 3 day old cultures in ice water for 10 minutes. This detached the trophozoites, which were then concentrated by centrifugation at 700g for 5 minutes. The pellet was resuspended in TY1-S-33 and the concentration of trophozoites

estimated using a haemocytometer. The cell suspension was then adjusted to $1.2\text{--}1.5 \times 10^6$ trophozoites per ml with media. A 0.25ml aliquot of this suspension was then placed in a 1ml Nunc cryopreservation tube, and 0.25ml of a 15% (w/v) solution of DMSO in TY1-S-33 was added slowly. The tubes were then mixed and wrapped in paper and tissue, and placed in a polystyrene container, which was placed in a -80°C freezer. This slowed the rate of freezing down, protecting the trophozoites from the violent effects of rapid cooling. When frozen some of the tubes were transferred to liquid nitrogen and the rest were stored in the -80°C freezer (Phillips *et al* , 1982).

2.3.2 Propagation and Preparation of *G. intestinalis* Isolates for DNA Extraction.

A 1ml aliquot of *G. intestinalis* Hast. 76 culture, stored in cryopreservation at -70°C , was thawed rapidly in a 37°C water bath. The contents were placed in a 50ml centrifuge tube and filled with media (TY1-S-33) that had been warmed to 37°C . The cells are washed three times (1050g for 3 minutes). (Instead of using media for the washing, PBS or sterile water could have been used.) It is important that the dimethyl sulphoxide (DMSO) is diluted out as fast as possible.

Once the cells had been washed, the pellet was resuspended, and then transferred to a 50ml tissue culture flask. The flask was then filled up with TY1-S-33, and placed at 37°C . The cells were grown for a week, and then subbed every three days.

The culture was scaled up to a 300ml culture, which was then centrifuged at 1050g for 10 minutes, the supernatant discarded and the pellet was resuspended in 30ml of sterile PBS. The cells were recentrifuged and the deposit was resuspended in 1.0ml of Tris-EDTA Solution. This cell suspension was placed in a 12ml NalgeneTM centrifuge tube and then stored at -70°C or used immediately for DNA extraction.

2.3.3 DNA Extraction

The pelleted preparations (see 2.3.1) of *G. intestinalis* isolates suspended in 1.0ml of Tris-EDTA Solution and stored at -70°C were thawed in a 37°C waterbath. 0.1ml of SDS (10% w/v) and 0.1ml of Pronase Type XIV (10mg/ml) were added to each isolate and incubated at 50°C overnight to ensure that all the cells were lysed and that their proteins were digested. A 0.1ml aliquot of RNase was added to the lysate and incubated for 60minutes at 50°C to digest the RNA. An aliquot of sodium perchlorate was then

added to give a final concentration of 1M and the lysate was incubated at 50°C for a further 60 minutes.

The phenol/chloroform/iso-amyl alcohol (25:24:1 v/v/v) solution was mixed with one tenth its volume of STE Buffer. Air was bubbled through the preparation to facilitate mixing. The solution was cooled to room temperature and used to deproteinate the lysate.

An equal volume of phenol/chloroform/iso-amyl alcohol solution was added to the lysate, mixed repetitively inverting (approximately 30 times) the centrifuge tube which was then left for 5 minutes until the interface reformed, the mixture was then centrifuged at 5,000g for 10 minutes at room temperature. The upper aqueous layer, containing the DNA, was removed using a 5ml serological pipette, placed into another centrifuge tube and the DNA was re-extracted twice more with the phenol/chloroform/iso-amyl alcohol solution.

One twentieth of the volume of 5M NaCl was added, and then two times the total volume of absolute ethanol at -20°C was added. The centrifuge tube was then left at -20°C overnight. The tube was then centrifuged at 16,000g for 30 minutes. The supernatant was removed, and absolute ethanol (at -20°C) was gently decanted into the tube. The absolute ethanol was gently tipped off, so as not to disturb the pellet. The pellet was then dried in the hot room, and once dried, resuspended in an equal volume of Tris-EDTA Buffer.

2.3.4 Spectrophotometric Determination of the Purity and Concentration of the DNA.

0.1ml of DNA was diluted with 1.9ml of TE Buffer, gently mixed so as not to shear the DNA and placed into a quartz cuvette and the optical density was measured using a Shimadzu UV-160A automatic scanning spectrophotometer which was zeroed against TE Buffer.

Readings were taken at 230nm, 258nm, 260nm, 270nm, 280nm and 300nm. Spectral ratios $260/230 = 1.8$ to 2.5 and $260/280 = 1.8$ to 2.0 were regarded as satisfactory. If the purity was unsatisfactory the DNA was re-extracted as previously described. If the absorbance at 270nm was greater than at 260nm this indicated phenol contamination of the DNA so the sample was re-ethanol precipitated and re-assayed.

The concentration of the DNA was determined as described by Brenner and Falkow (1971).

i.e.

$$\text{Concentration} = \frac{\text{OD}_{258\text{nm}} - \text{OD}_{300\text{nm}} \times \text{dilution factor}}{20}$$

A typical spectrophotometric profile is shown below:

Wavelength (nm)	230	258	260	280	300	<u>260</u> 280	<u>260</u> 230
<i>G. intestinalis</i> Hast 68 - diluted 1/100 (OD Units)	0.240	0.578	0.570	0.345	0.091	1.65	2.38

Therefore the DNA concentration of *G. intestinalis* isolate Hast 68 diluted 1/100 is:

$$\frac{(0.578-0.091) \times 100}{20} = 2.44\text{mg/ml}$$

The DNA was then diluted to give a stock concentration of 1µg/µl.

2.3.5 RAPD Analysis.

a) Optimisation of PCR Conditions for Amplification.

Before beginning the RAPD analysis of the 11 isolates of *G. intestinalis*, it was first necessary to optimise the PCR conditions. This was achieved by carrying out a number of PCR runs where one reagent or physical parameter was varied. The optimum condition was then selected and used in subsequent reactions. The annealing temperature used in all the reactions was 35°C.

b) RAPD Analysis.

The standard amplification reaction contained 1x Taq polymerase reaction buffer (20mM Tris-HCl [pH8.4]; 50mM KCl), 250µm each of dATP, dCTP, dGTP and dTTP, 50pg of each primer, 100ng of template DNA, 90µM of MgCl₂ and 2.5 units of DNA Taq polymerase. The reaction was made up to 20µl by the addition of sterile distilled water.

The amplification was performed in a Perkin Elmer Cetus DNA Thermal Cycler (9600), programmed for 1 cycle of: 2 minutes at 95°C, 30 seconds at 35°C and 30 seconds at

72°C; 30 cycles of: 30 seconds at 94°C, 30 seconds at 35°C and 30 seconds at 72°C; and 1 cycle of: 30 seconds at 94°C, 30 seconds at 35°C and 5 minutes at 72°C, using the fastest available transitions between each temperature.

2.3.6 Gel Electrophoresis.

See appendix

2.4 Results

a) Optimisation of PCR Conditions for Amplification.

The PCR reaction was optimised by individually varying the PCR reagents and the annealing temperature. In this context, optimisation implies the production of clear bands, small enough in number to be readily resolvable, but large enough in number to allow isolates to be differentiated. The results are shown as follows: cycle number (Figure 1); dNTP concentration (Figure 2); buffer concentration (Figure 3); DNA concentration (Figure 4); MgCl₂ concentration (Figure 5); primer concentration (one primer used only) (Figure 6); DNA Taq polymerase concentration (Figure 7).

It was concluded from the results that for our purposes (attempting to distinguish isolates), the optimal PCR conditions are as follows:

1x Taq polymerase reaction buffer (20mM Tris-HCl [pH8.4]; 50mM KCl)

250µm each of dATP, dCTP, dGTP and dTTP

50pg of each primer

100ng of template DNA

90µM of MgCl₂

2.5 units of DNA Taq polymerase

The reaction mixture should be diluted to 20µl by the addition of sterile distilled water.

The reproducibility of the results obtained, using two isolates and one primer, in these conditions was examined. The results shown in Figure 8.

FIGURE 1: Optimisation of PCR conditions - cycle number.

Standard PCR conditions were used with isolate Whang 48 DNA and primer GC100.

The first cycle: 95°C, 2 minutes; 35°C, 30 seconds; 72°C, 30 seconds

The final cycle: 94°C, 30 seconds; 35°C, 30 seconds; 72°C, 5 minutes

The intermediate cycle (94°C, 30 seconds; 35°C, 30 seconds; 72°C, 30 seconds) was repeated from 5 through to 60 times.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, 5 cycles; Lane 3, 10 cycles; Lane 4, 15 cycles; Lane 5, 20 cycles; Lane 6, 25 cycles; Lane 7, 30 cycles; Lane 8, 35 cycles; Lane 9, 40 cycles; Lane 10, 45 cycles; Lane 11, 50 cycles; Lane 12, 55 cycles; Lane 13, 60 cycles; Lane 14, 1 kb ladder.

NOTE: Faint bands were seen after 20 cycles, but 25 cycles produced clearer bands and the clarity of bands increased up to 30 cycles. Hence 30 cycles was used as the standard in subsequent experiments.

The figure quoted for the cycle number refers to the repeated cycles and does not include the first and final cycles which differed somewhat from the repeated cycle.

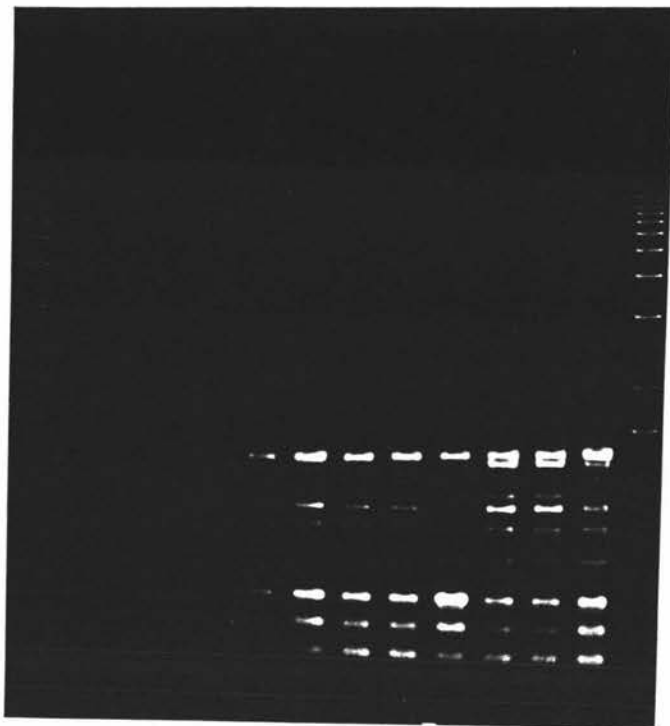
FIGURE 2: Optimisation of PCR conditions - concentration of dNTPs.

Standard PCR conditions were used with isolate Hast 68 DNA and primer GC90. Varying volumes of 1.25mM dNTPs were added to the reaction mixture. The concentration of dNTPs referred to is the final concentration in the reaction mixture.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, 0µM dNTPs; Lane 3, 62.5µM dNTPs; Lane 4, 125µM dNTPs; Lane 5, 250µM dNTPs; Lane 6, 375µM dNTPs; Lane 7, 1 kb ladder.

NOTE: Amplification of DNA peaked in lane 5 (250µM dNTPs), but waned when higher dNTP concentrations were used. It was concluded that 250µM is the optimum concentration of dNTPs.

1 2 3 4 5 6 7 8 9 10 11 12 13 14



1 2 3 4 5 6 7

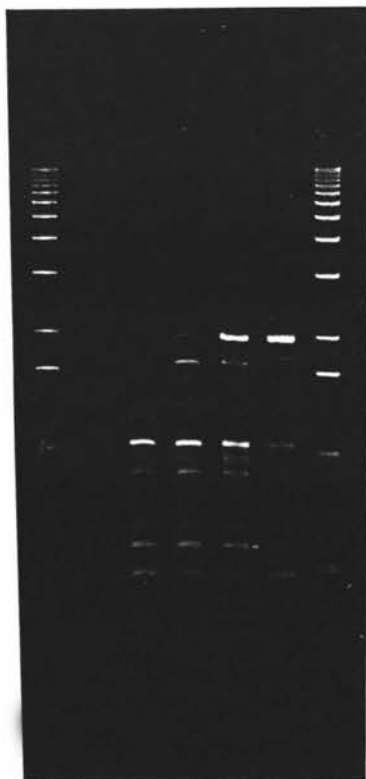


FIGURE 3: Optimisation of PCR conditions - buffer concentration.

Standard PCR conditions were used with isolate Hast 68 DNA and primer GC90. The buffer concentration was varied, from zero added to 2x the manufacturers concentration.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, 0x buffer; Lane 3, 0.25x buffer; Lane 4, 0.5x buffer; Lane 5, 0.75x buffer; Lane 6, 1.0x buffer; Lane 7, 1.25x buffer; Lane 8, 1.5x buffer; Lane 9, 1.75x buffer; Lane 10, 2.0x buffer; Lane 11, 1 kb ladder.

NOTE: The buffer was supplied at 10x concentration by the manufacturer. At very low and very high concentrations, the banding patterns were very faint or not visible.

Activity was clearest in lane 6 (1x buffer), so it was concluded that the recommended buffer concentration was the optimum.

FIGURE 4: Optimisation of PCR conditions - the amount of DNA added.

Standard PCR conditions were used with isolate Hast 68 DNA and primer GC90. The amount of DNA added was varied, from zero added to 1 μ g.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, no DNA; Lane 3, 1pg DNA; Lane 4, 10pg DNA; Lane 5, 100pg DNA; Lane 6, 1ng DNA; Lane 7, 10ng DNA; Lane 8, 100ng DNA; Lane 9, 1 μ g DNA; Lane 10, 1 kb ladder.

NOTE: At low DNA concentrations, no DNA amplification was detected but it was first seen in lane 5 (100pg DNA). Activity peaked in lane 8 (100ng DNA), and then decreased.

It was concluded that 100ng of DNA was the optimum amount for the conditions used. The lower activity at the highest DNA concentration tested may be due to interference by proteins and polysaccharides in the DNA stock.

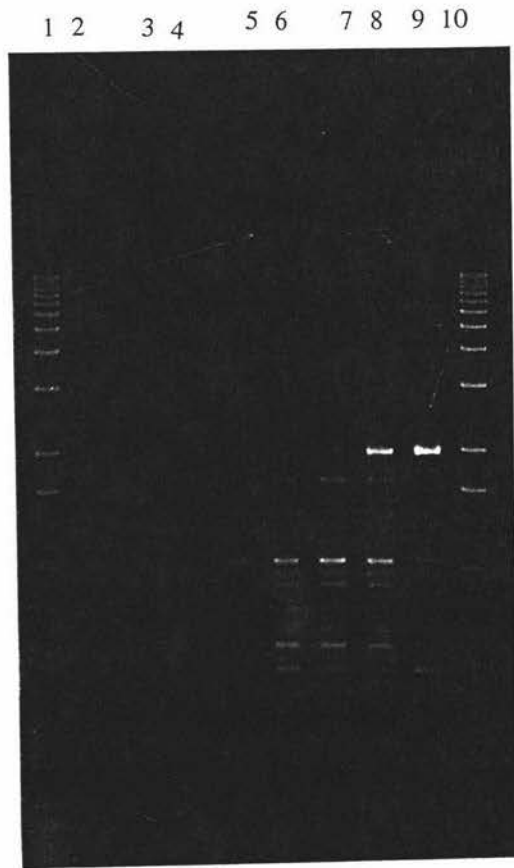
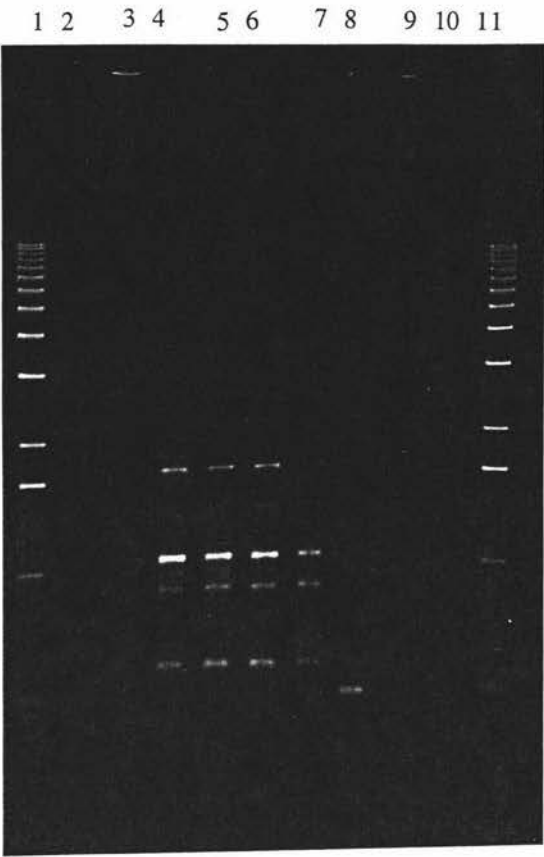


FIGURE 5: Optimisation of PCR conditions - MgCl₂ concentration.

Standard PCR conditions were used with isolate Ham 7 DNA and primer GC100. The final MgCl₂ concentration in the reaction mixture was varied from 1mM to 8mM.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, 1mM MgCl₂; Lane 3, 2mM MgCl₂; Lane 4, 3mM MgCl₂; Lane 5, 4mM MgCl₂; Lane 6, 5mM MgCl₂; Lane 7, 6mM MgCl₂; Lane 8, 7mM MgCl₂; Lane 9, 8mM MgCl₂; Lane 10, 1 kb ladder.

NOTE: Above 3mM MgCl₂ the clarity of the bands diminished, and at higher concentrations some bands were lost entirely. It was concluded that the optimum MgCl₂ concentration was 1.5mM in the conditions used.

FIGURE 6: Optimisation of PCR conditions - amount of primer added.

Standard PCR conditions were used with isolate Hast 68 DNA and primer GC90. The amount of primer added was varied from 0pg to 200pg.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, 0pg primer; Lane 3, 0.5pg primer; Lane 4, 5.0pg primer; Lane 5, 25pg primer; Lane 6, 50pg primer; Lane 7, 100pg primer; Lane 8, 150pg primer; Lane 9, 200pg primer; Lane 10, 1 kb ladder.

NOTE: That 50pg (lane 6) of primer gave the optimal banding pattern so it was used in subsequent experiments.

1 2 3 4 5 6 7 8 9 10



1 2 3 4 5 6 7 8 9 10

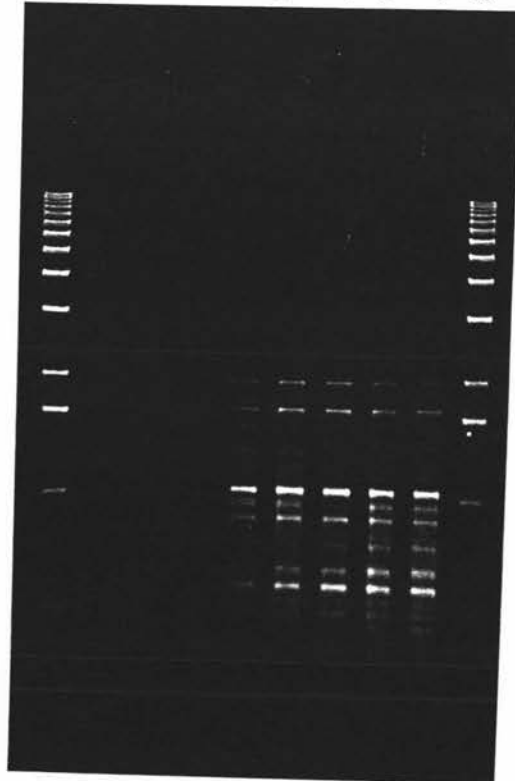


FIGURE 7: Optimisation of PCR conditions - amount of DNA Taq polymerase added.

Standard PCR conditions were used with isolate Hast 68 DNA and primer GC90. The amount of DNA Taq polymerase added was varied from 0 units to 5 units.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, 0units Taq; Lane 3, 1.25units Taq; Lane 4, 2.5units Taq; Lane 5, 3.75units Taq; Lane 6, 5units Taq; Lane 7, 1 kb ladder.

NOTE: 2.5 units produced all the bands. Hence, to economise, 2.5 units of DNA Taq polymerase were used as standard.

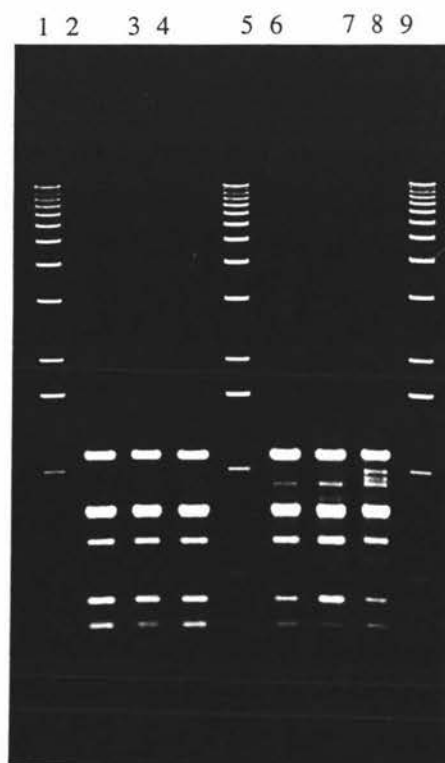
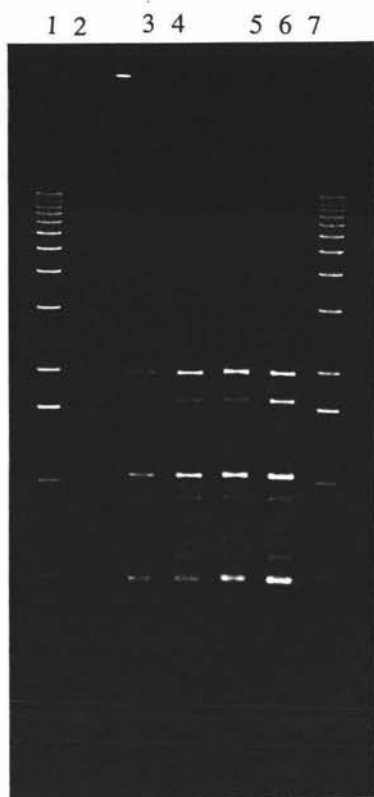
FIGURE 8: Examination of the reproducibility of the PCR reaction.

Standard PCR conditions (including all the parameters as optimised - see above) were used with isolates Hast 68 and Whang 48 DNA and primer GC70. Tests were repeated three times and compared.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, Hast 68 and GC70, test 1; Lane 3, Hast 68 and GC70, test 2; Lane 4, Hast 68 and GC70, test 3; Lane 5, 1 kb ladder; Lane 6, Whang 48 and GC70, test 1; Lane 7, Whang 48 and GC70, test 2; Lane 8, Whang 48 and GC70, test 3; Lane 9, 1 kb ladder.

NOTE: Test 2 was carried out using a different PCR machine to find if the results were similar when different machines were used.

Although slight variation was seen in minor bands, the major banding patterns remained the same in all 3 tests.



b) RAPD Analysis of 11 *G. intestinalis* Isolates using conditions optimised to show differences (see above).

The DNA of each of the 11 isolates was amplified by each of the 8 arbitrary 10-mer PCR primers. These results are shown in Figures 9 to 14 and summarised in Table 1. Note that two of the primers (GC30 and GC40) produced no bands in any of the isolates. The banding pattern of 8 of the isolates is similar. However three strains (Hast 68, Ham 84 and Hast 76) showed some variation from the other isolates. Thus primers GC50 and GC80 each produced three different patterns. However, taking the combined results of these two primers, four groups could be distinguished (ie Hast 68, Ham 84, Hast 76 and DNA from the other 8 isolates).

Two isolates that showed some variation, namely Hast 68 and Whang 48, were examined further by varying the annealing temperature, using primers GC70, GC80, GC90 and GC100. The results are shown in Figures 15 to 18.

Note that the differences in the banding patterns between the isolates increased as the annealing temperature increased.

To attempt to further increase the differences, Hast 68 and Whang 48 were examined using a mixture of 2 primers at an annealing temperature of 35°C and an annealing temperature of 50°C. The results are shown as follows: 35°C annealing temperature (Figure 19); 50°C annealing temperature (Figure 20).

This approach produced only minor banding pattern differences between the two isolates.

FIGURE 9: Examination of the heterogeneity of 11 isolates of *G. intestinalis* by RAPD analysis with the arbitrary 10-mer primer GC50.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, Hast 76; Lane 3, Bris/83/Hepu/106; Lane 4, Hast 86; Lane 5, Whang 48; Lane 6, Ham7; Lane 7, Whang 48; Lane 8, Wang 3; Lane 9, Hast 11; Lane 10, N.Ply 77; Lane 11, Ham 84; Lane 12, Hast 68; Lane 13, 1 kb ladder.

NOTE: All isolates show similar banding patterns but with some variation in intensity. Differences in band intensity were greatest with isolates Ham 84 and Hast 68 (lanes 11 and 12).

FIGURE 10: Examination of the heterogeneity of 11 isolates of *G. intestinalis* by RAPD analysis with the arbitrary 10-mer primer GC60.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, Hast 76; Lane 3, Bris/83/Hepu/106; Lane 4, Hast 86; Lane 5, Whang 48; Lane 6, Ham7; Lane 7, Whang 48; Lane 8, Wang 3; Lane 9, Hast 11; Lane 10, N.Ply 77; Lane 11, Ham 84; Lane 12, Hast 68; Lane 13, 1 kb ladder.

NOTE: All isolates show the same banding patterns but with slight variation in the intensities of the bands.

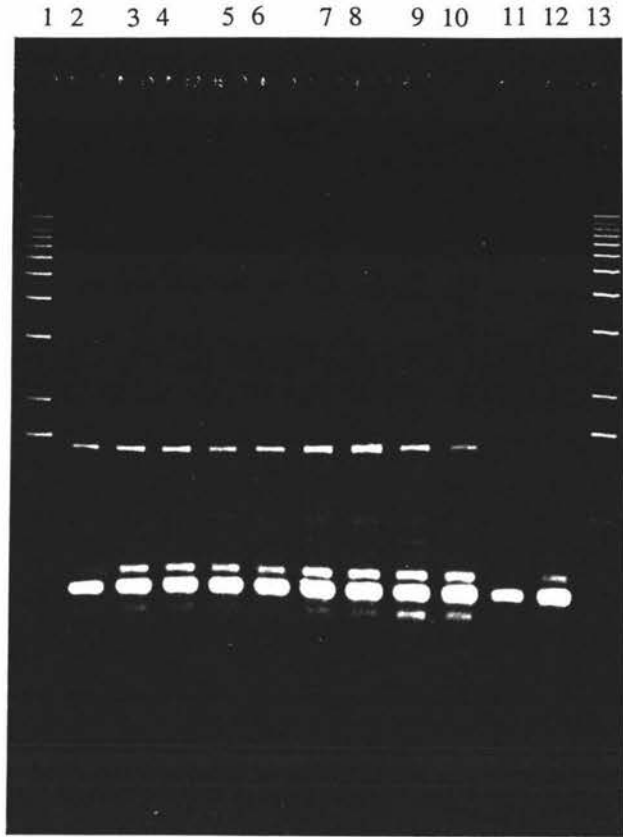


FIGURE 11: Examination of the heterogeneity of 11 isolates of *G. intestinalis* by RAPD analysis with the arbitrary 10-mer primer GC70.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, Hast 76; Lane 3, Bris/83/Hepu/106; Lane 4, Hast 86; Lane 5, Whang 48; Lane 6, Ham7; Lane 7, Whang 48; Lane 8, Wang 3; Lane 9, Hast 11; Lane 10, N.Ply 77; Lane 11, Ham 84; Lane 12, Hast 68; Lane 13, 1 kb ladder.

NOTE: All of the isolates show the same major bands. However Hast 68 (lane 12) shows a marked loss of intensity in the high molecular weight major band.

FIGURE 12: Examination of the heterogeneity of 11 isolates of *G. intestinalis* by RAPD analysis with the arbitrary 10-mer primer GC80.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, Hast 76; Lane 3, Bris/83/Hepu/106; Lane 4, Hast 86; Lane 5, Whang 48; Lane 6, Ham7; Lane 7, Whang 48; Lane 8, Wang 3; Lane 9, Hast 11; Lane 10, N.Ply 77; Lane 11, Ham 84; Lane 12, Hast 68; Lane 13, 1 kb ladder.

NOTE: All of the isolates show the same major bands, with the exception of Hast 68 (lane 12). This isolate is missing the top 3 (high molecular weight) bands present in the other isolates.

Hast 76 (lane 2) has an extra 2 high molecular weight bands. The smaller of these can be seen faintly with Ham 7, Whang 48 and Wang 3 (lanes 6-8).

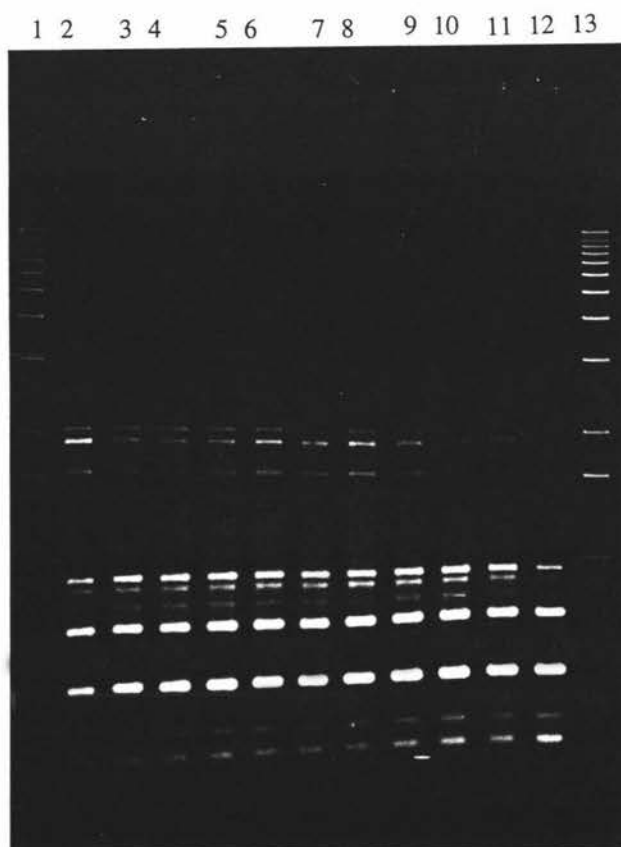
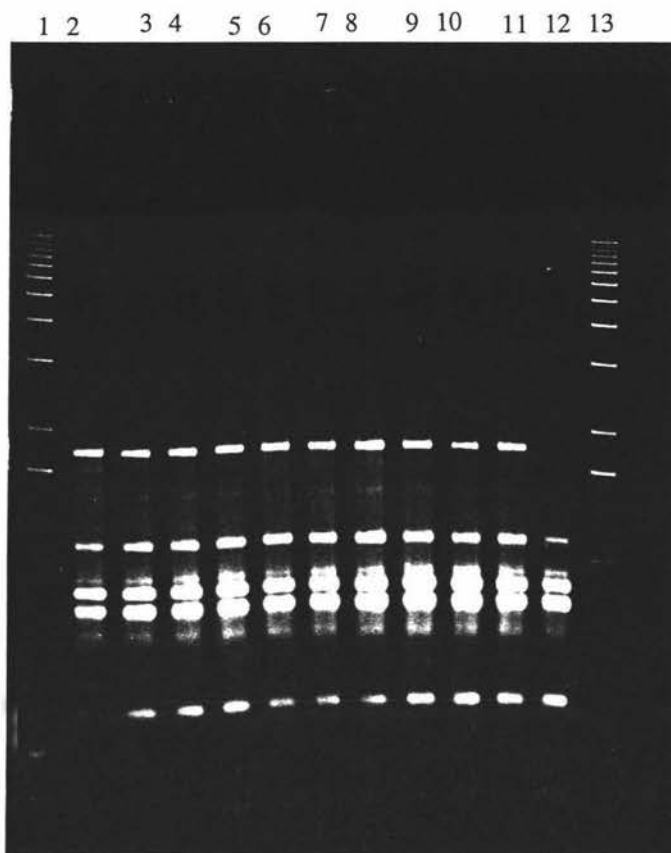


FIGURE 13: Examination of the heterogeneity of 11 isolates of *G. intestinalis* by RAPD analysis with the arbitrary 10-mer primer GC90.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, Hast 76; Lane 3, Bris/83/Hepu/106; Lane 4, Hast 86; Lane 5, Whang 48; Lane 6, Ham7; Lane 7, Whang 48; Lane 8, Wang 3; Lane 9, Hast 11; Lane 10, N.Ply 77; Lane 11, Ham 84; Lane 12, Hast 68; Lane 13, 1 kb ladder.

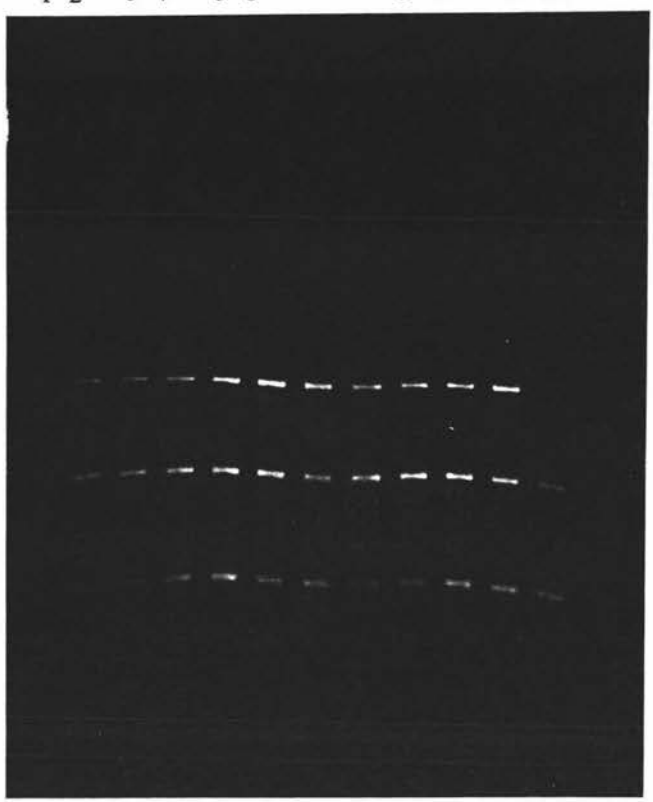
NOTE: All isolates show the same major bands. Hast 68 (lane 12) shows some variation in band intensity.

FIGURE 14: Examination of the heterogeneity of 11 isolates of *G. intestinalis* by RAPD analysis with the arbitrary 10-mer primer GC100.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, Hast 76; Lane 3, Bris/83/Hepu/106; Lane 4, Hast 86; Lane 5, Whang 48; Lane 6, Ham7; Lane 7, Whang 48; Lane 8, Wang 3; Lane 9, Hast 11; Lane 10, N.Ply 77; Lane 11, Ham 84; Lane 12, Hast 68; Lane 13, 1 kb ladder.

NOTE: All isolates show the same major bands.

1 2 3 4 5 6 7 8 9 10 11 12 13



1 2 3 4 5 6 7 8 9 10 11 12 13

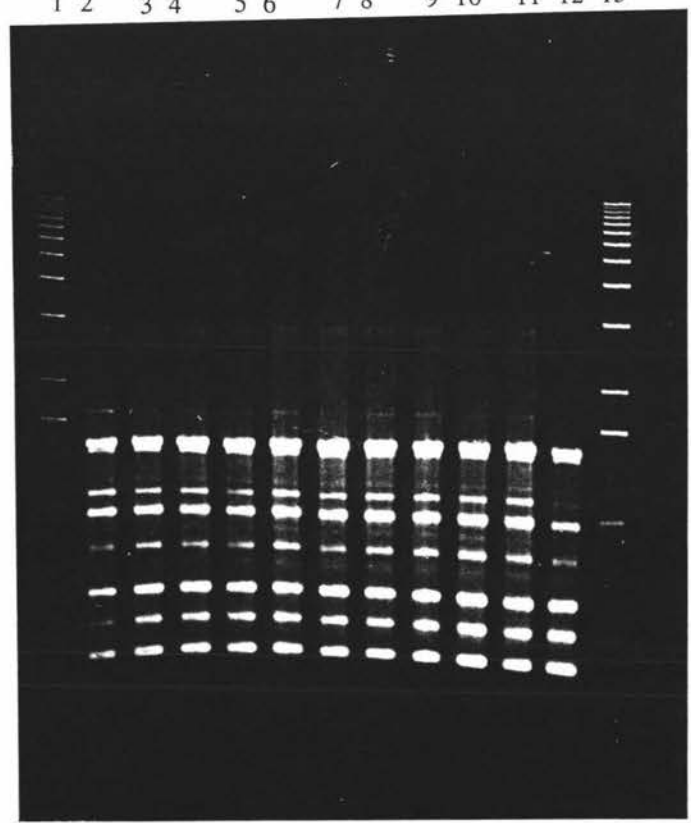


FIGURE 15: Examination of the heterogeneity of 2 isolates of *G. intestinalis* (Hast 68 and Whang 48) by RAPD analysis using primer GC70 - investigation of the effect produced by varying the annealing temperature.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, Hast 68, 35°C; Lane 3, Whang 48, 35°C; Lane 4, Hast 68, 40°C; Lane 5, Whang 48, 40°C; Lane 6, Hast 68, 45°C; Lane 7, Whang 48, 45°C; Lane 8, Hast 68, 50°C; Lane 9, Whang 48, 50°C; Lane 10, Hast 68, 55°C; Lane 11, Whang 48, 55°C; Lane 12, Hast 68, 60°C; Lane 13, Whang 48, 60°C; Lane 14, 1 kb ladder.

NOTE: As the temperature increased the differences became more marked, especially in the high molecular weight bands. However, the overall banding pattern of the 2 isolates remained very similar.

No amplification was seen when the annealing temperature was 60°C because this exceeded the T_m of the primer (Lanes 12 and 13).

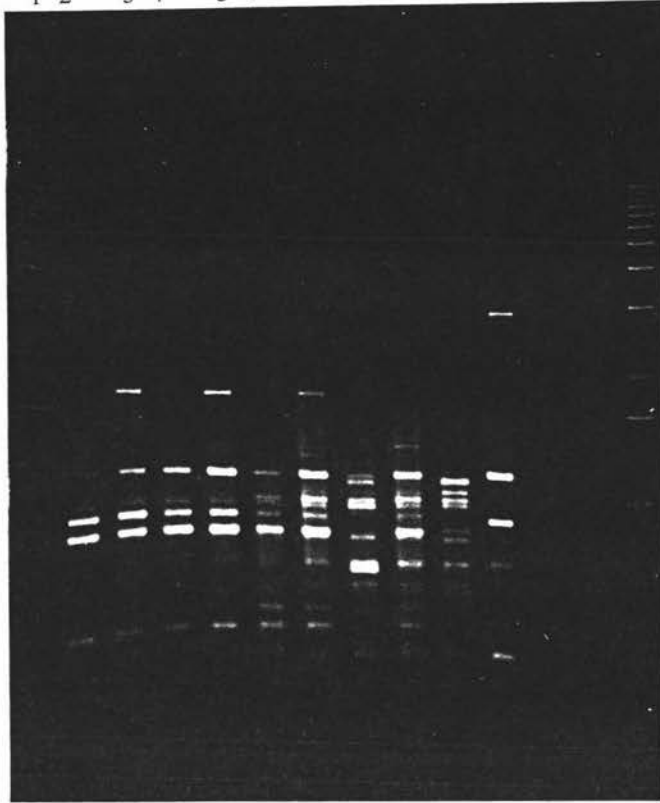
FIGURE 16: Examination of the heterogeneity of 2 isolates of *G. intestinalis* (Hast 68 and Whang 48) by RAPD analysis using primer GC80 - the effect of varying the annealing temperature.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, Hast 68, 35°C; Lane 3, Whang 48, 35°C; Lane 4, Hast 68, 40°C; Lane 5, Whang 48, 40°C; Lane 6, Hast 68, 45°C; Lane 7, Whang 48, 45°C; Lane 8, Hast 68, 50°C; Lane 9, Whang 48, 50°C; Lane 10, Hast 68, 55°C; Lane 11, Whang 48, 55°C; Lane 12, Hast 68, 60°C; Lane 13, Whang 48, 60°C; Lane 14, 1 kb ladder.

NOTE: Differences, particularly in the high molecular weight bands, were increased as the annealing temperature increased up to 55°C. However, the overall banding pattern of the 2 isolates remained very similar.

60°C exceeded the T_m of GC80 so no amplification occurred (Lanes 12 and 13).

1 2 3 4 5 6 7 8 9 10 11 12 13 14



1 2 3 4 5 6 7 8 9 10 11 12 13 14

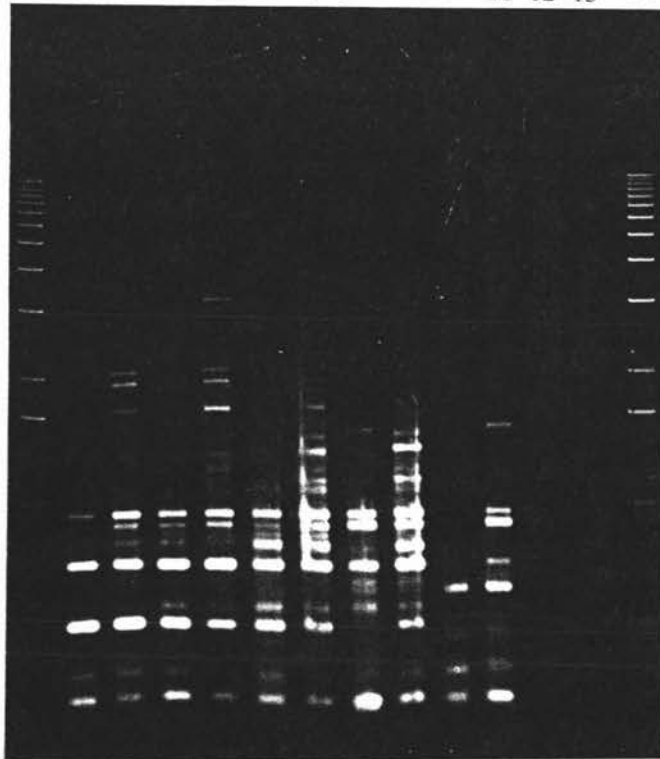


FIGURE 17: Examination of the heterogeneity of 2 isolates of *G. intestinalis* (Hast 68 and Whang 48) by RAPD analysis using primer GC90 - the effect of varying the annealing temperature.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, Hast 68, 35°C; Lane 3, Whang 48, 35°C; Lane 4, Hast 68, 40°C; Lane 5, Whang 48, 40°C; Lane 6, Hast 68, 45°C; Lane 7, Whang 48, 45°C; Lane 8, Hast 68, 50°C; Lane 9, Whang 48, 50°C; Lane 10, Hast 68, 55°C; Lane 11, Whang 48, 55°C; Lane 12, Hast 68, 60°C; Lane 13, Whang 48, 60°C; Lane 14, 1 kb ladder.

NOTE: Differences, particularly in the high molecular weight bands, were increased as the annealing temperature increased up to 55°C. However, the overall banding pattern of the 2 isolates remained very similar.

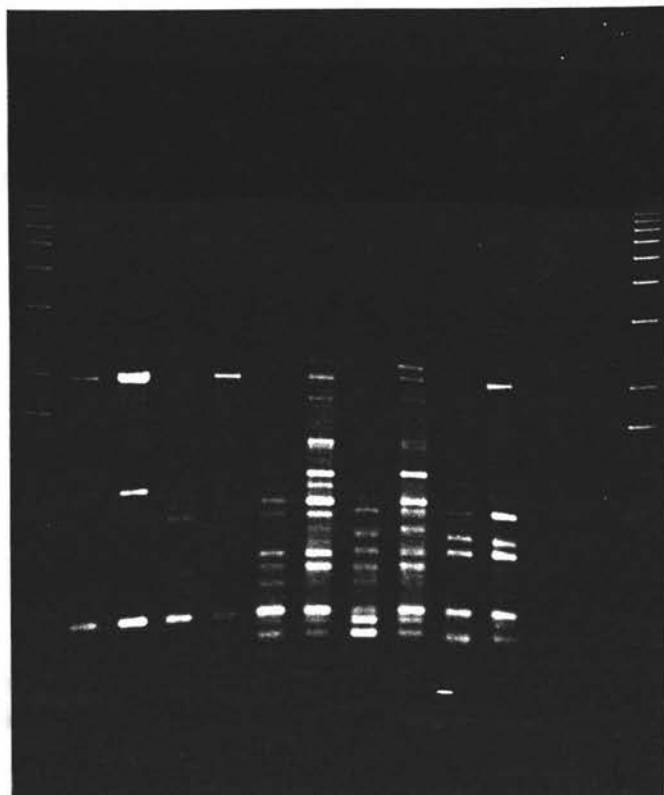
60°C exceeded the T_m of GC90 so no amplification occurred (Lanes 12 and 13).

FIGURE 18: Examination of the heterogeneity of 2 isolates of *G. intestinalis* (Hast 68 and Whang 48) by RAPD analysis using primer GC100 - the effect of varying the annealing temperature.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, Hast 68, 35°C; Lane 3, Whang 48, 35°C; Lane 4, Hast 68, 40°C; Lane 5, Whang 48, 40°C; Lane 6, Hast 68, 45°C; Lane 7, Whang 48, 45°C; Lane 8, Hast 68, 50°C; Lane 9, Whang 48, 50°C; Lane 10, Hast 68, 55°C; Lane 11, Whang 48, 55°C; Lane 12, Hast 68, 60°C; Lane 13, Whang 48, 60°C; Lane 14, 1 kb ladder.

NOTE: The 2 isolates showed little difference even when the annealing temperature was increased. The major bands were amplified in all cases but showed some variation in intensity.

1 2 3 4 5 6 7 8 9 10 11 12 13 14



1 2 3 4 5 6 7 8 9 10 11 12 13 14

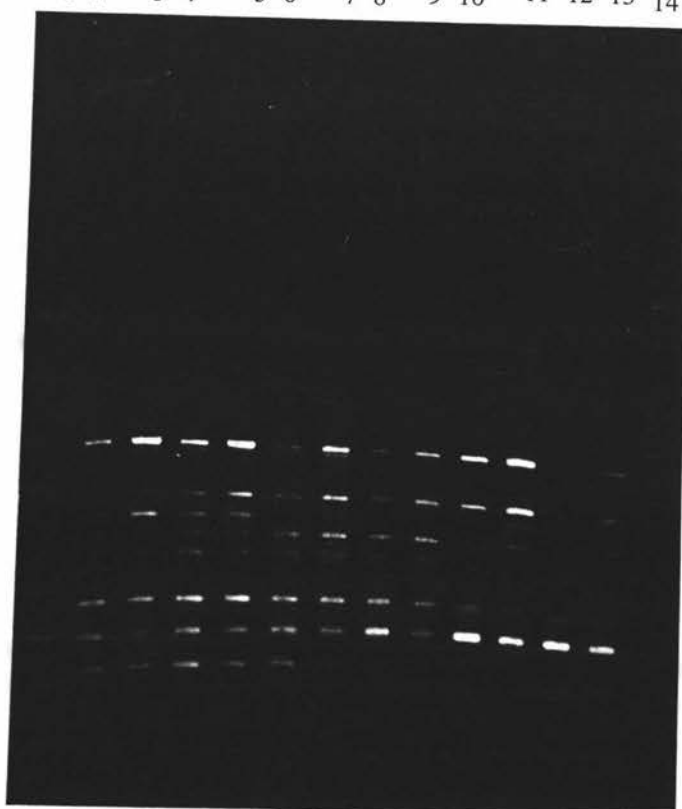


FIGURE 19: Examination of the heterogeneity of 2 isolates of *G. intestinalis* (Hast 68 and Whang 48) by RAPD analysis a combination of 2 arbitrary primers and an annealing temperature of 35°C.

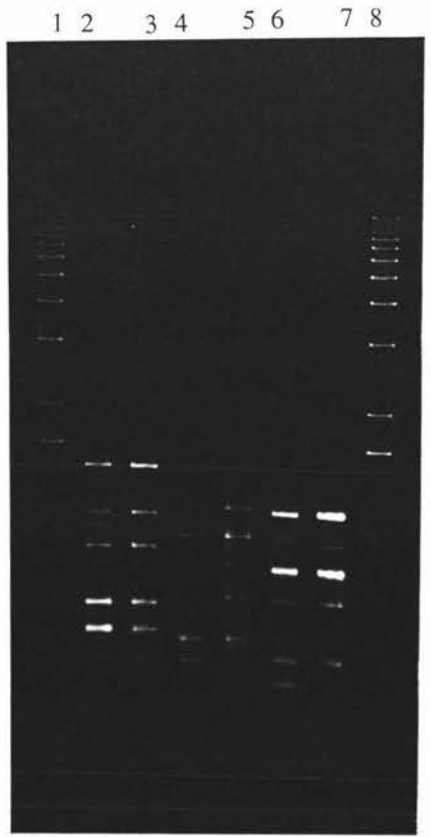
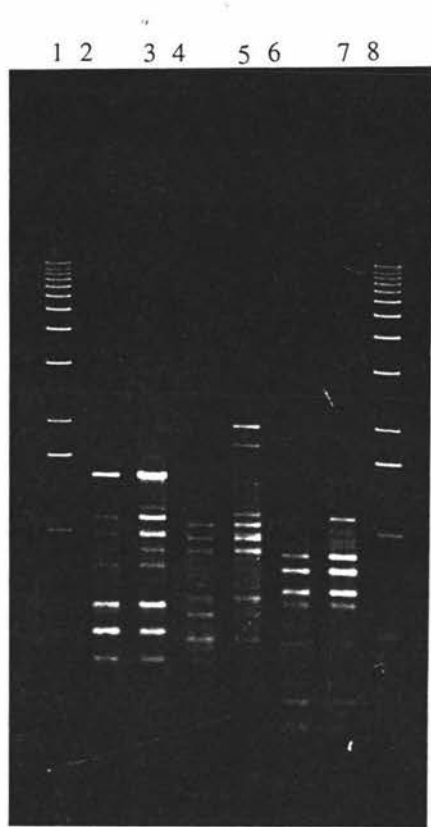
The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, Hast 68, GC30 and GC100; Lane 3, Whang 48, GC30 and GC100; Lane 4, Hast 68, GC40 and GC90; Lane 5, Whang 48, GC40 and GC90; Lane 6, Hast 68, GC50 and GC70; Lane 7, Whang 48, GC50 and GC70; Lane 8, 1 kb ladder.

NOTE: Some minor differences were detectable in the high molecular weight bands. However, the banding patterns between the 2 isolates were similar with all 3 primer combinations.

FIGURE 20: Examination of the heterogeneity of 2 isolates of *G. intestinalis* (Hast 68 and Whang 48) by RAPD analysis a combination of 2 arbitrary primers and an annealing temperature of 50°C.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, Hast 68, GC30 and GC100; Lane 3, Whang 48, GC30 and GC100; Lane 4, Hast 68, GC40 and GC90; Lane 5, Whang 48, GC40 and GC90; Lane 6, Hast 68, GC50 and GC70; Lane 7, Whang 48, GC50 and GC70; Lane 8, 1 kb ladder.

NOTE: No differences were seen with the combinations of GC30 and GC100, and GC50 and GC70. Some minor high molecular band differences were seen when the DNA was amplified using GC40 and GC90 as the two primers.



2.4 Conclusions.

Examination of the 11 isolates with GC60 (Figure 10) and GC100 (Figure 14) showed all the isolates to be similar. Thus while there were slight variations in the band intensity the overall banding patterns were identical.

The other 4 primers also gave banding patterns with little or no differences. Thus examination with all 5 primers showed the following isolates to be identical: Bris/83/Hepu/106, Hast 86, Whang 48, Ham 7, Whang 8, Wang 3, Hast 11 and N.Ply 77 (Figures 9, 11, 12, 13 and 14). These results therefore suggest that at least 8 of the 11 isolates tested can not be distinguished by this method but the method divided the 11 isolates into 4 groups - one containing 8 isolates and the other 3 containing 1 isolate each.

The results of all the tests done using single arbitrary primers with each of the 11 *G. intestinalis* isolates are summarised in Table 1. Each letter within the body of the table indicates a different banding pattern.

Initial investigations of the use of increased annealing temperature and use of two primers together had no apparent advantage.

This approach clearly shows promise for the purpose of distinguishing strains of *G. intestinalis*. However to achieve the objective of answering the question are animal and human strains the same or different it is necessary to examine a large number of animal and human isolates to establish if they represent a single population or alternatively represent several populations of host-specific strains.

Table 1: The 8 different random primers were used to amplify the DNA of 11 strains of *G. intestinalis*. Each unique banding pattern is designated by a letter. For example, GC50 showed 3 different banding patterns as illustrated in Figure 9.

Note: GC30 and GC40 produced no amplification.

GC60 and GC100 produced one banding pattern with all isolates.

The remaining primers produced two or three different patterns with the 11 isolates.

	GC30	GC40	GC50	GC60	GC70	GC80	GC90	GC100
Hast 68 DNA	no band	no band	a	d	e	g	j	l
Ham 84 DNA	no band	no band	b	d	f	h	k	l
Hast 76 DNA	no band	no band	c	d	f	i	k	l
DNA of other 8 isolates*	no band	no band	c	d	f	h	k	l

* Bris/83/Hepu 106, Hast 86, Whang 48, Ham 7, Whang 8, Wang 3, Hast 11, N.Ply 77.

Chapter 3

The development of *Giardia muris* -specific PCR primers.

3.1 Introduction

The current method used to detect *Giardia* cysts in human water supplies involves the filtration of a large volume (usually greater than 380 litres) of water. The filter is stripped, washed, the eluate centrifuged and the resuspended deposit is fractionated using a sucrose gradient so as to separate the cysts (if present) from most of the debris. The appropriate fraction is collected from the gradient and examined for the presence of cysts. This involves making a smear of cysts (if present) and staining with a fluorescein-labelled monoclonal antibody to a surface antigen of *Giardia*. However, this antigen is shared by *G.intestinalis* and *G. muris* so the test does not discriminate between the two species. Careful measurement of cyst dimensions using populations of *G. intestinalis* and *G. muris* cysts show that the two species vary slightly in size. However this difference is too small to allow individual cysts to be identified as to species. In a water sample only a few cysts are normally seen so their species cannot be identified. It is however generally accepted that *G. muris* does not infect humans so it is possible that some waters that may contain only *G. muris* cysts, are being unnecessarily condemned for human consumption.

It is desirable therefore to develop a test which could be used to reliably distinguish the two species. This test could be used not only to confirm a positive fluorescent test, but also to determine the species present.

The polymerase chain reaction is a highly sensitive method for detecting specific DNA sequences and hence can be used to detect and identify microorganisms, so it has the potential to detect one or a few *Giardia* cysts. If primers specific for each species were to be developed, PCR using appropriate primers should be able to detect *Giardia* cysts and also discriminate between the two species of *Giardia* even if few cysts were present. This section of the thesis is concerned with the development of PCR primer pairs specific for *G. muris*.

While there are many potential approaches to the production of *G. muris* -specific PCR primers, a prominent band was detected when *G. muris* DNA was amplified with a random PCR primer. This band, which was absent from *G. intestinalis* isolates, is about

350bp long. This is an appropriate size for detection by PCR. The following scheme was adopted in an attempt to produce a *G. muris* -specific primer pair:

1. Use a random primer to produce the band found upon amplification of *G. muris* DNA but not *G. intestinalis* DNA.
2. Clone the band in an *E. coli* based sequencing vector, pUC118.
3. Sequence the DNA fragment produce by the random primer.
4. Select DNA sequences, from each end of the fragment, suitable for use as PCR primers. These (unlike the random primer) should be of adequate length to ensure specificity.
5. Test the primer pair to investigate their specificity.

3.2 Materials

3.2.1 For identification of a *G. muris* -specific RAPD analysis product.

- 1) Sterile Distilled Water
- 2) dNTP's 1.25mM
- 3) DNA Taq Polymerase Buffer (x10)
As supplied by manufacturer (Gibco).
- 4) DNA 1 μ g/ μ l
G. muris DNA from a New Zealand isolate.
- 5) MgCl₂ 50mM
As supplied by manufacturer (Gibco).
- 6) Primer RC09 1nmol/ μ l
Supplied as dry pellet by Oligos etc, and diluted with sterile distilled water to a concentration of 1nmol/ μ l.

The primer sequence is as follows:

5' gAT AAC gCA C 3'

- 7) Taq Polymerase 5units/ μ l
As supplied by manufacturer (Gibco).

For polyacrylamide gel electrophoresis and photography of gel, see appendix.

3.2.2 Gel purification of the *G. muris* -specific RAPD analysis product.

- 1) Sterile scalpel blade
- 2) Shortwave ultraviolet light
- 3) 500 μ l sterile eppendorf tube
- 4) Needle
- 5) 1.5ml sterile eppendorf tube

3.2.3 To find restriction enzymes that do not cut the fragment.

- 1) Restriction enzymes as follows:

BamHI
EcoRI
HindIII
KpnI

- 2) Reaction buffers

All enzymes were used with the buffers recommended by the supplier.

3.2.4 Preparation of pUC118 vector.

- 1) SmaI enzyme
- 2) SmaI reaction buffer
- 3) pUC118 vector

From Carolyn Young, Dept Microbiology and Genetics Unit, Massey University.

- 4) DNA Taq polymerase
- 5) DNA Taq polymerase reaction buffer
- 6) dTTP 2mM
- 7) MgCl₂ 90μM

3.2.5 Ligation.

- 1) pUC118 T-vector as prepared 3.3.4
- 2) purified PCR product as prepared 3.3.2
- 3) T4 ligase
- 4) T4 ligase reaction buffer

3.2.6 Electroporation.

Materials as described in the Bio-Rad Pulse Controller Instruction Manual.

- 1) *E. coli* MC 1022 cells

From Carolyn Young, Dept Microbiology and Genetics Unit, Massey University.

3.2.7 Blue/White colour selection.

- 1) X-gal
Stock - 2% Xgal in DMF
Used at 0.3%
- 2) IPTG
Stock - 100mM IPTG in water
Used at 6mM
- 3) ampicillin - 100μg/ml final concentration

3.2.8 Alkaline lysis - Small scale plasmid preparation.

1) **Tris, EDTA, Glucose (TEG) buffer**

50mM Glucose

10mM EDTA

25mM Trizma base

pH 8.0 with conc. HCl

2) **NaOH/SDS**

0.2M NaOH

1% sodium dodecyl sulphate (SDS)

3) **Potassium acetate solution**

3M potassium acetate

2M glacial acetic acid

pH 4.8

4) **Phenol/chloroform 1:1**

5) **100% ethanol**

6) **70% ethanol**

7) **Tris, EDTA (TE) buffer**

10mM Trizma base

1mM EDTA

pH 8.2 with conc. HCl

3.2.9 Check for presence of inserts.

1) **Restriction enzymes as follows:**

EcoRI

BamHI

2) **Reaction buffer**

All enzymes were used with the buffers recommended by the supplier.

3.2.10 Large scale plasmid preparations.

- 1) Chloramphenicol - 150 μ g/ml final concentration
- 2) TE buffer
see 3.2.8
- 3) TEG buffer
see 3.2.8
- 4) Lysozyme
2mg/ml dissolved in TE buffer
- 5) NaOH-SDS solution
see 3.2.8
- 6) Isopropanol
- 7) 95% ethanol
- 8) Water saturated isobutanol
- 9) 0.3M NaOAc (pH 5.0)

3.2.11 Sequencing of the insert.

- 1) CsCl purified DNA template
As prepared in 3.3.10
- 2) Materials for sequencing as described in the Sequenase Version 2.0
DNA Sequencing Kit (USB)

3.2.12 Polyacrylamide gel electrophoresis.

- 1) for polyacrylamide gel
6% (w:v)
8M urea

3.2.13 Primer selection.

1) EMBL library on GCG computer

Genetics Computer Group (1991)

3.2.14 Primer testing.

see 2.2.5

1) Primer Gml 1nmol/μl

Supplied as dry pellet by Oligos etc, and diluted with sterile distilled water to a concentration of 1nmol/μl.

The primer sequence is as follows:

5' gAg gAA TCA TCA gAA CCT CgC 3'

2) Primer Gmr 1nmol/μl

Supplied as dry pellet by Oligos etc, and diluted with sterile distilled water to a concentration of 1nmol/μl.

The primer sequence is as follows:

5' CAT AAA TCA gTg CAg AgT gTT TC 3'

3) DNA 1μg/μl

The following *G. intestinalis* isolates supplied by The Medical Microbiology Dept, The Faculty of Medicine, University of British Columbia:

S1

S2

VANC/85/UBC/7

VANC/85/UBC/9

BE-1 (IP-OH 82:1)

BE-2 (IP-OH 83:1)

4) SacII restriction enzyme

5) SacII restriction enzyme reaction buffer

3.3 Methods

3.3.1 The identification of a *G. muris* -specific RAPD analysis product.

G. muris and *G. intestinalis* DNA was amplified in the standard amplification reaction as described in 2.3.5b using primer RC09. The products were then electrophoresed through an 8% polyacrylamide gel (appendix). A 350bp fragment was identified in the products for *G. muris* that was not present in the products for *G. intestinalis*.

3.3.2 Gel purification of the *G. muris* -specific RAPD analysis product (Heere, *et al.* 1990, modified).

A 5 μ l aliquot of the *G. muris* sample amplified with primer RC09 was electrophoresed through a 1.6% agarose gel and stained with ethidium bromide. The 350bp fragment specific for *G. muris* was then cut from the gel, using a sterile scalpel blade, under shortwave UV light (250nm), and placed in a 500 μ l sterile eppendorf tube. A small hole was made in the bottom of the tube with a hot needle, the 500 μ l eppendorf tube was then placed in a 1.5ml sterile eppendorf tube, and centrifuged at 6,500rpm at 4°C for 15 minutes. The fragment was precipitated out of the eluate by the addition of 0.1 volume NaOAc and 3 volumes of ethanol, spun, and the pellet resuspended in TE buffer.

3.3.3 To find restriction enzymes that do not cut the fragment.

The fragment purified from *G. muris* DNA in the RAPD analysis with primer RC09 was then subjected to restriction enzyme digestion, as described in the appendix. The digestion products were then agarose gel electrophoresed (appendix).

3.3.4 Preparation of pUC118 vector.

The vector pUC118 was cut with the restriction enzyme SmaI. The digest contained 2 μ g of pUC118, 24 units of SmaI and 1x SmaI reaction buffer in a total volume of 20 μ l. The digestion was incubated at 25°C for 2 hours and then 60°C for 15 minutes to inactivate the enzyme.

A dTTP overhang was added to each end of the linearised pUC118 in a reaction containing 2 μ g of linearised pUC118, 3 units of DNA Taq polymerase, 1x DNA Taq

polymerase reaction buffer, 2mM dTTP and 90 μ M MgCl₂ in a total volume of 40 μ l. The reaction was incubated at 70°C for 2 hours (Marchuk *et al* , 1990).

3.3.5 Ligation.

The purified *G. muris* PCR product was ligated to the T-tailed vector, in a reaction containing 200ng pUC118, 20ng purified PCR product, 1 μ l T4 ligase and 1x ligase reaction buffer in a total volume of 10 μ l. The ligation was incubated at 14°C for 16 hours.

3.3.6 Electroporation.

Electroporation of the ligated products into *E. coli* MC 1022 cells was carried out as described in the Bio-Rad Pulse Controller Instruction Manual, under conditions optimal for electroporation in *E. coli* (2.5V, 25 Ω , 25 μ F).

3.3.7 Blue/White colour selection.

Transformants were selected for by blue/white colour selection, making use of the *lac Z* gene in pUC118.

Luria agar was made in 3.5ml aliquots and autoclaved. Once cooled to 55°C, 50 μ l of X-gal (the chromogenic substrate in the presence of beta-galactosidase), 20 μ l of IPTG (which induces beta-galactosidase) and ampicillin to a final concentration of 100 μ g/ml were added. Each overlay was then poured over a Luria plate containing ampicillin, and allowed to set before plating the electroporated culture.

After incubation at 37°C for 1-2 days, there were two types of colonies present - blue and white. Antibiotic selection with ampicillin (100 μ g/ml) was used to ensure the presence of the plasmid in the *E. coli* transformant. The presence of an insert was detected by the inactivation of the beta-galactosidase gene in the pUC118 vector, making the colony white. Colonies where the insert was absent from the pUC118 vector had intact beta-galactosidase genes and were blue.

3.3.8 Alkaline lysis - Small scale plasmid preparation.

Each white colony (containing the vector and insert) was inoculated into 2ml of Luria broth containing ampicillin at 100 μ g/ml, and grown for 16 hours on a shaker at 225rpm

at 37°C. The colonies were also picked to a Luria agar plate containing ampicillin, and grown at 37°C for 16 hours, for later use.

A 1.5ml aliquot of the overnight culture was pipetted into a sterile eppendorf tube and centrifuged for 1 minute. The supernatant was removed with a sterile pasteur pipette, and the pellet resuspended in 100µl of TEG by vortexing. This suspension stood at room temperature for 5 minutes.

A 200µl aliquot of freshly prepared NaOH/SDS solution was added to the suspension and mixed by gently inverting several times. It then stood on ice for 5 minutes.

A 150µl aliquot of cold potassium-acetate solution was added and vortexed gently for 10 seconds, then stood on ice for 5 minutes.

The suspension was centrifuged for 5 minutes, and then the supernatant was transferred to a fresh tube. A equal volume of phenol/chloroform (1:1) was added, the suspension vortexed thoroughly, and spun for 2 minutes. The aqueous phase was transferred to a new tube, and 2 volumes of 100% ice cold ethanol were added. The solution was vortexed, stood at room temperature for 2 minutes, centrifuged for 5 minutes, and the pellet of precipitated DNA was washed with 70% ethanol. The pellet was dried and then resuspended in TE buffer. The DNA was stored at 4°C.

3.3.9 Check for presence of inserts.

The DNA from the alkaline lysis preparation was then analysed for the presence of an insert. A small volume, typically 3µl, of the DNA from each clone was run on a 1.6% agarose gel (appendix), alongside undigested pUC118 vector DNA. A number of clones appeared to contain the insert (their larger size meant that they ran more slowly than the pUC118 vector) and four of these were chosen for further analysis.

The 4 DNA samples thought to contain the insert, as well as 1 DNA sample that did not and one other DNA sample that gave an unexpected result, were digested with the enzymes BamHI and EcoRI to separate the insert from the vector. The digests contained 250ng of DNA, 5-10u each of BamHI and EcoRI in a restriction enzyme buffer compatible to both enzymes. The final volume was 25µl. These digests were then electrophoresed through a 1.6% agarose gel (appendix).

3.3.10 Large scale plasmid preparations.

The 4 clones containing the insert inoculated from the agar plate made earlier into 5ml of Luria broth containing ampicillin (100 μ g/ml), and grown for 16 hours with shaking at 37°C. The culture was then diluted 1:100 in Luria broth containing ampicillin, and grown for 3-5 hours, on a shaking platform at 225rpm at 37°C. Chloramphenicol was added to 150 μ g/ml, and the incubation continued for 16 hours at 37°C.

The cells were spun down in a GS-A tube at 9,000rpm for 5 minutes, then resuspended by vortexing in 50ml of cold TE. Another 75ml of TE was added, and the cells were spun again.

The supernatant was poured off, and residual liquid removed by pasteur pipette. The cells were resuspended in 8ml of TEG buffer, and transferred to a SS34 tube. A 1ml aliquot of freshly prepared lysozyme was added, and the suspension was stood on ice for 30 minutes. A 10ml aliquot of freshly prepared NaOH-SDS solution was added, and the suspension was mixed by gently inverting a few times. The suspension was then centrifuged at 15,000rpm for 1 hour at 4°C.

The supernatant was decanted into 12ml of isopropanol, mixed, and stood on ice for about 1 hour, or at 4°C overnight. Following centrifugation at 4,000rpm for 30 minutes at 4°C, the supernatant was poured off and discarded, and the pellet washed in 20ml of 95% ethanol, and spun at 3,000rpm for 20 minutes at 4°C. The supernatant was discarded, the pellet air dried slightly and resuspended in 9ml of TE.

The plasmid DNA was separated on a 5ml CsCl-ethidium bromide gradient (9.54g CsCl, 0.9ml ethidium bromide, per 9ml DNA solution) at 55,000rpm for 5 hours at 15°C in an ultracentrifuge.

After centrifugation, the lower, major, band was removed, under long wavelength UV light. The ethidium bromide was extracted 2-3 times with isobutanol that had been saturated with sterile distilled water. The DNA was dialysed against 3 litres of TE.

To ensure that the insert was present, a small amount, typically 200ng of each preparation was digested with BamHI and EcoRI (as described in 3.3 10), and electrophoresed through a 1.6% agarose (appendix).

3.3.11 Sequencing of the insert.

The sequencing reaction was carried out using the double stranded sequencing protocols, as described in the Sequenase Version 2.0 DNA Sequencing Kit (USB). A 2 μ g amount of CsCl purified DNA was used as a template.

3.3.12 Polyacrylamide gel electrophoresis.

The order of nucleotide bases was determined by electrophoresing 3 μ l of each sequencing reaction through a 6% polyacrylamide gel containing 8M urea at a constant power of 65W for 6 hours. This was repeated in a short run, where the gel was run for 2 hours. The gel was removed to a piece of 3MM Whatman filter paper, vacuum dried at 80°C for 40 minutes and exposed overnight to X-ray film (Kodak XAR).

3.3.13 Primer selection.

Primers were designed to match either end of the sequence, so that each of the primers had an equal T_m . The primer sequences were checked using the GCG library to ensure that there was minimal homology between pairs of primers and minimal internal homology.

3.3.14 Primer testing.

Once the primer sequences selected had been manufactured by Oligos etc, their specificity was tested. PCR reactions were carried out as described in 2.3.5.2, using DNA extracted from *G. intestinalis* and *G.muris*, and 50pg each of Gml and Gmr, for 30 cycles at 65°C. The amplified products were then examined by acrylamide gel electrophoresis (appendix). The amplified product should have been 307bp in length.

To ensure that the primers were amplifying the correct sequence, the sequence of the original PCR product was examined. The sequence data predicted that the enzyme SacII would cut the product to produce 2 bands, 224bp and 83bp. Digestion of the product, amplified by the *G. muris*-specific primer pair, with SacII could therefore be used to check that the correct sequence was being amplified. The amplified product of *G. muris* was then cut with this restriction enzyme, SacII. The digest products were then examined by acrylamide gel electrophoresis (appendix).

3.4 Results

3.4.1 Detection of a *G. muris* -specific RAPD analysis product.

When DNA from *G. muris* and several *G. intestinalis* isolates was amplified using RCO9 as a single random primer, several bands were detected in all cases. These varied in intensity. However, a prominent band was produced with *G. muris* DNA. This band was intense and on repeated experiments was always produced. It was not found in *G. intestinalis* DNA(Figure 21).

3.4.2 Gel purification of the *G. muris*-specific RAPD analysis product.

The 350bp product was excised from the gel and eluted ("gel purified"). The product was then electrophoresed to confirm its purity (Figure 22).

3.4.3 Restriction enzyme digests.

Since the required product had ultimately to be excised from the vector it was necessary to identify restriction enzymes which do not cut the 350bp product . Four enzymes (see legend, Figure 23) were examined and none of them cut it.(Figure 23).

3.3.4 Small scale plasmid preparation.

After preparation of the pUC118 vector, ligation, electroporation and blue/white colour selection, a small scale plasmid preparation was produced from each white colony. The preparations were run on a 1.6% agarose gel (Figures 24a and 24b). Six samples were selected for further analysis. The plasmids were cleaved using EcoRI and BamHI to excise the insert (350bp amplification product) . These digests were then run on a 1.6% agarose gel and in all cases two bands were seen (Figure 25). These corresponded to the vector (upper band) and the original *Giardia* insert.

FIGURE 21: RAPD analysis to distinguish *G. muris* and *G. intestinalis* .

DNA from *G. intestinalis* and *G. muris* was amplified using primer RC09 and a standard PCR reaction with 30 cycles.

The samples (left to right) are : Lane 1, 1 kb ladder; Lane 2, *G. intestinalis* ; Lane 3, *G. muris* ; Lane 4, 1 kb ladder.

NOTE: A 350 bp band produced by amplification of *G. muris* DNA is marked with an arrow. There is no corresponding product following the amplification of *G. intestinalis* DNA.

FIGURE 22: The purity of the *G. muris* specific RAPD analysis product.

The 350bp product amplified with RC09 from a *G. muris* isolate DNA was electrophoresed and the band was eluted. The purity of the eluted product was examined by gel electrophoresis.

The samples (left to right) are: Lane 1, *G. muris* amplification before purification of the 350bp product; Lane 2, the 350bp product of *G. muris* amplification after purification; Lane 3, 1 kb ladder.

Note that the eluted product gave a single well defined band (lane 2).



FIGURE 23: The identification of restriction enzymes that do not cleave the 350bp product.

To cleave the 350bp fragment from the vector, it was necessary to identify restriction enzymes with cleavage sites to the left and right of the SmaI (site to be used for ligation) site of pUC118, but which would not cut the product.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, BamHI digest; Lane 3, undigested fragment; Lane 4, EcoRI digest; Lane 5, undigested fragment; Lane 6, HindIII digest; Lane 7, undigested fragment; Lane 8, KpnI digest; Lane 9, undigested fragment; Lane 10, 1 kb ladder.

NOTE: None of the enzymes tested cleaved the fragment, so any of them could be used to excise the product from the plasmid preparation.

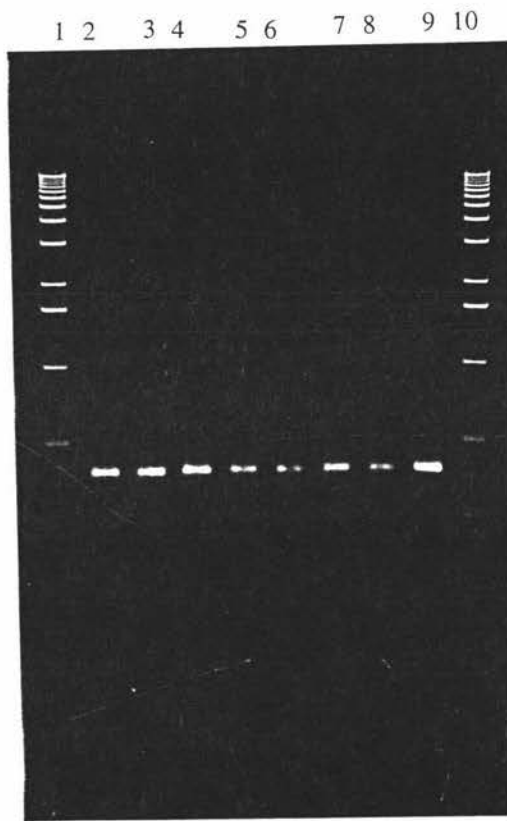


FIGURE 24: Examination of small scale plasmid preparations of electroporated *E. coli* cells to detect the presence of the insert.

After (possible) ligation of the 350bp PCR product into the pUC118 vector, electroporation of the vector into electrocompetent *E. coli* DH1 cells and blue/white colour selection of the resultant colonies, a small scale plasmid preparation from white colonies was examined to identify those clones which included the to identify those clones which included the RAPD analysis product.

Figure 24a: The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, pUC118; Lane 3, clone 1; Lane 4, clone 2; Lane 5, clone 3; Lane 6, clone 4; Lane 7, clone 5; Lane 8, clone 6; Lane 9, clone 7; Lane 10, clone 8; Lane 11, clone 9; Lane 12, clone 10; Lane 13, pUC118; Lane 14, 1 kb ladder.

Figure 24b: The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, pUC118; Lane 3, clone 11; Lane 4, clone 12; Lane 5, clone 13; Lane 6, clone 14; Lane 7, clone 15; Lane 8, clone 16; Lane 9, clone 17; Lane 10, clone 18; Lane 11, pUC118; Lane 12, 1 kb ladder.

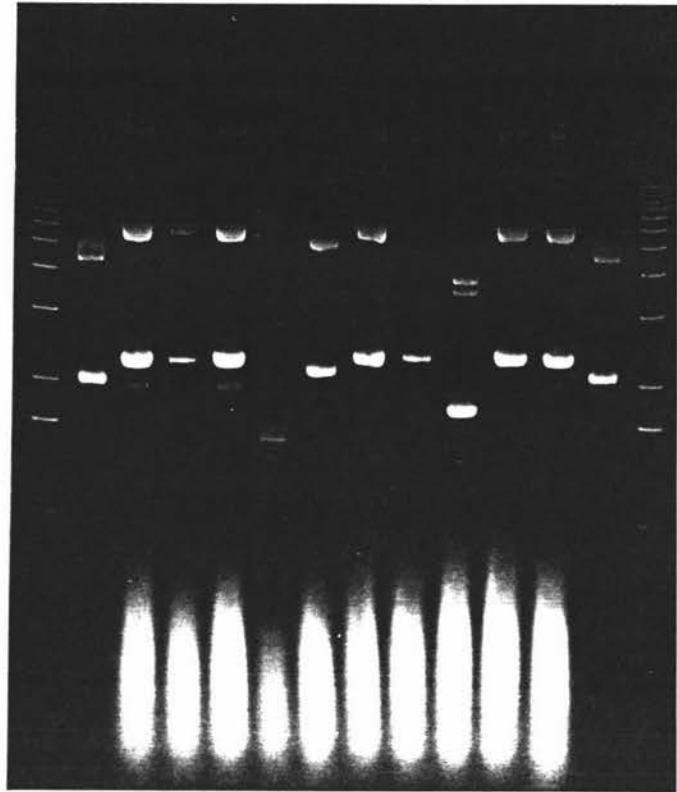
NOTE: Taking, from Figure 24a, lane 2 (no insert) and lane 3 (probably contains insert) as examples note that the prominent lower band of lane 3 (as indicated by an arrow) corresponds to a similar band in lane 2 (as indicated by an arrow). However, presumably because of the presence of an insert, the DNA band (lane 3) is slightly larger in molecular weight.

Similarly the following clones contain the 350bp product insert: 1, 2, 3, 6, 7, 9, 10, 11, 12, 15, 16, 17, 18. The others do not.

Further analysis (Fig 25) was carried out on clones 3, 9, 10 and 16 (contain insert); clone 14 (no insert); and clone 5, because it had an unexpected banding pattern.

The bright mass at the bottom of the gel is due to RNA.

1 2 3 4 5 6 7 8 9 10 11 12 13 14



1 2 3 4 5 6 7 8 9 10 11 12

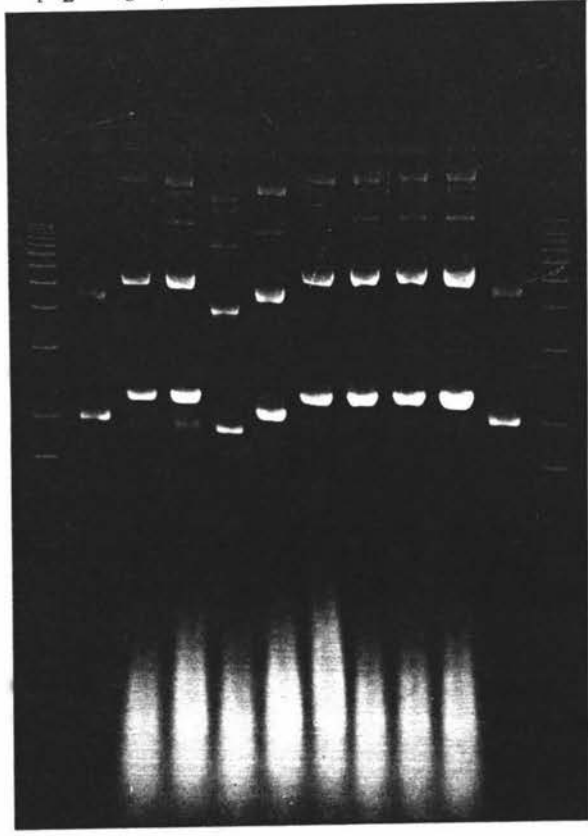


FIGURE 25: Detection of the insert in four (of six) of the small scale plasmid preparations by excision using restriction enzyme digestion. The fragment (if present) should be flanked by BamHI and EcoRI restriction sites in the plasmid.

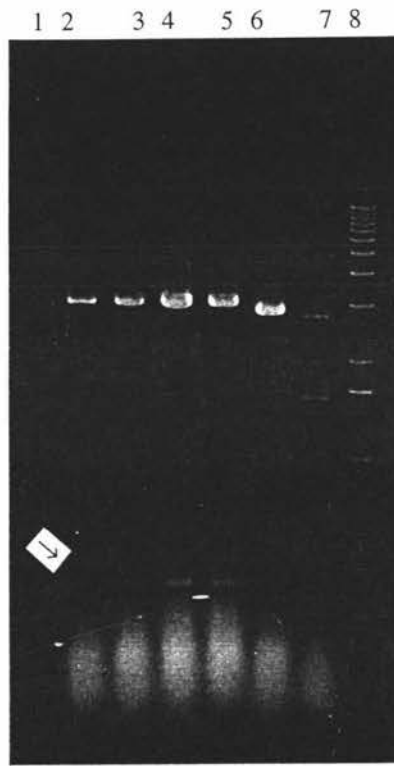
Clones 3, 5, 9, 10, 14 and 16 were digested with BamHI and EcoRI.

The samples (left to right) are: Lane 1, unligated purified product; Lane 2, clone 3; Lane 3, clone 9; Lane 4, clone 10; Lane 5, clone 16; Lane 6, clone 14; Lane 7, clone 5; Lane 8, 1 kb ladder.

NOTE: The insert (as indicated by an arrow) was (as expected from Fig 24a and Fig 24b) present in 4 of the clones. It was not found in the negative control or in the atypical clone.

The cleaved inserts (lanes 2 to 5) are slightly higher in the gel than the original purified fragment (lane 1). This is due to a slight increase in length because the excision sites are outside the two ends of the insert.

The bright mass at the bottom of the gel is due to RNA.



3.3.5 Large scale plasmid preparation.

Restriction enzyme digests were performed on the DNA after the large scale preparations to ensure that the insert was present (Figure 26).

3.3.6 Sequencing.

The *G. muris* -specific PCR product was sequenced as described. The sequencing gels are shown in Figures 27 and 28. The sequence data is presented in Figure 29.

3.3.7 Primer selection.

The primer pair selected from the sequence data is shown in Figure 30. Their selection is discussed in Chapter 4.

3.3.8 Primer testing.

Examination of the specificity of the selected *G. muris* -specific PCR primer pair was performed using the DNA from *G. muris* and six isolates of *G. intestinalis* (Figure 31). The results show that no amplification occurred with *G. intestinalis* DNA whereas a single band of the anticipated length was amplified with *G. muris* DNA.

3.3.9 Restriction enzyme digestion of the *G. muris* amplification product to confirm its identity.

Provided the DNA sequence of the product is as expected, it should be cleaved into 2 fragments by SacII. The result this digestion is shown in Figure 32. The fragment size, of 224bp and 83bp, are as expected

FIGURE 26: Restriction enzyme digestion to detect the presence of the insert in large scale plasmid preparations.

Clones 3 and 9 were digested with BamHI and EcoRI to cleave the 350bp insert from the vector.

The samples (left to right) are: Lane 1, 1kb ladder; Lane 2, undigested plasmid from clone 3; Lane 3, digested plasmid from clone 3; Lane 4, undigested plasmid from clone 9; Lane 5, digested plasmid from clone 9; Lane 6, 1 kb ladder.

NOTE: The insert (as indicated by an arrow) was present in both of the clone. Both were subsequently sequenced.

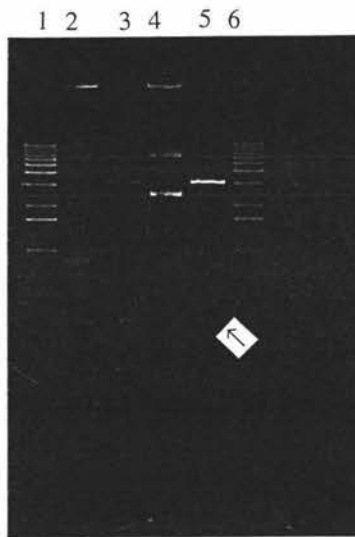


FIGURE 27: Long run polyacrylamide sequencing gel.

Clones 3 and 9 were used in a sequencing reaction and electrophoresed on a long run polyacrylamide gel. The results are shown.

The samples (left to right) are: Lane 1, clone 3, + strand, GATC bases; Lane 2, clone 3, - strand, GATC bases; Lane 3, clone 9, + strand, GATC bases; Lane 4, clone 9, - strand, GATC bases.

1

2

3

4

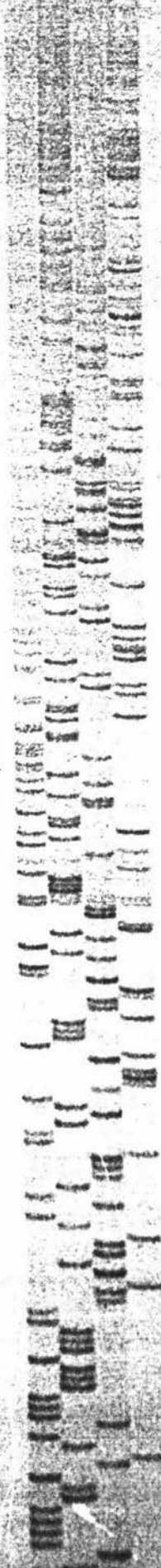


FIGURE 28: Short run polyacrylamide sequencing gel.

Clones 3 and 9 were used in a sequencing reaction and electrophoresed on a short run polyacrylamide gel. The results are shown.

The samples (left to right) are: Lane 1, clone 3, + strand, GATC bases; Lane 2, clone 3, - strand, GATC bases; Lane 3, clone 9, + strand, GATC bases; Lane 4, clone 9, - strand, GATC bases.

NOTE: The sequence of the insert in both clones was determined from a combination of two gels. Both sequences (see Fig 29) were identical.

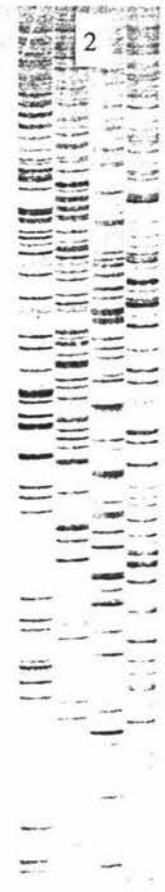
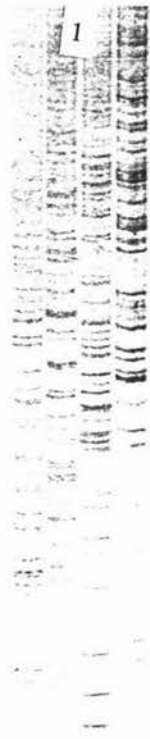


FIGURE 29: Nucleotide sequence of the *G. muris* specific product.

The nucleotide sequence of one strand of the DNA, as read from the sequencing gels is shown below. The sequence obtained for the other DNA strand complemented exactly.

Note: The sequence of the primer RC09 at each end of the fragment is marked, as is the SmaI recognition site.

	Primer RC09				Primer gml
1	GATCCCCTGA	<u>TAACGCACTA</u>	AGGATTGCGG	AGGTGTGTCA	<u>GAGGAATCAT</u>
51	<u>CAGAACCTCG</u>	CGCCCGTTTT	CATGAAGTGT	GGGATCTCCA	TCTCAGAGAC
101	TGACGATCAT	GTCAGTGAGG	AAGTCAATGG	GGAAGTCAGT	GGGAAAGAAA
151	GGTTCATTC	AGTGATTTTCG	GATAGTCCCT	CGAAACTTCC	TGGTAGTACC
201	TTGGAAAGCG	TCGAGCAAGT	TAGTGAGGTG	GGAAGGACAA	GGCTCATGGA
	SacII site				
251	<u>CGCCGCGGCT</u>	GATGGAGACG	TGGATAAGGT	CTNTCCACGT	GCGCTTCTCG
				Primer gmr	
301	GAGTTTGGAC	AGGTAGACAA	<u>TAAGAGAAAC</u>	<u>ACTGCACTGA</u>	<u>TTTATGCTTT</u>
		Primer RC09			
351	AGAGCTGCTA	GCCCTGGGTG	<u>CGTTATCAGG</u>	G	

FIGURE 30: Possible *G. muris* -specific primer pair sequences.

A left and a right primer was chosen, to constitute a possible *G. muris* -specific PCR primer pair. Care was taken to ensure that the primers were of equal T_m , had minimal internal homology and had minimal homology between the pair.

gml: 5' gAg gAA TCA TCA gAA CCT CgC 3'
(21-mer)

gmr: 5' CAT AAA TCA gTg CAg AgT gTT TC 3'
(23-mer)

FIGURE 31: Examination of the specificity of selected Gml and Gmr primer pair.

To examine if the primer pair Gml and Gmr could distinguish *G. muris* and *G. intestinalis* DNA of a *G. muris* isolate and 6 *G. intestinalis* isolates.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, *G. muris*; Lane 3, *G. intestinalis*, S1; Lane 4, *G. intestinalis*, S2; Lane 5, *G. intestinalis*, VANC/85/UBC/7; Lane 6, *G. intestinalis*, VANC/85/UBC/9; Lane 7, *G. intestinalis*, BE-1 (IP-OH 82:1); Lane 8, *G. intestinalis*, BE-1 (IP-OH 83:1); Lane 9, 1 kb ladder.

NOTE: A product is seen only following the amplification of *G. muris* DNA, and not following amplification of DNA from any of the *G. intestinalis* isolates. Subsequently this primer pair is referred to as a *G. muris*-specific primer pair.

FIGURE 32: Confirmation of the identity of the *G. muris*-specific amplification product. This was done by restriction enzyme digestion.

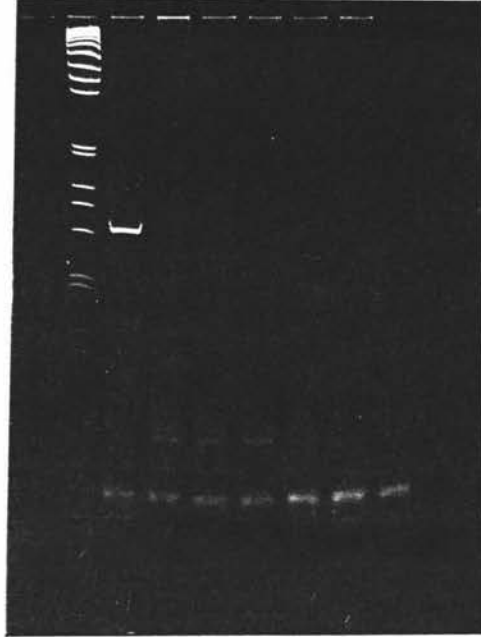
The *G. muris* product amplified using the primers Gml and Gmr was cut using SacII.

The samples (left to right) are: Lane 1, 1 kb ladder; Lane 2, uncut 307bp product; Lane 3, digested product; Lane 4, 1 kb ladder.

NOTE: That since the primers are slightly internal to the original sequence ends the product is now 307bp long (original 350bp). This is due to the fact that the new specific primer sequences are not at the very ends of the fragment, but are a short distance in at either end.

The two expected restriction products, 224bp and 83bp (lane 3), were detected.

1 2 3 4 5 6 7 8



1 2 3 4



3.4 Conclusions

PCR amplification of *G. muris* DNA with the primer RC09 resulted in the formation of a 350bp product. This product was not present after amplification of *G. intestinalis* DNA. It could therefore be used to distinguish the two species of *Giardia*. However, RC09 is a 10-mer primer, and therefore of low specificity.

To increase the product specificity longer (and paired) primers are required. To facilitate primer selection the product was sequenced, and 21 and 23-mers primer sequences were selected. These sequences were tested on *G. muris* DNA, and on DNA from 6 isolates of *G. intestinalis*. The results indicate that the primers amplified a *G. muris*-specific DNA sequence but no amplification of *G. intestinalis* DNA occurred. Restriction enzyme digestion (Figure 32) indicated that the product amplified was the expected sequence.

Chapter 4

General Discussion

This thesis had two major aims:

1. To investigate the use of random amplification of polymorphic DNA (RAPD) analysis to distinguish strains of *G. intestinalis*. This could be used in subsequent studies to investigate the possible host-specificity of *G. intestinalis*.
2. To develop a method by which *G. muris* and *G. intestinalis* cysts in water can reliably be distinguished. This could be used at least in the context of small rural supplies to avoid condemnation of supplies containing *G. muris* but not *G. intestinalis*.

The first question was addressed in Chapter 2. PCR was chosen as a potential means for distinguishing *G. intestinalis* isolates because of its advantages: the DNA primers can be selected for the purpose required and only a small amount of DNA is needed for analysis. RAPD analysis has been applied by a number of workers (Berg *et al*, 1994; Farber and Addison, 1994; Rasmussen *et al*, 1994; Bardakci and Skibinski, 1994; Kell and Griffin, 1994) to distinguish individual strains of many species including eukaryotic species.

The primer sequences chosen for this study were first used by Williams *et al* (1990), who were interested in the heterogeneity of soybean DNA. They recommended a set of oligonucleotide 10-mers, that ranged from 0% to 100% in G+C content. Each primer was similar to the "next" primer but had a single base change. This gave a set of primers ranging from AT rich to GC rich. Each primer was then used individually to amplify soybean DNA.

The results of their study indicated that a single base change in the primer composition can cause a complete change in the set of amplified DNA segments. The primers even though used with eukaryote DNA produced a limited number of bands, which could be resolved by agarose gel electrophoresis.

The present study examined the use of these primers with *G. intestinalis* DNA and the system was optimised with respect to cycle number, dNTP concentration, buffer concentration, DNA concentration, MgCl₂ concentration, primer concentration, DNA Taq

polymerase concentration, and annealing temperature. Following optimisation of the amplification system (see Chapter 2), the DNA of eleven human-sourced isolates of *G. intestinalis* was examined by RAPD analysis using eight arbitrary 10-mer primers.

The results indicated that RAPD analysis can be applied to distinguish strains of *G. intestinalis*. Thus using primers GC50 and GC80 were able to assign eleven isolates to three different groups, although interestingly, other related (one base change) primers failed to distinguish the groups. Therefore we concluded that RAPD analysis has the potential not only to trace the epidemiology of individual strains in humans but it also has the potential to indicate whether or not human and animal strains belong to one or a number of populations, ie, it has the potential to investigate host-specificity.

However, to pursue this point it would be necessary to study isolates from a range of animals and compare them to human isolates. While it was not possible to undertake this work in this thesis, this approach is being undertaken in this laboratory and it should shed light on the question of host-specificity.

The second and major section of this thesis was concerned with the development of a *G. muris* -specific primer pair, such as could be used to identify *G. muris* in water and distinguish it from *G. intestinalis*. Ideally if PCR is to be used for this purpose the following are needed:

1. *Giardia* genus-specific primer pair. This could be used to confirm a positive immunofluorescent test, and to demonstrate that DNA was extracted and was available for the PCR reaction.
2. *G. intestinalis* -specific primer pair. This could be used to test for the presence of *G. intestinalis*
3. *G. muris* -specific primer pair. This could be used to test for the presence of *G. muris*.

Theoretically, the last two primer pairs would be adequate to make the required distinction even in the absence of the genus-specific primer pair. However, it is preferable to confirm a positive result, and concomitantly to confirm that *Giardia* DNA was extracted and is available for amplification with species specific primers. The table below outlines the four possible results that could be obtained following PCR with the three primer pairs on a *Giardia* positive (by fluorescent staining) water sample.

Primer pair	1	2	3	4
<i>Giardia</i> genus	-	+	+	+
<i>G. intestinalis</i>	-	+	-	+
<i>G. muris</i>	-	-	+	+

These results would be interpreted as follows:

1. DNA was not extracted from the cyst, or the original IFT gave a false positive result.
2. *Giardia intestinalis* only, was present.
3. *Giardia muris* only, was present.
4. *Giardia intestinalis* and *Giardia muris* were both present.

PCR primer sequences specific for *Giardia* genus (Mahbubani et al, 1991) and *G. intestinalis* (Mahbubani et al, 1992) are already available. Chapter 3 of this thesis was, therefore, concerned with the development of primer pairs specific for *G. muris*.

The initial experiment used a random primer (RC09) to amplify a band specific for *G. muris*. Several products including a 350bp band of *G. muris* DNA were amplified. While segments of *G. intestinalis* DNA were also amplified using this primer, there was no 350bp band produced. However, RC09 is a 10-mer primer, so if used in an environmental sample, it would amplify sequences of varying lengths, some of which could be similar in length to the 350bp band, from extraneous DNA from other sources. Consequently, to develop specific primers the 350bp product was sequenced to allow the selection of a primer pair suitable for PCR and long enough to be specific for the target band of DNA even in the presence of extraneous DNA extracted from water-borne organisms. This required that each primer of the pair had to be of sufficient length to ensure with a high degree of probability that they are specific for *G. muris*. It is generally accepted that a sequence of 15-25 mer is likely to fulfil this criterion for any organism. Hence primer sequences around 20bp long were needed.

Two primers, gml (*G. muris* left) and gmr (*G. muris* right) were selected. The melting temperatures (T_m) of the 21-mer sequence gml(5' gAg gAA TCA TCA gAA CCT CgC 3') was estimated as follows:

$$\begin{aligned}
 T_m(\text{gml}) &= 4(\text{G}+\text{C})+2(\text{A}+\text{T}) \\
 &= 4(11)+2(10) \\
 &= 44+20 \\
 &= 64^\circ\text{C}
 \end{aligned}$$

As selected initially, the 21-mer gmr had an estimated T_m of 58°C. However, the addition of one thymine plus one cytosine nucleotide at the 3' end increased the sequence length to 23bp and the T_m to 64°C thus matching the T_m of the primers. The final gmr sequence used was 5' CAT AAA TCA gTg CAg AgT gTT TC 3'.

If primers self-anneal PCR would not amplify DNA at its optimum rate so each primer was checked for internal homology using the computer program EMBL. This showed that although internal loops would form with both gml and gmr at low stringency, the annealing temperature to be used in the PCR reaction would break the bonds.

The computer programme also predicted that there would be no formation of primer-dimers with gml or with gmr, due either to self annealing or annealing to the other primer in the pair.

While this primer pair was likely to be long enough to be specific in the sense that it is unlikely to amplify DNA of unrelated organisms the relationship between *G. muris* and *G. intestinalis* is sufficiently close that species-specificity could not be guaranteed. Thus it is possible that the 350bp sequence in *G. muris* could, with the exceptions of the two 10bp sequences at either end, be present in the DNA of both *G. muris* and *G. intestinalis*. Should this have proved to be the case, it would have been necessary to determine longer sequences of both organisms to find non-homologous sequences. However these primers gave no amplification of the DNA from *G. intestinalis*. That is they were species-specific in this context.

The development of the *G. muris* -specific primer therefore achieved our aim, that is to produce the third set of primer pairs (*Giardia* genus-specific; *G. intestinalis* -specific; *G. muris* -specific). This set of three pairs will, in principle, allow water to be tested for the presence of individual species of *Giardia*.

However, before this is actually applied in the field, some further work should be conducted as follows:

1. Sequence the PCR products to ensure that the actual band amplified is the predicted one.
2. The conditions for lysis of *Giardia* cysts should be optimised to ensure that DNA is available for amplification, even if only a few cysts are present.

3. Experiments to estimate the minimum number of cysts which will reproducibly give an amplified band need to be undertaken.
4. The primer pairs should be used in association with DNA extracted from organisms in random water samples (without *Giardia*) to ensure that the primer pair does not amplify DNA from other organisms.

These investigations are presently being undertaken by the author and others in the laboratory. They are expected to lead to a procedure which will reliably indicate if water samples contain *G. muris* only. If *G. muris* but not *G. intestinalis* is present in the water supply such water, although presently unacceptable for human consumption, may be accepted for human consumption at least for small isolated human populations in non-urban areas.

Appendix

Basic Procedures

1. Ethanol Precipitation of DNA

To precipitate DNA, 0.1 vol of 3M NaOAc pH 5.5 and 3 vol of cold (-20°C) 95% ethanol were added to the solution and the mixture stood for at least 2 hours at -20°C. The DNA was then pelleted by centrifugation for 15 minutes at 4°C in a microcentrifuge, or for 30 minutes at 4°C at 6000K in a Heraeus Christ centrifuge, washed in 70% ethanol, centrifuged for 5 minutes as before, dried for 5-10 minutes under vacuum in a Savant Speedvac Concentrator, and resuspended in TE buffer.

2. Restriction Enzyme Digests

Typically 50-250ng of DNA was digested in a total volume of 25 µl with 10U of enzyme in a commercially prepared buffer. The digest was incubated for 2 hours at 37°C, then for 10 minutes at 60°C to stop the reaction.

3. Agarose Gel Electrophoresis

DNA fragments were size fractionated by electrophoresis through a horizontal agarose gel matrix (1.6% Biorad Ultrapure DNA grade agarose in E-buffer (96.88g trizma base, 7.44g EDTA (disodium salt), 8.2g sodium acetate, pH to 7.8 with glacial acetic acid, distilled water to 2000ml. Store at 4°C and dilute 1/10 with distilled water when required)), in an Horizon 11.14 gel electrophoresis apparatus. A 5ml aliquot of each sample was mixed with 5ml of bromophenol blue dye (0.1% bromophenol blue w:v, 80% glycerol v:v) and loaded. The agarose gels were run at 80 volts for 3 hours, and DNA detected by staining with ethidium bromide at 5µg/ml for 10 minutes, destaining in E-buffer for 10 minutes and viewing on a short wave transilluminator. Photographs were taken with Polaroid Type 665 film.

The amplification products were analysed by electrophoresis in 1.6% agarose gels run at 80 volts for 3 hours in an Horizon 11.14 gel electrophoresis apparatus, and detected by staining with ethidium bromide. The gel was photographed under ultra violet (UV) light.

The BRL 1kb ladder was included in the gels in lanes adjacent to the samples as size markers.

BRL 1kb ladder

Each molecular weight given below represents the molecular weight of a band in the 1kb ladder used throughout this thesis.

12216

11198

10180

9162

8144

7126

6108

5090

4072

3054

2036

1636

1018

517

506

396

344

298

4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

DNA fragments were size fractionated through a vertical 8% polyacrylamide gel matrix. The gel was run for 3.5 hours at 100 volts, and DNA detected by staining with ethidium bromide at 5 μ g/ml for 45 minutes, destained in E-buffer for 10 minutes and view on a short wave transilluminator. Photographs were taken with Polaroid Type 665 film.

5. Luria broth

1.0% (w:v) tryptone, 0.5% (w:v) yeast extract, 0.5% (w:v) NaCl, pH 7.3 with NaOH.

6. Luria agar

1.5% (w:v) agar in Luria broth.

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