

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**DIFFERENTIATION OF STRAINS OF *GIARDIA INTESTINALIS* BY
IDENTIFICATION OF RESTRICTION FRAGMENT LENGTH
POLYMORPHISMS (RFLP's) AND THE CONSTRUCTION OF A GENE
LIBRARY.**

A Thesis Presented in Partial Fulfilment of the
Requirements for the Degree of
Master of Science in Microbiology

at

Massey University, Palmerston North
New Zealand

Shalome Anitta Campbell

1993

ABSTRACT

Giardia intestinalis is a flagellate protozoan which infects the gastrointestinal tract of humans and other mammals such as cats, dogs and farm animals. The organisms involved have been assigned to a single species, which might be taken to suggest the absence of host specificity, yet there is little epidemiological evidence to suggest that human infections are derived from nonhuman sources. This suggests some degree of host specificity which in turn implies the existence of different strains of *Giardia intestinalis*. Consequently, two related questions of public health interest can be raised: do animal strains of *Giardia intestinalis* infect humans and if not, how can cysts of human and animal origin detected in water samples be distinguished? If all or even some strains from animals fail to infect humans then it is probable that water may often be unnecessarily condemned as unsuitable for human use. Conversely, if animal strains infect humans then exclusion of animals from water catchment areas would be desirable. To clarify this situation, it is desirable to be able to distinguish individual strains of *G. intestinalis* and this thesis represents a preliminary attempt to do so using the techniques of molecular genetics.

Initial experiments attempted to detect Restriction Fragment Length Polymorphisms (RFLP's) produced by digests of total genomic DNA using a range of restriction endonucleases. The results were difficult to interpret because an excessive number of bands were produced. However, some denser bands, probably representing repetitive DNA sequences, were relatively well resolved and could allow comparisons between strains to be made. This repetitive DNA is GC-rich and was separated from most of the nonrepetitive genomic DNA by CsCl centrifugation in the presence of Hoescht 33258 stain. Using this approach, GC-rich fractions of DNA from several strains of *G. intestinalis* were compared using a variety of restriction endonucleases. Most did not reveal differences but digestion with some restriction endonucleases revealed minor differences. This demonstrated the potential usefulness of this approach in distinguishing between strains of *Giardia* but the procedure was laborious and required large amounts of DNA which could only be produced by the

culturing of organisms in bulk. This is not yet possible in the case of many strains of *G. intestinalis*, so it was concluded that an alternative approach using digests of total genomic DNA followed by electrophoresis, Southern blotting and hybridisation with specific DNA probes would represent both a better theoretical and practical approach. A desirable preliminary step to facilitate this approach is the production of a *Giardia* gene library and the latter part of this thesis describes this process.

The major problem encountered with the production of the library was that the DNA produced by a range of extraction methods was sheared and was present only in low concentration. This problem was traced to the presence of an excessive amount of polysaccharide which appeared to be strongly associated with unsheared DNA and hence caused it to be trapped at the interphase during phenol/chloroform extractions. As only the supernatant was retained, the high molecular weight DNA was largely lost during such extraction steps. This difficulty was overcome by precipitating the DNA with isopropanol at an early stage in the extraction method. This reagent does not precipitate the polysaccharides present in the cell lysate so that these are removed with the supernatant. The precipitated DNA was redissolved and, following conventional purification procedures, represented a high yield of unsheared DNA. Using this DNA, a library was made using LambdaGEM-11 Xho Half-site armsTM (Promega). Experiments showed that the library is representative of the genome of *G. intestinalis* and exceeded by 6-fold the number of clones required for a 99.9% probability that any particular sequence of interest is present.

The availability of such a library should permit the selection of suitable clones for use as probes to hybridise with total genomic digests to reveal differences between strains of *G. intestinalis*. The ability to distinguish strains would allow investigations of host specificity which may have implications for the formulation of testing procedures designed to prevent human infections of *G. intestinalis* but avoiding the unnecessary condemnation of water supplies containing *Giardia* strains that do not infect humans. The development of strain-specific probes would also serve as a useful epidemiological tool in tracing the source of infection within a community.

ACKNOWLEDGEMENTS

*God has made a way where there seemed to be no way
He works in ways we cannot see
He has made a way for me
He is my guide, He holds me closely to His side
With love and strength for each new day
He has made a way. (Don Moen)*

My first thanks goes to the One who always has faith in me - no matter what.

I would like to thank the Department of Microbiology and Genetics for the use of facilities that have made this project possible. I would also like to thank my supervisor Assoc. Prof. John Clarke for guidance and support and for valuable help in the writing up of this thesis.

Thanks to Prof. Barry Scott and Yasuo Itoh of M.G.U. for help and inspiration in the construction of the gene library and Dr. George Ionas for help in the initial stages of this project. Thanks also to members of the *Giardia* group (past and present) - Phil, M & M's, Mitch, Jody, Dan, Morg, Kirsty and Erica for putting up with me, the Guess What?'s and sick jokes. You guys made the lab a fun place to be in (and ABBA Kirsty!).

Special awards for keeping sanity go to my great friends Merie, Sheree, Paul, Terence and Morgan - even if I destroyed yours. Awards for keeping humility go to Treacle, Roach, Ron, Stingle, Smurf, Jackie, Mitch, Paul, Vicki, Gail, Lo, Neroli and Garry, all the folk at C.C.C. (esp. the Worship Team) and Diana.

To my greatest support team - Dad, Mum, Cath, Ed, Cherith, Alan, Logan, Aaron, Nathan, Josiah, my grandparents, the Primes and all the other rellies - I thank God for the privilege of having such a great family.

TABLE OF CONTENTS

	Page
TITLE PAGE	i
ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	v
LIST OF FIGURES	ix
LIST OF TABLES	xii
CHAPTER 1: <u>Introduction</u>	1
CHAPTER 2: <u>Historical Review</u>	4
2.1 The Biology of <i>Giardia</i>	4
2.1.1 Historical Background	4
2.1.2 Taxonomy and Nomenclature	4
2.1.3 Life Cycle and Morphology	5
2.1.4 Cultivation	8
2.2 Giardiasis	11
2.2.1 Pathogenesis and Clinical Illness	11
2.2.2 Immunity	12
2.2.3 Diagnosis and Treatment	13
2.3 Epidemiology	16
2.3.1 Transmission	16
2.3.2 Host Specificity	19
2.4 Species Determination and Strain Variation	21
2.4.1 Species Determination	21

2.4.2 Strain Differentiation	22
CHAPTER 3: <u>Optimization of the Conditions Required for</u> <u>Large-Scale Harvesting of <i>G. intestinalis</i></u> <u>Trophozoites</u>	26
3.1 Introduction	26
3.2 Materials and Methods	27
3.2.1 Retrieval of Frozen Trophozoite Cultures	27
3.2.2 Subculture of <i>Giardia intestinalis</i> Trophozoites	27
3.2.3 Treatment of Contaminated Cultures	28
3.2.4 Preparation and Harvesting of Large-Scale Trophozoite Cultures	30
3.2.5 Counting Trophozoite Population Numbers	32
3.2.6 Cryopreservation of <i>Giardia</i> trophozoites	33
3.3 Results	35
3.4 Discussion	38
CHAPTER 4: <u>Detection and Characterization of Restriction</u> <u>Fragment Length Polymorphisms in the</u> <u><i>G. intestinalis</i> Genome</u>	39
4.1 Introduction	39
4.2 Materials and Methods	40
4.2.1 Propagation of <i>G. intestinalis</i> isolates for DNA Extraction	40
4.2.2 DNA Extraction	41

4.2.3	Isolation and Removal of the GC-rich Fraction of DNA	44
4.2.4	Removal of CsCl by Microdialysis	45
4.2.5	Determination of DNA Concentration and Purity	45
4.2.6	Restriction Enzyme Digestion of <i>G. intestinalis</i> DNA	46
4.2.7	Agarose Gel Electrophoresis of Restriction Enzyme Digests	48
4.2.8	Determination of the Size of DNA Fragments	49
4.3	Results	50
4.4	Discussion	61
CHAPTER 5: <u>Construction of a Gene Library from <i>G. intestinalis</i> strain NP77 DNA</u>		68
5.1	Introduction	68
5.2	Materials and Methods	69
5.2.1	Preparation of High Yield Trophozoite Cultures	69
5.2.2	Extraction of High Molecular Weight DNA	69
5.2.2a	Ionas (1989)	69
5.2.2b	Nash <i>et al</i> (1985)	71
5.2.2c	Strauss (1990)	73
5.2.2d	Sambrook <i>et al</i> (1989)	74
5.2.2e	van Keulen (personal communication)	76
5.2.3	Examination of the State of the DNA	79
5.2.4	Determination of the DNA Concentration and Purity .	79
5.2.4a	Spectrophotometric Method	79
5.2.4b	Concentration Standards	79
5.2.4c	Fluorometric Method	80

5.2.5	Establishing Conditions for Partial Digestion of High Molecular Weight DNA	82
5.2.6	Large-Scale Preparation of Partially Digested DNA	87
5.2.7	Partial Fill-In Reaction for Genomic DNA	89
5.2.8	Ligation of Insert to Vector Arms	91
5.2.9	Small-Scale Packaging of Ligated DNA	93
	5.2.9a Packaging of the Library	96
	5.2.9b Titre of the Library	96
5.2.10	Large-Scale packaging of Ligated DNA	98
5.2.11	Amplification of the Library	99
5.2.12	Storage of the Library	101
5.2.13	Demonstration of the Representation of the Library	101
5.2.14	Determination of Insert Size	107
5.3	Results	109
5.4	Discussion	116
CHAPTER 6: <u>General Discussion</u>		121
APPENDIX		127
BIBLIOGRAPHY		132

LIST OF FIGURES

		Page
Figure 1:	<i>G. intestinalis</i> trophozoites cultured <i>in vitro</i>	7
Figure 2:	<i>G. intestinalis</i> cysts from (a) human faeces and (b) an <i>in vitro</i> culture	9
Figure 3:	Comparison of the yield of <i>G. intestinalis</i> strain Hast 11 trophozoites with and without cooling of the culture to 0°C before harvest	37
Figure 4:	Bam HI digestion of total genomic DNA from ten New Zealand isolates of <i>G. intestinalis</i>	52
Figure 5:	Representation of the results obtained when total genomic DNA was centrifuged with CsCl in the presence of Hoescht dye 33258	53
Figure 6:	Bam HI digestion of total genomic DNA, AT-rich DNA and GC-rich fraction DNA from <i>G. intestinalis</i> strain Hast 76	54
Figure 7:	Bam HI digestion of the GC-rich fraction of DNA from each of ten New Zealand isolates of <i>G. intestinalis</i>	55
Figure 8:	Sma I digestion of the GC-rich fraction of DNA from each of ten New Zealand isolates of <i>G. intestinalis</i>	55

Figure 9:	Apa I digestion of the GC-rich fraction of DNA from each of ten New Zealand isolates of <i>G. intestinalis</i>	56
Figure 10:	Hinf I digestion of the GC-rich fraction of DNA from each of ten New Zealand isolates of <i>G. intestinalis</i>	56
Figure 11:	Bgl II digestion of the GC-rich fraction of DNA from each of ten New Zealand isolates of <i>G. intestinalis</i>	57
Figure 12:	Hae III digestion of the GC-rich fraction of DNA from each of ten New Zealand isolates of <i>G. intestinalis</i>	57
Figure 13:	Fok I digestion of the GC-rich fraction of DNA from each of ten New Zealand isolates of <i>G. intestinalis</i>	58
Figure 14:	Kpn I digestion of the GC-fraction of DNA from each of ten New Zealand isolates of <i>G. intestinalis</i>	58
Figure 15:	Taq I digestion of the GC-rich fraction of DNA from each of ten New Zealand isolates of <i>G. intestinalis</i>	59
Figure 16:	Organisation of the <i>G. intestinalis</i> rRNA genes as a 5.56 kb repeating unit showing the presence of both constant and variable regions	66

Figure 17:	The complete nucleotide sequence of the <i>G. intestinalis</i> rRNA gene repeat	67
Figure 18:	Pilot-scale partial Sau 3A I digestion of intact genomic DNA from <i>G. intestinalis</i> strain NP 77	86
Figure 19:	Comparison of total genomic DNA extracted from <i>G.intestinalis</i> trophozoites using the methods of Ionas, Strauss and van Keulen	110
Figure 20:	Large-scale partial Sau 3A I digestion of intact genomic DNA from <i>G. intestinalis</i> strain NP 77	111
Figure 21:	Hind III digestion of 12 clones from the <i>G. intestinalis</i> strain NP 77 gene library	114

LIST OF TABLES

Table 1:	Optimization of the conditions required for growth and harvest of <i>G. intestinalis</i> trophozoites	36
Table 2:	Observed and expected band size from restriction enzyme digestion of the <i>G. intestinalis</i> rRNA gene repeat contained within the GC-rich fraction of DNA	60
Table 3:	Titre of the small-scale library	112
Table 4:	Titre of the large-scale and amplified libraries	113
Table 5:	Average size of inserts present in the gene library	115

CHAPTER 1: INTRODUCTION.

Giardia is an aerotolerant anaerobic protozoan parasite of man and many other species of animals including mammals, birds and reptiles. On morphological grounds, three species have been proposed by Filice (43): *Giardia intestinalis*; *Giardia muris* and *Giardia agilis*. In addition, three more species; *Giardia psittaci*, *Giardia microti* and *Giardia ardeae* have recently been added to the list (33, 34, 39).

G.intestinalis is found in a wide range of mammalian hosts including humans while *G.muris* does not apparently infect humans but is found in animals especially rodents. Initially, it was thought that *Giardia* strains infected only one or a few host species which might imply that a different species or subspecies of *Giardia* is associated with each mammalian host (89). However in more recent studies, isolates from humans were shown to infect rats, mice, gerbils and other mammals at least under experimental conditions (89, 109). In contrast to this, other studies have shown *Giardia* isolates from humans were not easily transmitted to cats or dogs although cats and dogs are commonly and naturally infected with *Giardia*. Furthermore, there is little epidemiological evidence to support the conclusion that animal strains can be transmitted to humans with the possible exception of strains infecting beavers (1). This suggests that *G. intestinalis* strains show some degree of host specificity. If *Giardia* strains are host specific, it might reasonably be expected that some differences between strains would be found. However, there have been no consistent differences detected between *G. intestinalis* isolated from human and animal sources.

The lack of a test to distinguish between strains of *Giardia* means that any cysts detected in the environment, especially in water, have to be regarded (possibly incorrectly in many instances) as a danger to human health. Epidemiological evidence supports the conclusion that person to person transmission occurs primarily by the faecal-oral route. However, *Giardia* may be transmitted by food or may be waterborne (16).

While it is generally agreed that transmission by water occurs less often than transmission by other routes, it is nevertheless established that large outbreaks of giardiasis have been caused by contaminated water supplies (26a, 26b). This is unlikely to affect large conurbations where water supplies are adequately treated. However, small New Zealand towns with limited resources may fail to control and maintain water quality so such communities carry some risk of infection with waterborne *Giardia* cysts.

Cysts have traditionally been detected in water by a combination of filtration and staining. Recent improvements in filtration and staining techniques especially the application of immunological methods has increased the efficiency of the detection of cysts and at present, the mere detection of cysts in water is enough to justify the conclusion that it is unsuitable for use as potable water. However, this conclusion may be unjustified because the cysts may have been derived from an animal source and may be incapable of infecting humans. This thesis is concerned with this possibility and represents preliminary steps in a larger project designed to investigate the possibility of discriminating between various strains of *G. intestinalis* so that it may become possible to determine if cysts in water are or are not potentially infective for humans. To date, two general approaches, a classical biological approach and a molecular approach, have been employed in an effort to distinguish between *Giardia* strains.

The biological approach to strain differentiation has utilized such strategies as isoenzyme analysis (7, 12, 24, 80), drug sensitivity (17, 76), virulence and infectivity (4, 87), *in vitro* growth rates (13, 81), morphological differences using light and electron microscopy (33, 34, 39, 43), ability to axenize (81) and transmission studies (35, 65). In general, this approach was not successful although some minor strain variation was detected (16, 109, 118).

More recent approaches have employed molecular techniques such as the analysis of surface antigens (88, 113) and total proteins using SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (73, 109). The *Giardia* genome

has also come under intense scrutiny with the use of such techniques as electrophoretic karyotyping (21, 112), Polymerase Chain Reaction (PCR) eg. to amplify ribosomal RNA (rRNA) gene sequences (115, 118) and phage M13 fingerprinting (114). These techniques are discussed in greater detail elsewhere (see Chapter 2: Historical Review).

Another technique is to detect restriction fragment length polymorphisms (RFLP's) which are formed when variation occurs in the sequence of a gene resulting in the creation or loss of specific restriction endonuclease sites. Noncoding regions of a gene are more likely to have varying sequences or allelic forms as random changes are less likely to have an adverse biological effect and consequently these regions do not have the same restriction on maintaining genetic sequence as coding regions. Thus strains may be differentiated by digestion of their DNA with restriction enzymes if changes have occurred within the genome. However, a few difficulties arise with this when using total genomic DNA digests as an excessive number of bands are generated (31, 37, 50, 89, 92). Because of this, following the initial use of total genomic DNA digests, we investigate here the possibility of examining a subcomponent of the total genomic DNA, namely the GC-rich fraction. However, this approach proved to be laborious and requires the *in vitro* culture of *Giardia* strains which in many, indeed most, cases is not yet achievable. Consequently, we also undertake at least the initial steps of what we believe to be a more productive approach namely Southern blotting of digests of total genomic DNA and hybridisation with selected probes. This, however, clearly requires the availability and selection of appropriate probes, the development of which would be greatly facilitated by the availability of a gene library. A major part of this thesis is concerned with the production of such a library.

CHAPTER 2: HISTORICAL REVIEW

2.1 THE BIOLOGY OF *GIARDIA*

2.1.1 Historical Background

Giardia intestinalis (syn. *duodenalis*, *lamblia*, *enterica*) was first discovered by Antony van Leeuwenhoek who, in 1681, observed the organism in his own stools (7, 16, 83). However, van Leeuwenhoek did not associate the organisms he saw were associated with the symptoms of disease he experienced. It was not until almost 200 years later that a physician, Vilem Lambl, described the organism he had found in children with dysenteric diarrhoea and associated the two (7, 16, 83). The cyst form of *G. intestinalis* was first noted by Grassi (1879) who first thought they might be coccidia and later associated them with the flagellated form of the organism (83).

Despite its early discovery, *Giardia* has only recently become universally accepted as a human pathogen. This delay occurred because *Giardia* infections are noninvasive and asymptomatic carriers are very common (1, 83). However, the occurrence of waterborne outbreaks of diarrhoea, the discovery of severe giardiasis in patients with hypogammaglobulinaemia and the fulfillment of Koch's postulates in experimental human infections have confirmed its pathogenicity (1, 87).

2.1.2 Taxonomy and Nomenclature

Giardia belongs to the phylum Protozoa, subphylum Sarcomastigophora, class Zoomastigophora, order Diplomonadida and family Hexamitidae (70).

Filice (1952) divided the genus *Giardia* into three groups based largely on morphological criteria with an emphasis on the appearance of the median bodies: *Giardia intestinalis*, *Giardia agilis* and *Giardia muris* (43). Three additional species,

Giardia psittaci (33), *Giardia microti* (39) and *Giardia ardeae* (34), have recently been recognised as separate *Giardia* species. *G. intestinalis* is found in approximately forty different animal species including humans and other mammals, birds, and rodents (1, 16, 31, 53, 115). *G. muris* is primarily found in rodents and *G. agilis* is found only in amphibians (1, 16, 31, 53, 115). *G. ardeae* was isolated from the Great Blue Heron; *Ardea herodias* (34), *G. microti* was isolated from voles (39) and *G. psittaci* was isolated from a parakeet (33). The methods of species classification will be discussed later (see 2.4 Differentiation of *Giardia*).

2.1.3 Life Cycle and Morphology

Giardia has a diphasic lifestyle and exists in two forms; the binucleate trophozoite which is the vegetative stage and the quadrinucleate cyst which is the resistant, dormant stage (1, 104).

After a viable cyst is ingested it passes through the acidic stomach into the small intestine. Here excystation occurs within thirty minutes of ingestion to release two trophozoites from each cyst. The trophozoites attach, each by means of an adhesive disc, to the epithelial cells lining the upper small intestine and their activities are responsible for causing the symptoms of giardiasis (1, 5, 53).

The trophozoites multiply by binary fission. The nuclei divide first so that the cell has 4 nuclei followed by the flagella, sucking disc and finally the daughter cells separate. Encystment usually occurs in the large intestine and during this process the cell divides so that each newly formed cell has only two nuclei. As many as 100 million cysts can be shed by an infected host per day although cysts may be shed intermittently (71).

The Trophozoite Structure

The *G. intestinalis* trophozoite is approximately 10-12 μm long, 5-7 μm wide and 2-4 μm thick (1, 41, 71). The body of the trophozoite is pear-shaped, dorsoventrally

flattened and bilaterally symmetrical (Fig. 1),(41, 71). The cytoskeleton consists of a funis (body), a median body, a concave sucking disc on the ventral surface and four pairs of flagella (1, 41, 71). Golgi apparatus, two identical nuclei and lysosomal vacuoles are also present. Glycogen and ribosomal granules are present in the cytoplasm but mitochondria, peroxisomes, smooth endoplasmic reticulum and nucleoli have not yet been identified (1).

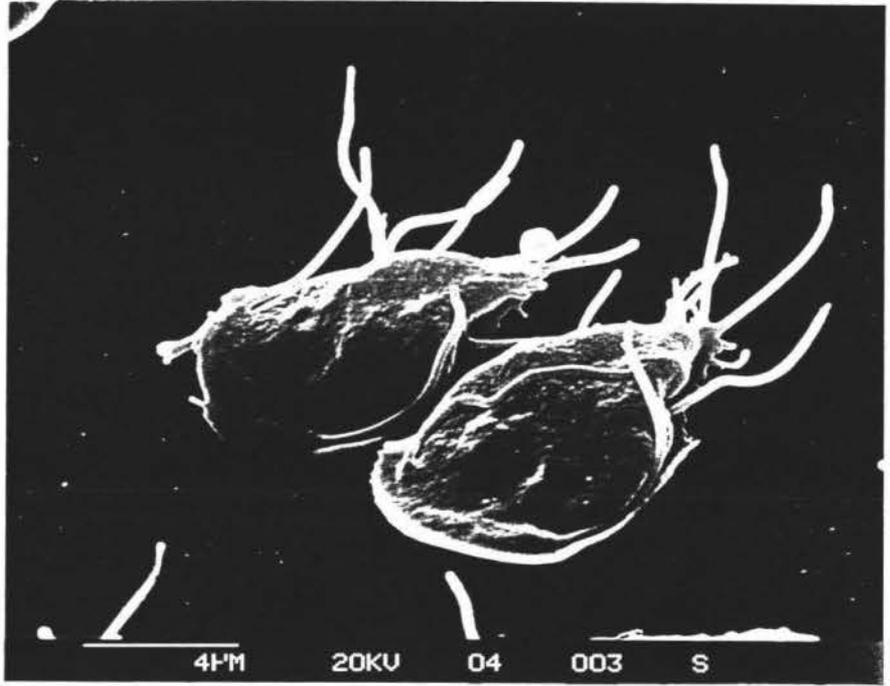
The median bodies lie behind the sucking disc as a pair of large, curved, tranverse, darkly staining organelles. Median bodies are unique to *Giardia* and are composed of microtubules arranged either irregularly or in ribbons. It has been suggested that they may help support the posterior end of *Giardia* or may be involved in energy metabolism. (1, 71).

The large ventrally located (concave surface) sucking disc is used to attach the trophozoite to surfaces and acts as a suction cup in attachment to the intestine (1, 71). The disc appears to be fairly rigid and is comprised of microtubules, cross-bridges attached to the microtubules and unique structures called microribbons which are perpendicular to both the microtubules and the cross-bridges. The microtubules contain tubulin whereas the microribbons contain a set of *Giardia*-specific proteins, found only in the ventral disc, called giardins (27, 28, 94).

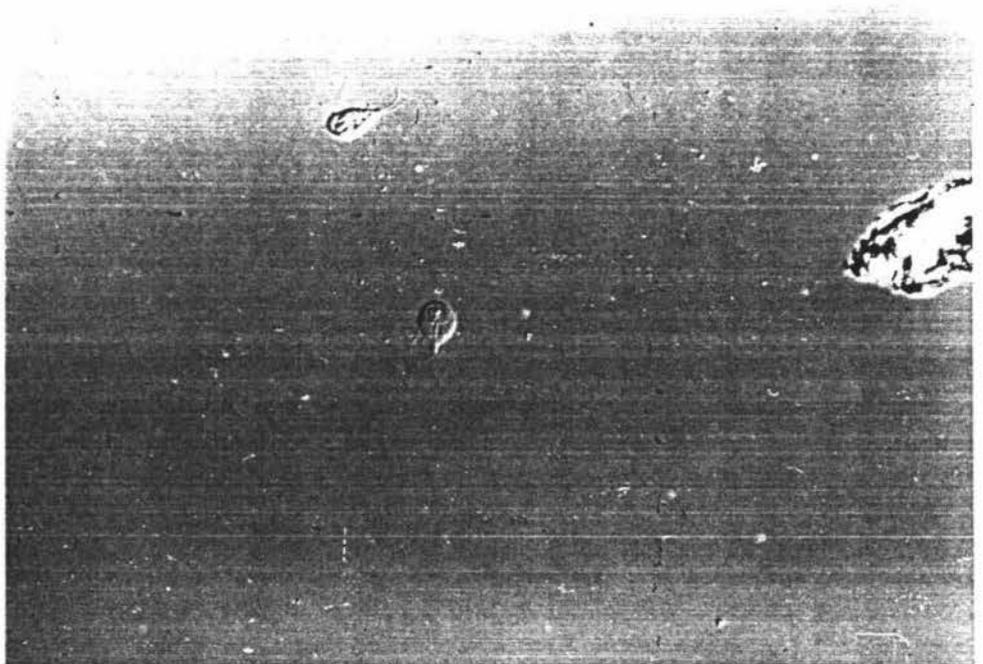
The four pairs of flagella (anterior, caudal, posterior and ventral) are comprised of microtubules in the typical eukaryotic '9+2' arrangement (1, 41). Although flagellar motion is associated with motility the failure to find a selective inhibitor of flagellar function has meant the role of flagella is not yet well known (1). Studies have shown that adherence can occur in the absence of flagellar movement and that antibiotics which inhibit the adherence of *Giardia* spp. have no apparent effect on flagellar function (1). These results indicate that flagella are not important in adherence.

The two nuclei of *Giardia* trophozoites are morphologically identical and symmetrically positioned on either side of the midline (1). The nuclei each contain a haploid genome, the same number of chromosomes and replicate at approximately

Fig. 1: *G. intestinalis* trophozoites from an *in vitro* culture: (a) Scanning Electron Micrograph showing the edge of the ventral sucking disc and the flagella (scale and magnification shown) (b) Nomarsky Differential Interference Microscope (400x magnification). The nuclei, suction disc and flagella are visible.



1a



1b

the same time (2, 117). Furthermore, both nuclei appear to be transcriptionally active and also contain copies of ribosomal RNA (rRNA) genes (63, 109). Thus *Giardia* spp. are unique in possessing two nuclei that are equivalent by all criteria. This raises the question of the selective advantage gained by the maintenance of two nuclei and the possibility of the exchange of genetic material (1).

The Cyst Structure.

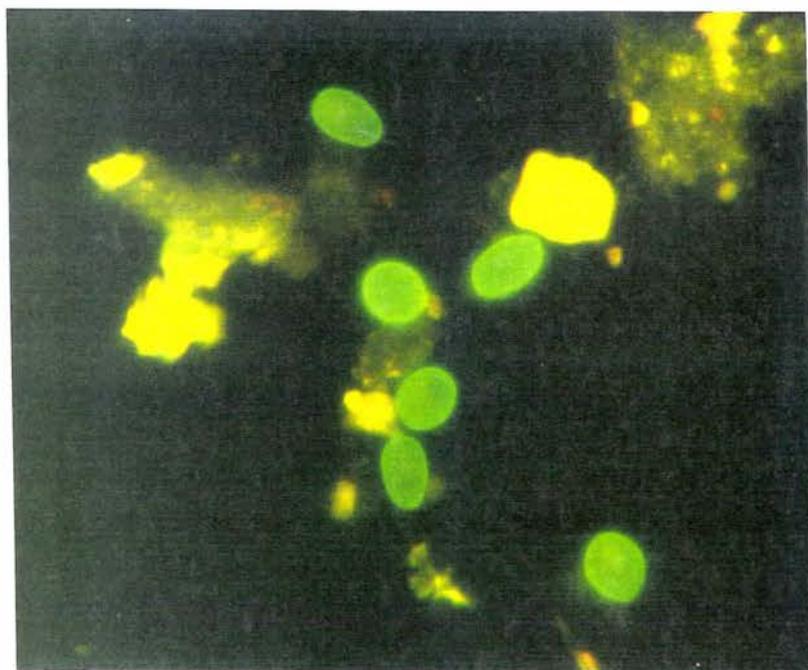
G. intestinalis cysts are oval or elliptically shaped and range from 6-10 μm in size (1, 107). The cyst wall is approximately 0.3-0.5 μm thick, smooth in appearance and composed of a layer of fibrils arranged in a felt-like web. The cyst has a fibrous (filamentous) outer wall and an electron-dense inner membrane (Fig.2).

Proteins and sugars excluding chitin are found to be present in the cyst wall. The presence of a major sugar component, N-acetylgalactosamine (GalNAc), in cysts but not in trophozoites suggests that the synthesis of this outer cyst wall sugar during encystation may be a key biochemical process of encystation (1, 58). Inside the cyst wall, the organelles and structures found in the trophozoite can also be found. However, some are modified in shape (1, 41). Two or four nuclei, depending on whether nuclear division has occurred, are found at one pole of the cyst. Axonemes of the flagella, vacuoles, ribosomes and fragments of the ventral disc are also present (1, 41).

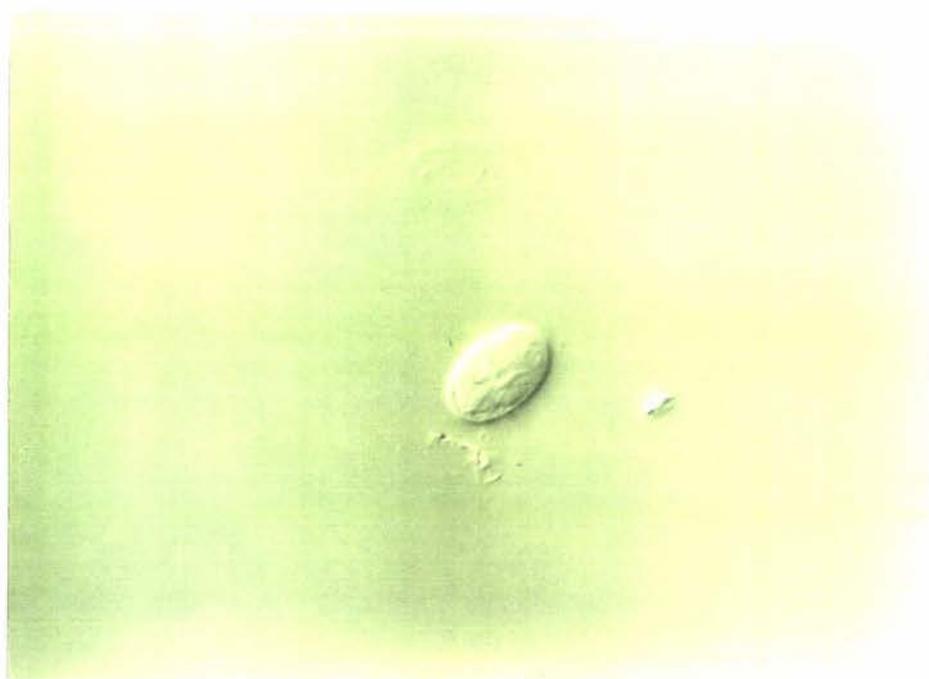
2.1.4 Cultivation

The establishment of *in vitro* or *in vivo* cultures of *Giardia* require the isolation of the organism from an infected host.

Fig. 2: *G. intestinalis* cysts: (a) Fluorescent Light Microscope using fluorescently-labelled antibody to the walls of cysts isolated from human faeces (400x magnification) (b) Nomarsky Differential Light Microscope showing an *in vitro* cultured cyst. The axonemes and suction disc are clearly visible (1000x magnification).



2a



2b

In Vitro Culture.

The development of an axenic (free of exogenous cells) culture medium for the *in vitro* growth of *G. intestinalis* trophozoites, TY1-S33 medium (82), has enabled the cultivation of large quantities of *G. intestinalis* trophozoites required for DNA extraction and analysis, metabolic activity and other studies.

The trophozoites form a monolayer when incubated anaerobically in tissue culture flasks, containing TY1- S33 medium, at 37°C and can be maintained following subculture and the addition of fresh medium to the flask (see Chapter 3). The pH (6.85-7.0) of the medium, as well as incubation temperature, is also important (45). Modified TY1-S33 medium, the medium used in this laboratory, contains bovine serum, bile salts, yeast extract, cysteine, ferric ammonium citrate and ascorbic acid (see appendix). Cysteine and low oxygen concentration are absolute requirements for axenic cultivation of trophozoites as without the presence of cysteine at required levels, the trophozoites are unable to attach to the culture flask and are also unable to divide (85).

Although axenic cultures have been successfully established for *G. intestinalis* and *G. ardeae* isolates, no satisfactory methods are available for culturing *G. muris*, *G. agilis* or *G. psittaci in vitro* nor for culturing the cysts of any *Giardia* spp. found in water samples (71). The development of axenic cultures from *G. intestinalis* cysts requires the cysts to undergo excystation. A variety of methods are available (18, 40, 42, 103, 106), many of which require the use of an acid medium to induce excystation followed by final incubation in a growth medium at 37°C. A different method of excystment is normally used for *G. muris* isolates due to the physiological differences between *Giardia* species (40).

G. intestinalis trophozoites can undergo encystment *in vitro* by the replacement of growth medium with modified TY1-S33 medium containing additional bile to a final concentration of 5 mg/ml.

In Vivo Culture

Animal models have been successfully used to cultivate *G. intestinalis* and *G. muris*. Most frequently used are laboratory strains of rats, mice, and hamsters. However, it is essential that such animals are found to be free of *Giardia* themselves before cultivation of strains or species under investigation can begin.

G. intestinalis can be cultured *in vivo* following the inoculation of 'Giardia-free' neonatal mice with trophozoites or 'Giardia-free' adult mice with large quantities of cysts ($> 10^3$ cysts) (79). *G. muris* can also be cultured *in vivo* by the inoculation of mice with cysts ($> 5 \times 10^3$ cysts) (79). The cysts are collected from the faeces of the infected mouse whereas the trophozoites are harvested following sacrifice of the mouse and removal of its intestine.

2.2 GIARDIASIS

2.2.1 Pathogenesis and Clinical Illness

For many years *G. intestinalis* was considered to be either nonpathogenic or an opportunistic pathogen for humans because of the large numbers of asymptomatic carriers (71). Children are more frequently affected with giardiasis than adults. Factors other than age, such as infective dosage, host type, enteric bacterial infection, malnutrition, variable pathogenicity among strains of *Giardia* and reduced gastric acidity, may also play an important role in the determination of the degree of infection and the manifestation of the clinical symptoms of giardiasis (57, 71).

A minimum of ten viable ingested cysts are required for infectious dose (99). The incubation period (from infection until the symptoms occur) is usually 14 days, although this period may often last less than one week (1) and the prepatent period before *Giardia* can be detected in the stools is 10-31 days (71). Acute giardiasis usually lasts 3-4 days and is characterised by many symptoms; the most common of which is diarrhoea with loose, foul-smelling stools that are greasy, frothy or bulky

(1). Other symptoms that usually accompany the disease are, in descending order of occurrence: weakness, weight loss, abdominal cramps, nausea, greasy stools, abdominal distention, flatulence, vomiting, belching and fever (44). The diarrhoea is thought to be caused by low-grade inflammation of the intestine brought about by the attachment of the parasites to the mucosa. Most of the other symptoms experienced appear to be secondary to the diarrhoea and either caused by it or, in severe cases, by dehydration (1, 44, 71).

The dense colonisation of trophozoites on the surface of columnar epithelial cells at the base of the villi of the intestine interferes with the absorption of fats and other nutrients by limiting the area available to absorb nutrients. This causes an increase in cell turnover leading to an accumulation of mucous and cytoplasm covering the intestine. Such malabsorption can lead to steatorrhea, vitamin deficiencies (particularly vitamin B₁₂), interference with protein absorption, disaccharidase deficiency, hypocarotinaemia, low serum folate levels, increased fat content of the faeces and, in children, a failure to thrive particularly in developing countries. (1, 44, 71). There are also reports that *Giardia* may cause low levels of all forms of gamma globulin (hypogammaglobulinemia) and diseases of the bile tract, liver, pancreas, and large bowel (6, 95). In addition, *Giardia* has been implicated with nodular lymphoid hyperplasia, mesenteric adenitis and rectal bleeding (1, 44).

Several studies of chronic giardiasis have been demonstrated where the patients carried the parasite for up to 5 years. In such cases, constipation was common alternating with episodes of diarrhoea. Also associated with this syndrome were nasal allergies, asthma and an intolerance to milk (44). Fortunately giardiasis, although extremely uncomfortable, is not fatal.

2.2.2 Immunity

The humoral immune response appears to be critical for the elimination of infection both in human and animal models of giardiasis. While it is likely that secretory IgA is the most important component of this response it may also be possible that the

parasite may evade the immune response by the production of *Giardia* IgA protease and antigenic variation (1). Whether the antibody response in humans is T-cell dependent is unknown although the antibody response is cell-mediated (CD4) in *G. muris* infected mice (1).

Important forms of nonimmune protection include the intestinal mucous layer, intestinal motility and human breast milk for infants.

2.2.3 Diagnosis and Treatment

Diagnosis

The most commonly used method for the diagnosis of giardiasis is by direct microscopic examination of the stool for cysts and trophozoites (1, 71).

The detection of *Giardia* cysts in faeces requires multiple examinations of faecal samples as some hosts excrete cysts intermittently. Two to three faecal samples should be collected one to three days apart (75). In addition, the symptoms of illness may begin approximately 1-7 days before the excretion of cysts begins so the examination of faecal samples from such patients should be repeated where diagnosis of giardiasis has not been established (1, 71). Because cysts may be excreted intermittently by an infected host, only a positive result has any true meaning as a negative test does not prove that the patient is *Giardia* free.

Although a variety of techniques are available for the recovery and detection of *Giardia* cysts from faecal samples, the simplest, most commonly used, method involves the direct microscopic examination of faecal smears stained with a non-specific stain such as Trichrome or Grams iodine. The concentration of *Giardia* cysts with the use of zinc sulphate, percoll or sucrose gradients before staining is also common.

Giardia cysts can also be detected by a number of stains that are available

commercially. These stains include a Direct Fluorescent Antibody assay (DFA) (Cellab; Meridian), an Enzyme Linked Immuno Sorbent Assay (ELISA) (Cellab) and an Indirect Fluorescent Antibody assay (IFA) (Meridian). An Enzyme Immuno Assay (EIA) (ProSpecT/*Giardia*TM test kit; Alexon Biomedical Inc.) specific for GSA65, a 65 kilodalton trophozoite antigen (100), is also available (79). The IFA and EIA assays are reported to be more specific and less labour intensive than the use of phase contrast microscopy to detect cysts stained non-specifically with iodine or trichrome or not stained at all (1, 102).

Treatment.

There are four main drug families that are used in treating giardiasis. These include quinacrine (AtabrineTM); the nitroimidazoles: metronidazole (FlagylTM), tinidazole (FasigynTM), nimorazole (NaxoginTM) and ornidazole (TiberalTM); paromomycin (HumatinTM) and furazolidone (FuroxoneTM). Each of these drugs is effective in curing the disease yet all, except Humatin, have some serious side effects (1).

Quinacrine is a highly effective (80-90% success rate) antimalarial drug that is usually administered as a 5-10 day treatment programme (1, 44). While gastrointestinal side effects are commonly experienced, other side effects such as nausea, vomiting, dizziness, severe skin rash and discolouration of the urine may also occur.

The broadspectrum nitroimidazoles are very effective for the treatment of giardiasis (62). However, their mechanism of action involves their reduction to either radicals or to intermediate compounds that bind to DNA or protein molecules. Consequently, these drugs are carcinogenic and should not be used for long term treatment.

Metronidazole is more than 90% effective when given as a 5-day treatment but concerns have been raised over its mutagenic qualities in bacteria and its ability to cause tumours in rats and mice (1, 44). Frequent side effects experienced with the use of this drug include nausea, headaches, dry mouth and metallic taste, skin rash

and vomiting (1, 44). Tinidazole is effective as a single dose treatment. However, although this drug is well tolerated by many patients (1), side effects such as allergic reactions, stomach upset, furry tongue and dark urine may occur (1, 44).

Nimorazole is administered as either a single dose (2g) or as a 12 hourly treatment over 36 hours. Commonly experienced side effects when using this drug include skin reactions, drowsiness, nausea and pyrosis (1). Ornidazole is given as either a single dose or a 2-day treatment (1). As with the other nitroimidazoles, patients undergoing ornidazole treatment for giardiasis may experience side effects such as headaches, dizziness and gastrointestinal intolerance.

Furazolidone is used for the treatment of giardiasis in small children because it is available in a liquid form. However, as with the nitroimidazoles, furazolidone is a suspected carcinogen as it binds to DNA and also inhibits respiration (1, 44). Paromycin (humatin) is used when the patient is pregnant as this drug has a minimal toxic effect on the patient and the foetus (1).

Patients who fail to respond to treatment usually respond to a second course of treatment either with the same drug or a new drug (1). Other factors such as rapid reinfection of the host, failure of the patient to comply with the treatment instructions, inactivation of the drug by superinfection with other organisms or any variations in the pharmacokinetics of drugs may also play a role in the failure of some drug treatments to eradicate infection.

The development of other effective drug treatments is hindered by the inability to perform clinical studies on many potential drugs. Such clinical studies are in turn limited by the difficulty in culturing *Giardia* spp. in patient samples and the lack of standardization of testing (1, 44). Ultimately, the development of an effective, single dose treatment drug with no/minimal side effects is desirable.

2.3 EPIDEMIOLOGY

2.3.1 Transmission

Transmission of giardiasis is facilitated by the diphasic lifestyle of *Giardia*. Trophozoites colonising the jejunum of the host encyst as they are passed through the distal portion of the small intestine and colon. These cysts remain viable in moist faeces until they are ingested. Infection follows ingestion of as few as ten viable cysts of *Giardia* (55, 99).

Although giardiasis is primarily transmitted person to person by the faecal-oral route, it may also be food or waterborne (1, 55). Mechanical transmission by insects has also been demonstrated experimentally where cysts have survived passage through the intestines of flies (99). However, the importance of this mode of transmission in the epidemiology of giardiasis is unknown at the present time (16).

(i) Direct Faecal-Oral Transmission

Since infection can occur with only a few *Giardia* cysts, populated areas with inadequate sanitation and a high proportion of susceptible individuals are at high risk of direct faecal-oral transmission of *Giardia*.

Studies in developing countries and in the poorer areas of developed countries support the importance of the faecal-oral mode of transmission especially in conditions of poverty, overcrowding, and where water supply and sanitation are inadequate (55). Childcare centres, preschools and kindergartens are also important to the spread of *Giardia* where despite good hygiene practises, young children show exploratory behaviour and place their fingers in their mouths (16, 55). Consequently, children in child-care centres not only have a greater chance of acquiring the infection but are subsequently likely to carry such infections to their families (16).

The possibility of transmission of *Giardia* by flies moving from faeces to food has

also been reported from India (46). In addition, the increased prevalence of giardiasis in homosexual males is most probably the result of faecal-oral transmission following certain sexual behaviour (96, 105).

The role of asymptomatic carriers may also serve as an important reservoir of *Giardia* cysts in the environment. Such people are less likely to seek medical treatment and so serve to continuously contaminate the environment (55).

(ii) Foodborne Transmission

Foodborne transmission of giardiasis occurs when faecally contaminated food is consumed. This contamination can be direct or indirect from faeces. Specifically, the cysts may be carried to food by water or by the use of wastes derived from human excrement in the fertilisation of garden and field crops especially since *Giardia* cysts have been shown to survive for as long as three months (93).

Only 3 reports of common-source outbreaks of eating contaminated food have been published (16) although a number of foods have been shown to harbour cysts including strawberries, Christmas pudding and lettuce (16). Contamination of food by food handlers who practise substandard hygienic practices may play a role in the transmission of disease in developing countries (55). The foodhandlers may act as reservoirs for the transmission of disease. Infected children may also transmit the cysts by sharing their food with their friends or younger siblings (16, 55). In addition, *Giardia* cysts may survive on moist foods which are not cooked prior to eating (49).

(iii) Waterborne Transmission

Waterborne giardiasis, the least common mode of transmission, currently ranks number one as a cause of waterborne illnesses and number four as a cause of waterborne outbreaks in the United States although its significance as a disease-causing agent did not become firmly established until the 1960's (5, 26a, 54).

In developed countries, waterborne outbreaks of giardiasis have occurred primarily in areas where water supplies were contaminated either by a failure of filtration systems or by direct contamination of water supplies with sewage (25). Craun (1986) compiled valuable statistical data from in excess of 42 waterborne outbreaks of giardiasis in the United States reported over the past 25 years and showed that the majority of these resulted from the consumption of untreated surface water or surface water in which disinfection was the only treatment (26b). 12 % of the outbreaks resulted from ineffective or malfunctioning filtration (26b).

Watershed investigations and animal surveys have indicated that wild and domestic mammals represent a significant reservoir of *Giardia* cysts in areas devoid of any substantial human activity (1, 5, 56). Beavers, in particular, have been implicated in several waterborne outbreaks (5, 56). However, proving that the beavers were the source of contamination has not yet been possible because there is, at present, no means to identify the species of origin of a cyst detected in a water sample (1, 5, 56).

(iv) Control Measures

Measures to control *Giardia* infections should be directed towards:

1. Prevention of the contamination of water and food with *Giardia* cysts.
2. Destruction or removal of cysts which manage to reach food or water.
3. Reduction in the number of asymptomatic carriers that are chronic cysts passers.
4. Improvement in personal hygiene to reduce the risk of person to person transmission (55).

Prevention of the contamination of water or food with cysts is dependant on the availability and the use of safe faecal waste disposal systems, the treatment of human faeces used as fertilisers to kill cysts, and the protection of sources of drinking water from contamination with infected material, whether derived from humans or potential animal reservoirs. In developing countries, many opportunities remain for human

sewage to contaminate the water supply; the major contribution of which is the indiscriminate defaecation habits of many people in developing countries because they do not have latrines (55). Most importantly, improved methods of water and waste management systems require financial and educational support. This is seldom forthcoming for developing countries where such assistance is most needed.

Unfortunately, *Giardia* cysts are not routinely susceptible to the usual levels of chlorine that are recommended for drinking water (26b) therefore treatment of infected water supplies involves the combined use of disinfection and filtration (59, 74). As such methods are impractical in most developing countries, due to cost and the lack of piped drinking water, the simple method of boiling drinking water is sufficient if fuel supplies are available (55).

Treatment of all people with recognised symptomatic giardiasis and symptomatic cyst carriers is important to eliminate reservoirs of infection. Improved personal hygiene by better preparation and storage of food and water, handwashing and control of insects which may transfer cysts from faeces to food also serve as measures of control. Such measures not only reduce the number of sources for community spread, but also reduces the opportunity of direct person to person spread of giardiasis.

2.3.2 Host Specificity

A controversial aspect of *Giardia* epidemiology is that of host specificity, particularly whether or not *Giardia* exists as a zoonosis (zoonosis = disease of animals that is transmissible to humans). Two schools of thought have emerged on this issue. One school adopted the view that, with rare exceptions, the *Giardia* of mammals are host specific (52) thereby excluding animals as sources of human infection. The other school holds that *Giardia* are not highly host specific and bases this belief on demonstration of the apparent transfer of *Giardia* infection between different animal species and the apparent lack of host specificity in human isolates of *G. intestinalis* (35, 84).

Most of the earlier cross-transmission studies have been criticized, either because of assumptions made about the cyst sources, the status of the host (care was not taken to prove that the recipient animals were free of their own *Giardia* before the experiments began) or inadequate controls (10). However, *Giardia* infections have been successfully established in other selected species. The Mongolian gerbil can readily support the establishment of patent host infections from cysts derived from several host species (9). In addition, beavers and muskrats were successfully infected with cysts derived from human faeces but only by using large numbers of viable cysts (35). Because of their association with waterborne outbreaks of giardiasis, the beaver and the muskrat must be considered as possible intermediate reservoirs for *Giardia* spp. capable of infecting humans. However, because of the evidence for their harbouring different *Giardia* species from those that have been described for humans, as well as the potential contributions from other unevaluated sources such as birds and humans, it is not possible at this time to assign a major role in waterborne transmission to these animals (35).

Current methods of detection of cysts found in water cannot differentiate between the various species of *Giardia* based on their host origin or recognise different strains within a species. This may lead to false conclusions about the origin of the cysts and condemnation of the water as a unsuitable for drinking because many animals have indogenous infections of *Giardia* species that are totally unrelated to the species producing giardiasis in humans (11). As most cysts are morphologically indistinguishable from each other, an investigator cannot tell whether the cysts detected in a water sample originated from a human or some other species of mammal or bird; they are simply *Giardia* cysts of unknown origin. One exception is that *G. microti* cysts are very different from those of humans and other animals in that the mature cyst contains two completely formed trophozoites and presents a considerably different appearance when viewed microscopically (11). Consequently, the resolution of whether or not giardiasis is a zoonotic infection will depend on clarification of the issue of species determination.

2.4 SPECIES DETERMINATION AND STRAIN VARIATION

There are two important questions in current *Giardia* research. Firstly, is cross transmission of *Giardia* between animals and humans possible (ie. is *Giardia* a zoonosis?) and secondly, is it possible to distinguish between strains of *Giardia* which do infect humans from those that do not and thereby trace the source of infection within a community? Species determination and strain variation are essential starting points in answering these questions and have been extensively examined using a variety of methods.

2.4.1 Species Determination.

Determination of *Giardia* species has classically been made by two predominant methods: (i) host of origin and (ii) morphology by light microscopy, primarily by the appearance of the median body (1).

Species determination by host of origin is probably invalid because *Giardia* spp. from different hosts (eg. cat, beaver and guinea pig) frequently appear identical by a number of methods including morphology and genetic analysis, whereas different isolates from the same host species can be markedly different (89). Contradictory results from experimental transmission of *Giardia* isolates from one host to another have also posed difficulties for species determination by host of origin alone (35, 65).

In contrast to the difficulties of species determination by host of origin, electron microscopy of the cyst and trophozoite has improved the ability to detect morphological differences between organisms that appear the same under the light microscope. Using such methods, the number of species has increased from those proposed by Filice (43) (see section 2.1.2) with the recognition of *G. psittaci* (33), *G. microti* (39) and *G. ardeae* (34).

2.4.2 Strain Differentiation.

The classical biological approach to strain differentiation has utilised such strategies as drug sensitivity (17, 76), virulence and infectivity (4, 87), *in vitro* growth rates (13, 81), morphological differences using light and electron microscopy (33, 34, 39, 43), ability to axenise (81), isoenzyme analysis (7, 12, 24, 80) and transmission studies (35, 65). In general, this approach was not successful although some minor strain variation was detected (16, 109, 118). These studies have included isolates from humans, cats, beavers and guinea pigs and seem to show that differences are not associated with host of origin (1).

More recent approaches to both strain and species identification have employed molecular techniques such as electrophoretic karyotyping (21, 112), the analysis of surface antigens (88, 113), SDS-Polyacrylamide Gel Electrophoresis (PAGE) analysis of total proteins and Western blotting (73, 109), Polymerase Chain Reaction (PCR) eg. using primers to amplify ribosomal RNA (rRNA) sequences (115, 118) and DNA fingerprinting (114). Restriction Fragment Length Polymorphisms (RFLP's) are also an important technique employed (31, 37, 50, 89, 92, 111).

Electrophoretic karyotyping has separated *G. intestinalis* isolates into two distinct groups (112) but such large grouping is not useful epidemiologically. However, pulsed-field separations of the chromosomes of *G. intestinalis*, *G. muris* and *G. ardeae* demonstrated marked differences in the sizes and apparent number of chromosomes, and at very high stringency, little cross-hybridization of total DNA or specific DNA probes was observed among the three species (21). Consequently, electrophoretic karyotyping is a more useful method for species determination than for strain differentiation.

SDS-PAGE of total *Giardia* proteins have so far yielded no conclusive differences between *G. intestinalis* isolates. Other methods, such as the analysis of isoenzymes, have also experienced difficulties in detecting differences among *Giardia* isolates. Isoenzyme analysis did not reveal host differences although four distinct groups were

detected (7). Consequently, all of the experimental data so far accumulated suggests that *G. intestinalis* isolates from different host species are similar (1).

Restriction enzyme digestion and Southern blotting of total genomic DNA from a variety of *G. intestinalis* isolates showed great similarities (89). This suggests that all the strains are closely related. However, the large genomic size of *G. intestinalis* (8×10^7 bp) creates smears when digested with restriction enzymes making isolation of individual bands of interest difficult.

Although *G. intestinalis* in humans and other animals is similar by a number of criteria, this does not necessarily imply that cross infection can occur (16). Therefore a much more sensitive method is required to identify strains. To this end, several techniques involving the analysis of DNA at the molecular level have been examined such as the use of phage M13 fingerprinting, the examination of the *Giardia* ribosomal RNA genes and the construction of genomic libraries.

Phage M13 fingerprinting of digested total genomic DNA of *G. intestinalis* showed results comparable to those seen in higher eukaryotes but has yet to yield significant differences between isolates (114).

Recent techniques have utilised and revealed the potential usefulness of rRNA gene sequences in determining the source of infection in outbreaks and may also facilitate studies of virulence and infectivity (31, 115, 118). The ribosomal RNA (rRNA) genes of *G. intestinalis* are unusual for several reasons (5):

1. The RNA's themselves are the smallest yet reported for any eukaryotic organism.
2. The genes encoding them are found as a small tandemly repeated unit of 5.6 kb in size.
3. The genes are extraordinarily GC-rich, even in regions which are highly conserved between all other eukaryotic rRNA genes.
4. The sequence and restriction map of these genes are also known.

5. rRNA encoding chromosomes in *G. intestinalis* undergo a high frequency of rearrangement (67). However, the significance of this finding is unknown.
6. rRNA genes are present on the ends of at least six telomeres (67).

Total genomic DNA of *G. intestinalis* can be separated into two distinct populations of different densities by centrifugation through caesium chloride gradients containing Hoescht dye 33258 (92). The upper, less dense, band is AT-rich compared to the lower, more dense population and comprises approximately 90% of the total DNA encoding single copy DNA and structural genes such as alpha and beta tubulin (92). The lower, more dense, band is GC-rich and comprises approximately 10% of the total DNA. This GC-rich fraction is enriched for rRNA sequences (92) therefore its isolation and analysis using restriction enzyme known to cleave within the rRNA sequence raise its potential usefulness in characterising differences between *Giardia* isolates. This technique will be examined later in this thesis (see Chapter 4).

PCR has been used to amplify a segment of the 18S rRNA gene sequence of *G. intestinalis* to analyse whether polymorphism is present among isolates. By sequencing and hybridisation to sequence-specific probes, the *G. intestinalis* isolates could be divided into three groups based on their rRNA gene sequence (118). However, additional sequence analysis is required so that isolates can be separated into more than three groups.

PCR has also been successfully used to demonstrate significant differences between species of *Giardia*. *G. intestinalis* isolates show different banding patterns to those of *G. muris* isolates after amplification of rRNA genes using random arbitrary GC-rich primers (Ionas *et al*, in press). Similarly, a comparison of the rRNA genes of *G. intestinalis*, *G. muris* and *G. ardeae* revealed significant differences (115). The rRNA genes of *G. muris* and *G. ardeae* are significantly larger (7.6 kb) than those of *G. intestinalis* (5.6 kb) primarily because of a much larger intragenic spacer region in *G. muris* and *G. ardeae*. Differences were also found in the coding regions for the 5.8S rRNA and the 3' region of the LS rRNA. PCR analyses, using primers specific for different parts of the beta-giardin gene, also distinguished both *G. muris* and *G.*

ardeae from various isolates of *G. intestinalis* (78) but failed to detect any differences between the human-derived and animal-derived isolates of *G. intestinalis* (36).

The construction of total genomic libraries play a crucial role in the analysis of genes of interest with respect to size, composition, sequence and copy number of genes (15, 20, 31, 32, 47, 51, 77, 78, 115). A genomic library also offers many possibilities in both species determination and strain differentiation of *Giardia* isolates. The isolation of suitable species-specific (host-specific) and strain-specific clones could be used to create DNA probes useful as epidemiological tools and thus provide many of the long-awaited answers to the question of zoonotic transmission of *Giardia*. This thesis is concerned with the production of a gene library from *G. intestinalis* as preliminary approach in the identification of such probes.

3.2 MATERIALS AND METHODS

3.2.1 Retrieval of frozen trophozoite cultures

Materials

1) TY1-S33 media preincubated to 37°C (see appendix).

2) 50 ml Falcon™ tissue culture flask

3) Frozen *G. intestinalis* strain Hast 11 trophozoite culture (see 3.2.5)

This is a New Zealand strain isolated from a human faecal sample and obtained for this study from the Massey University Dept. of Microbiology and Genetics Culture Collection.

Method

A frozen trophozoite culture stored at -80°C was quickly thawed in either hot tap water or a 37°C waterbath and transferred to a 50ml Falcon™ culture flask 3/4 full of TY1-S33 media. After addition of the trophozoites, the flask was completely filled with media to exclude oxygen and incubated on its side for 30 min at 37°C. By this time many of the trophozoites had attached. The media was then discarded and the flask containing the trophozoites refilled with media. This was incubated for a further 4 hours at 37°C. After this time, the media was again discarded and the flask refilled. This ensured that all the dimethyl sulphoxide (DMSO), used to preserve the trophozoites, had been diluted out. The flask containing the trophozoites was reincubated on its side at 37°C and the culture fed every 3 days by discarding the old media and refilling the flask with fresh TY1-S33 media. Such cultures formed a complete monolayer of cells and were regularly subcultured.

3.2.2 Subculture of *Giardia* trophozoites

Materials

- 1) TY1-S33 media preincubated to 37°C (see appendix).
- 2) 50ml or 750ml Falcon™ tissue culture flasks.

Method

5ml of an exponential growth phase (rapidly growing) trophozoite culture was inoculated into a 50ml Falcon™ culture flask. TY1-S33 media was added to completely fill the flask and thereby remove any oxygen present. The flask containing the trophozoites was incubated at 37°C, the culture fed every 2-3 days (see 3.2.1) and eventually subcultured to another flask.

3.2.3 Treatment of Contaminated Cultures

Materials

Modified TY1-S33 medium for contamination was used. This contained TY1-S33 medium with the following antibiotics added depending on the nature of the contamination.

- 1) TY1-S33 medium - see appendix
- 2) Tetracycline (Tc) 10 mg/ml

Tetracycline	100 mg
Methanol to	10 ml

This stock solution was stored at -20°C. Tetracycline was added to TY1-S33

medium to produce a final concentration of 10 µg/ml.

3) Streptomycin (Str) 50 mg/ml

Streptomycin sulphate	250 mg
Sterile Water	5.0 ml

This stock solution was sterilized by passage through a sterile 0.2µ filter and stored at -20°C. Streptomycin was added to TY1-S33 medium to produce a final concentration of 50 µg/ml.

4) Chloramphenicol (Cm) 50 mg/ml

Chloramphenicol	250 mg
Ethanol	5.0 ml

This stock solution was stored at -20°C. Chloramphenicol was added to TY1-S33 medium to produce a final concentration of 50 µg/ml.

5) Norfloxacin (Nor) 200 mg/ml

Norfloxacin (Sigma N-9890)	2.0 g
Ethanol	10 ml

As the norfloxacin did not dissolve in ethanol, a suspended solution was formed. This was vigorously shaken to produce an even suspension before use. Norfloxacin was added to TY1-S33 medium to produce a final concentration of 10 µg/ml. This stock solution was stored at -20°C.

6) Amphotericin B 10 mg/ml

Amphotericin B	100 mg
Sterile Water	10 ml

This stock solution was sterilized after passage through a sterile 0.2µ filter

and stored at -20°C. Amphotericin B was added to TY1-S33 medium to produce a final concentration of 10 µg/ml.

Method

Although culture manipulations were carried out aseptically in a Class 2 Biohazard Cabinet, contamination of trophozoite cultures did occasionally occur particularly if the frozen stock cultures were contaminated. Tetracycline, streptomycin and chloramphenicol were added to TY1-S33 media when bacterial contamination occurred. However, the most common bacterial contaminant that occurred in stock cultures, a Gram-negative rod, was treated with the addition of a norfloxacin/ streptomycin combination (using the concentrations described above). Fungal contamination was treated with the addition of amphotericin B to culture medium.

Treatment: Old media was discarded and the culture flask washed several times with fresh TY1-S33 media. Preincubated antibiotic modified media was then added and the flask incubated at 37°C. This procedure was repeated twice daily, then daily as necessary.

3.2.4 Preparation and harvesting of large-scale trophozoite cultures.

Materials

- 1) TY1-S33 Media preincubated to 37°C - see appendix.
- 2) 750ml Falcon™ tissue culture flasks.
- 3) 60ml of *G. intestinalis* strain Hast 11 trophozoites in the exponential phase of growth.

4) Phosphate Buffered Saline (PBS) - see appendix

5) Tris-EDTA (TE) Buffer - see appendix

6) 50 ml Falcon™ centrifuge tubes

Method

2ml aliquots of *G. intestinalis* strain Hast 11 trophozoites in the late log phase of growth were inoculated into each of 28, 750ml Falcon™ tissue culture flasks containing TY1-S33 medium and incubated for 1-7 days at 37°C.

4 flasks were removed from incubation at each 24 hour interval. Of these 4 flasks, 2 were incubated in ice water for 10 minutes prior to harvesting to detach trophozoites from the walls of the flask while the other 2 flasks were used immediately for harvesting. All 4 flasks were shaken vigorously to detach the trophozoites and a 5 ml sample was removed for counting.

Cultures were removed from each flask and centrifuged at 2500g for 10 min to pellet the cells. TY1-S33 media was added to fill each of the flasks and these were reincubated at 37°C.

Pellets from each flask were resuspended in 10ml of PBS and centrifuged at 2500g for 10 min. After a second wash with PBS, each pellet was resuspended in 5ml of sterile TE buffer and either used immediately for DNA extraction or stored at -70°C until further use.

3.2.5 Counting trophozoite population numbers.

Materials

- 1) PBS - see appendix
- 2) Haemocytometer and coverslips
- 3) Phase contrast microscope
- 4) Hand tally counter

Method

A clean cover slip was applied to the haemocytometer and each haemocytometer chamber loaded with 8-10 μ l of well-mixed trophozoite suspension by capillary action. At each of the four corners of the chamber was a 1mm² ruled area divided into 16 squares in which *Giardia* trophozoites were counted.

The trophozoite numbers were counted with a hand tally counter. Counting trophozoites twice was avoided by counting only those touching the top and left boundary lines of each square. Each of the four 1mm² areas were counted in this manner. A total of four to six different haemocytometer chambers were loaded, counted and then averaged for each *Giardia* trophozoite suspension to achieve counting precision. If trophozoite numbers were too high to be counted, i.e. usually numbers greater than 300 trophozoites per 1mm² area, then a 2 or 3 fold dilution of the trophozoite suspension was made using PBS.

The formula for determining the number of *Giardia* trophozoites per 1 ml of suspension was as follows:

$$\frac{\text{number of cysts counted}}{\text{number of sq. mm counted}} \times \frac{10}{1 \text{ mm}} \times \frac{\text{dilution factor}}{1} \times \frac{1000 \text{ mm}^3}{1 \text{ ml}} = \text{number of cysts per ml}$$

A graph was constructed showing the effect of time and chilling of flasks on the number of trophozoites harvested (see Fig.3).

3.2.6 Cryopreservation of *Giardia* trophozoites

Materials

- 1) *Giardia intestinalis* strain Hast 11 trophozoites grown *in vitro* to late log phase.
- 2) Dimethyl sulphoxide (DMSO)
- 3) TY1-S33 medium - see appendix

Method

Giardia trophozoites were harvesting by centrifuging at 2000g for 5 min. The pellet was resuspended in TY1-S33 medium and the number of trophozoites estimated using a haemocytometer (refer to section 3.2.5). The cell concentration was adjusted to 1.2 - 1.5 x 10⁶/ml with TY1-S33 medium (or trophozoite cultures were used as concentrated as possible).

0.25 ml of the cell suspension was pipetted into a labelled 1ml Nunc™ cryopreservation tube and 0.25ml of DMSO added very slowly to each tube. Tubes were carefully mixed, wrapped in laboratory tissue (to avoid rapid freezing of the trophozoites) and placed in a polystyrene container. The

polystyrene container was then placed in the -80°C freezer. After 24-48 hours, the tubes were removed from the container and stored in a labelled freezer box at -80°C until further use.

Note: As these cultures were used as a backup source, it was important to check that cryopreserved cultures were not contaminated or lethally affected by DMSO by retrieving some of the frozen cultures 1-4 weeks after freezing (refer to section 3.2.1).

3.3 RESULTS

The optimal conditions for growth and harvest of large numbers of *G. intestinalis* trophozoites were investigated by incubating 750 ml culture flasks containing strain Hast 11 for a period of 1-7 days. Flasks removed at 24 hour intervals were examined microscopically for the presence or absence and intactness of the cell monolayer (Table 1). A 5 ml sample was removed from each flask either immediately after microscopic examination or after incubation in ice water for ten min and the trophozoite numbers counted using a haemocytometer. These results are illustrated in Fig. 3. Trophozoite suspensions were centrifuged at 2500 g for 10 min and the presence or absence of a pellet noted (Table 1).

TABLE 1: Optimization of the conditions required for growth and harvest of *G. intestinalis* trophozoites (A = ice treatment; B = no treatment).

TIME OF INCUBATION (DAYS)	STATE OF MONOLAYER	ICE WATER INCUBATION YES/NO	NUMBER OF CELLS/ml	PELLET FORMATION
0	None formed	No	355	No
1A	Present; not complete	Yes	1.53×10^4	No
1B	Present; not complete	No	1.16×10^4	No
2A	Present; not complete	Yes	6.2×10^4	Yes but too small
2B	Present; not complete	No	4.1×10^4	Yes but too small
3A	Complete monolayer	Yes	2.78×10^5	Yes
3B	Complete monolayer	No	2.2×10^5	Yes
4A	Complete monolayer	Yes	8.1×10^5	Yes
4B	Complete monolayer	No	6.4×10^5	Yes
5A	Complete monolayer	Yes	1.07×10^6	Yes
5B	Complete monolayer	No	9.0×10^6	Yes
6A	Incomplete monolayer	Yes	1.22×10^6	Yes
6B	Incomplete monolayer	No	1.14×10^6	Yes
7A	Incomplete monolayer	Yes	1.02×10^6	Yes
7B	Incomplete	No	1.11×10^6	Yes

NOTE: Bands smaller than 0.5 kb were difficult to detect in gel photos.

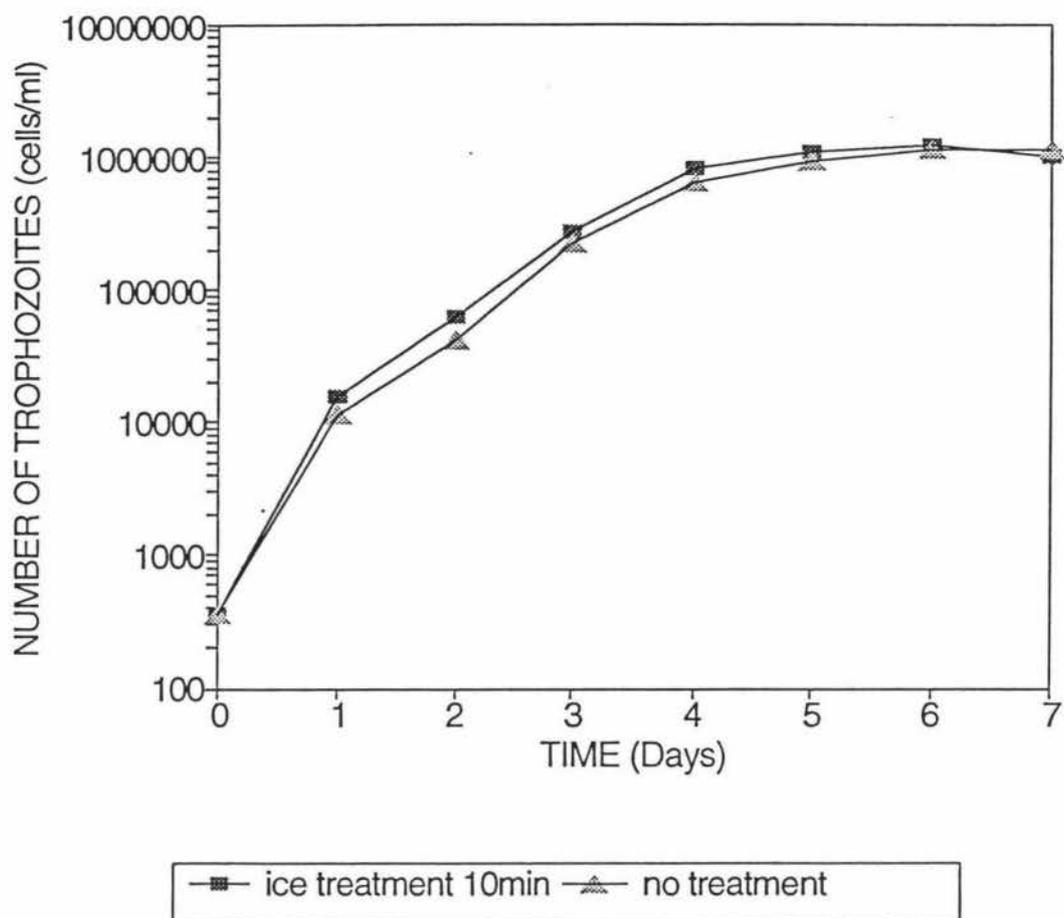


Fig. 3: Comparison of the yield of *G. intestinalis* strain Hast 11 trophozoites taking \triangle trophozoites in suspension and \blacksquare total trophozoites following cooling of the culture to 0°C to suspend attached organisms.

Note: (a) the increase in the number of trophozoites in suspension following cooling to 0°C is minimal and (b) under these conditions the yield is maximal after 4 to 5 days.

3.4 DISCUSSION

The investigation outlined in this thesis required the *in vitro* production of large quantities of *G. intestinalis* trophozoites. To investigate the conditions which maximised the yield of trophozoites, *G. intestinalis* strain Hast 11 with typical growth characteristics was chosen for a preliminary investigation of its growth curve.

It was concluded from the conditions used that the maximum yield of trophozoites in the log phase of growth, 2.78×10^5 , was reached after approximately three days and the rate of growth subsequently diminished after this time (Fig. 3). However, maximum viability was maintained for a further two days so the cultures could be harvested either at day 3 or could be delayed for a further two days. At this stage of growth the lower flask surface is totally covered with trophozoites (13, 19) and many organisms are found in the supernatant.

Because many trophozoites are attached, it is standard practise to cool the culture flask to detach cells for harvest. However, under these conditions the increase in yield was minimal (Fig. 3) and as the attached trophozoites at this stage of growth represent such a small proportion of the total it was concluded that it was unnecessary to cool cultures for harvest. Instead, high numbers of trophozoites could be achieved by simply decanting medium containing suspended trophozoites. This also had the further advantage that a fresh culture of trophozoites could be prepared merely by replacing the medium and reincubating the flask at 37°C and in such conditions (data not shown) trophozoites could be harvested two days later.

Although detailed studies were not conducted on other strains, pilot experiments showed that the results obtained in this chapter could be extrapolated to that of others.

**CHAPTER 4: DETECTION AND CHARACTERISATION OF RESTRICTION
FRAGMENT LENGTH POLYMORPHISMS (RFLP's) IN THE
GIARDIA INTESTINALIS GENOME.**

4.1 INTRODUCTION

Host specificity of *Giardia* strains has long remained as a central unanswered epidemiological question. If *Giardia* is host specific, it might reasonably be expected that some differences between strains would be found. However, there have been no consistent differences detected between strains of *G. intestinalis* isolated from human and animal sources. In this context, identification of individual strains would be a useful starting point.

Much of current *Giardia* research centres on techniques designed to detect genetic variation among different *Giardia* strains. In this chapter we examine the use of restriction enzymes to detect Restriction Fragment Length Polymorphisms (RFLP's) in the *Giardia* genome. Initial experiments attempted to detect RFLP's in total genomic DNA but in further experiments only repetitive sequences were examined.

4.2 MATERIALS AND METHODS

4.2.1 Propagation of *G. intestinalis* isolates for DNA extraction

Materials

- 1) TY1-S33 Medium - see appendix
- 2) Phosphate Buffered Saline (PBS) - see appendix
- 3) Tris-EDTA solution - see appendix
- 4) Frozen aliquots of *G. intestinalis* strains Hast 11, Hast 76, Hast 86, Hast 68, NP 77, Ham 7, Ham 84, Whang 8, Whang 48 and Wang 3. These ten New Zealand strains of *G. intestinalis* were isolated from human faeces and obtained from the Massey University Department of Microbiology and Genetics Culture Collection.

Method

A 0.5 ml aliquot of a *G. intestinalis* culture stored at -70°C was rapidly thawed, inoculated into TY1-S33 medium in a 50 ml Falcoln™ tissue culture flask and incubated at 37°C until a complete monolayer of trophozoites had just formed (at this stage the trophozoites are in the exponential growth phase). 5 ml of this culture was removed and inoculated into each of four 750 ml Falcoln™ tissue culture flask containing TY1-S33 medium and incubated at 37°C until a complete monolayer had just formed (usually 3-4 days).

Each 750 ml culture of trophozoites was harvested by centrifuging at 5000g for 10 minutes in a GSA rotor. The supernatant was discarded and the pellet resuspended in 20 ml of sterile PBS and recentrifuged at

5000g for 10 minutes in the Heraeus Christ® centrifuge. The preparation of trophozoites was washed twice more with sterile PBS, suspended in 5 ml of TE buffer and either stored at -70°C or used immediately for DNA extraction.

4.2.2 DNA extraction

Materials

1) Sodium Dodecyl Sulphate (SDS) 10%

SDS (Sigma)	10.0 g
Distilled Water to	100.0 ml

This solution was stored at room temperature without autoclaving.

2) Pronase Type XIV 20 mg/ ml

Pronase (Sigma)	0.2 g
Distilled water to	10.0 ml

This solution was preincubated at 37°C for 3 h to self-digest contaminants, especially DNase activity, and filtered through a sterile 0.2 µ filter to remove precipitates. This was then stored at -20°C.

3) RNase (Ribonuclease 1) 10 mg/ ml

RNase from bovine pancreas (Sigma)	0.10 g
RNase buffer (see appendix)	10.0 ml

This solution was preincubated at 100°C for 15 min to destroy DNase activity. It was then stored at -20°C.

4) 5M Sodium perchlorate

Sodium perchlorate	61.22 g
Distilled water to	100.0 ml

This solution was stored at room temperature after autoclaving.

5) 10x Saline Tris-EDTA (STE) Buffer - see appendix

6) Phenol/Chloroform/Isoamyl alcohol - see appendix

7) Dialysis Tubing

10 mm flat width dialysis tubing was cut to approximately 20 cm lengths and boiled in 5% sodium carbonate solution. This process was repeated until no colour could be detected (approximately 3-4 times). The tubing was then boiled in distilled water once; in 0.001 M EDTA once; rinsed in distilled water and then boiled in distilled water. The dialysis tubing in the distilled water was then allowed to cool before being stored at 4°C.

8) 1 M Tris-HCl (pH 7.5) - see appendix.

9) 0.2 M EDTA - see appendix.

10) TE Buffer (for dialysis)

1 M Tris-HCl	40.0 ml
0.2 M EDTA	20.0 ml
Distilled water to	4000.0 ml

This buffer was freshly prepared before use.

11) *G. intestinalis* trophozoites as prepared in section 4.2.1.

Method

Method

DNA was extracted from *G. intestinalis* trophozoites using the method of Ionas (55a).

Pelleted preparations (see section 5.2.1) suspended in 5 ml of TE buffer were thawed in a 37°C waterbath. 0.5ml SDS (10%) and 0.5ml Pronase type XIV (10mg/ml) were added to each trophozoite suspension and incubated at 50°C overnight to lyse the cells and digest their proteins. 0.1ml RNase was added to the lysate and incubated for 60 minutes to digest the RNA. 5M Sodium perchlorate was then added to give a final concentration of 1M and the lysate was incubated at 50°C for a further 60min.

Phenol was melted in a 70°C water bath and added to chloroform and isoamyl alcohol to give a ratio of 25:24:1. This solution was mixed with one tenth of its volume of STE buffer. Air was bubbled through the preparation to facilitate mixing. The solution was cooled to room temperature and used as follows to deproteinate the lysate. An equal volume of phenol/choloroform/isoamyl alcohol solution was added to the lysate, gently mixed by repetitively inverting the centrifuge tube and centrifuged at 5000 g for 10 min at room temperature.

The upper (aqueous) layer, containing the DNA was removed using a 5ml autopipette fitted with a sterile tip, the point of which was removed, placed into another centrifuge tube and re-extracted twice more with the phenol/choloroform/isoamyl alcohol solution or until a white precipitate was no longer visible at the interface. The DNA was then placed into dialysis tubing and dialysed at 4°C against 4 L (total 16 L) of TE buffer with three changes over a 48 hour period. After dialysis the DNA was placed into a sterile universal bottle and stored at 4°C.

4.2.3 Isolation and removal of the GC-rich fraction of DNA.

Materials

1) Hoescht 33258 stain (1 mg/ml)

Hoescht 33258 0.01 g

Sterile water 10.0 ml

This was stored at 4°C in the dark (ie. with tin foil around the glass bottle).

2) Caesium chloride (research grade)

Method

A protocol based on that of Ortega-Pierres *et al* (92) but with modifications was used.

7 g of CsCl was added to 7 ml of the DNA preparation to give a total volume of approximately 11 ml. 140 µl of Hoescht 33258 stain was added to this and the refractive index was checked using a refractometer. CsCl was then added until the refractive index reached 1.4. The solution was divided equally into 2 centrifuge tubes and centrifuged at 55 K for 48 hours in the TST 60.4 swing bucket rotor (Sorvall®, Du Pont).

The DNA separated into 2 distinct bands visible under long wave UV light. Each band was carefully removed using a pasteur pipette, transferred into separately labelled eppendorf tubes and stored at 4°C.

4.2.4 Removal of CsCl by microdialysis

Materials

1) TE buffer - see appendix.

Method

CsCl was removed from DNA preparations using the method of microdialysis as described by Ward and Jarvis (116).

A petri dish containing a 1.0 cm flea (stirring bar) and approximately 40 ml TE buffer was placed on a magnetic stirrer. A membrane disc (Millipore type VS, pore size 0.25 μm , 47 mm diameter) was floated on the surface and the DNA containing caesium chloride were placed in drops on the filter. 100 μl drops of DNA (no more than 7 drops/filter) were placed symmetrically on the filter so as to keep the filter balanced and afloat. The DNA was dialysed for 45 mins, then removed from the filter to a labelled eppendorf tube and stored at 4°C.

4.2.5 Determination of DNA concentration and purity

Materials

1) TE Buffer - see appendix.

Method

(a) Spectrophotometric Method

50 μl of DNA was diluted with 950 μl of TE buffer and mixed gently so as not to shear the DNA. The optical density at 260 nm and 280 nm was measured using the Shimadzu® automatic spectrophotometer

zeroed against the dialysis buffer (viz. TE buffer = blank)

The following parameter allowed the DNA concentration to be determined from the 260 nm reading:

If: $E_{260} = 1$ then the DNA concentration = 50 $\mu\text{g/ml}$

i.e. E_{260} absorbance $\times 50 \times$ dilution factor (20)
= DNA concentration in $\mu\text{g /ml}$.

To determine if the purity was adequate, the absorption of the diluted sample at 260 nm and 280 nm was noted and the 260/280 ratio calculated. A ratio of greater or equal to 1.8 was considered to represent DNA of adequate purity.

4.2.6 Restriction enzyme digestion of *G. intestinalis* DNA

Materials

A total of 10 different enzymes were used in this section:

Bam HI, Eco RI, Sma I, Fok I, Hinf I, Bgl II, Hae III, Kpn I, Apa I and Taq I. The restriction buffers (10x concentrated) used were supplied with purchase of the enzyme from the manufacturer.

- 1) 3M Sodium acetate - see appendix.
- 2) 95 % Ethanol - see appendix.
- 3) 70 % Ethanol - see appendix.

4) Tris-EDTA (TE buffer) + 0.05 % SDS + 20 % glycerol (Running Buffer).

1.0 M Tris-HCl (Ph 7.5)	0.10 ml
0.2 M EDTA (Ph 7.2)	0.05 ml
Glycerol	2.00 ml
10 % SDS	0.05 ml
Distilled water to	10.0 ml

This solution was stored at room temperature.

5) Bromophenol Blue Dye (10x concentration) - see appendix.

Method

Aliquots calculated to contain 30 μg of DNA were added to 1.5 ml eppendorf tubes containing 1/10th of the final volume of 10x reaction buffer plus sterile water to dilute to the final volume desired (not usually more than 150 μl). Restriction endonuclease was added to the mixture to give an concentration of 30 units. (Note that 1 unit of restriction enzyme is the amount required to digest 1 μg of DNA in 1 hr using the conditions specified by the manufacturer). The eppendorf tube, which contained the reaction mixture, was pulsed in the microfuge to ensure that all of the reaction mixture was at the base of the tube. It was then incubated at the temperature specified by the manufacturer for two hours. The reaction was stopped by heating at 65°C for 10 minutes for those enzymes sensitive to heat inactivation or by cooling to 0°C.

To concentrate the digest to a volume small enough to fit in the well of an agarose gel, the DNA was precipitated with ethanol by adding 1/10th of the volume of 3M Sodium acetate and mixed gently, followed by 2.5 volumes of cold (-20°C) 95 % ethanol. After gentle mixing this was

placed at -20°C or 4°C overnight.

The DNA was pelleted by centrifuging at 15000g for 30 minutes in a microfuge at 4°C. The supernatant was discarded and 1 ml of 70 % ethanol was added. The DNA was spun again for a further 30 minutes at 15000g (room temperature) to wash the proteins and salt from the surface of the pellet. After discarding the supernatant, the DNA pellet was dried under vacuum for 5-15 minutes in the Speedvac (dessicator). The pellet containing DNA was resuspended in 35 µl of running buffer. 5 µl of bromophenol blue dye was added and mixed well. This 40 µl suspension was then used for electrophoresis.

4.2.7 Agarose gel electrophoresis of restriction enzyme digests.

Materials

1) Tris Borate Buffer (TBB) - see appendix.

2) 1.0 % Agarose gel

Agarose (AR grade)	1.0 g
TBE buffer	100 ml

The agarose was dissolved by heating in the microwave (Med High; 4 min) and cooled to 50°C before use.

3) Ethidium bromide (10 mg/ml) - see appendix.

Method

The agarose gel was prepared as described in Sambrook *et al* (101). Digested DNA was size fractionated through a 1% agarose gel (size 20 x 15 x 0.6 cm³) in TBE buffer at 80 V for 4-5 hours. 10 µl of BRL 1

kb ladder was also run in each end lane to act as standard fragment sizes with which to compare the size of bands produced from the digests. Gels were stained with ethidium bromide for 10 minutes, destained with water for 1 hour and then photographed under shortwave UV light.

4.2.8 Determination of the size of DNA fragments.

Materials

- 1) 1 kb ladderTM (BRL)

Method

10 μ l samples of the BRL 1 kb ladder were run alongside the DNA samples on agarose gels (see section 4.2.7). Following staining of the gel with ethidium bromide and subsequent photography of the gel under shortwave UV light, the mobility of each DNA band was measured as the distance from the middle of the well to the centre of the band. Fragment lengths were determined graphically from a plot of relative mobility (mm) versus fragment size (kb) on semilog graph paper.

4.3 RESULTS

A. Total genomic DNA

Total DNA from ten New Zealand strains of *G. intestinalis* isolated from human sources were digested with the restriction enzymes Bam HI, Alu I, Apa I and Sph I. Digested DNA was separated by agarose gel electrophoresis and the results indicate that in all cases a very large number of fragments were produced which led to smearing and hence diminished the resolution. This is illustrated with the Bam HI digest (Fig. 4).

B. Fractions of DNA

Total genomic DNA mixed with CsCl and Hoescht dye 33258 were centrifuged. The DNA separated into a higher buoyant density (GC-rich) band and a lower buoyant density (AT-rich) band (Fig. 5).

Total genomic DNA, AT-rich DNA (total DNA minus the GC-rich component) and GC-rich fraction DNA from *G. intestinalis* strain Hast 76 were digested with Bam HI and the products of digestion were run on agarose gels. The results (Fig. 6) show that the AT-rich DNA digest produced a smear as many bands were present. The GC-rich DNA digest showed three bands (of sizes 2.4 kb, 1.85 kb and 1.3 kb) and there was an absence of smearing. The total genomic DNA, while showing smearing, produced three bands more evident than the others. The sizes of these three bands corresponded to the three bands present in the GC-rich DNA digest. This is illustrated in Fig. 6.

The GC-rich fraction of DNA from each of ten strains of *G. intestinalis* was digested with Apa I, Fok I, Hae III, Hinf I, Sma I and Taq I restriction enzymes known to have restriction sites within the variable (spacer) regions of the 5.56 kb rRNA gene repeat. In addition, restriction enzymes Kpn I, Bgl II and Bam HI with recognition sites anywhere within the 5.56 kb rRNA repeat were used. These results are shown

in Figures 7-15 and summarised in Table 2.

Note that no differences in banding patterns of the ten strains were detected using Bam HI, Sma I, Apa I, Hinf I, Bgl II or Hae III (Figs. 7-12). However, digestion with enzymes Taq I, Kpn I and Fok I (Figs. 13-15) revealed distinctive differences in the number and intensity of the bands present between the strains. All experiments were performed at least twice and gave consistent results.

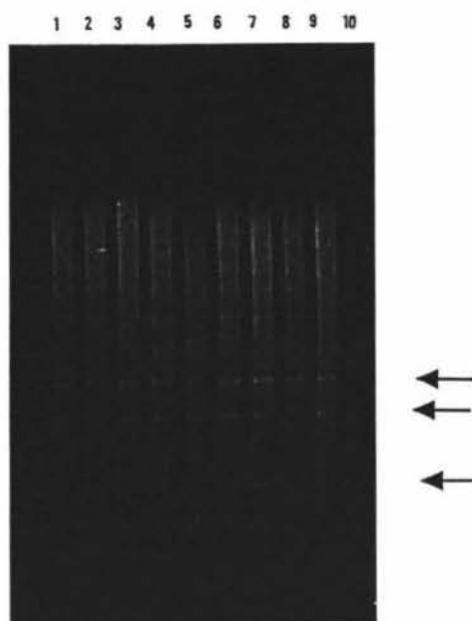


Fig. 4: Bam HI digestion of total genomic DNA from ten New Zealand isolates of *G. intestinalis*: NP 77 (lane 1); Ham 84 (lane 2); Wang 3 (lane 3); Ham 7 (lane 4); Hast 68 (lane 5); Hast 11 (lane 6); Whang 8 (lane 7); Whang 48 (lane 8); Hast 86 (lane 9); Hast 76 (lane 10).

Note that many bands were generated. This caused smearing and consequent loss of resolution of the bands, nevertheless three bands are clearly visible. No differences were detected between the isolates.

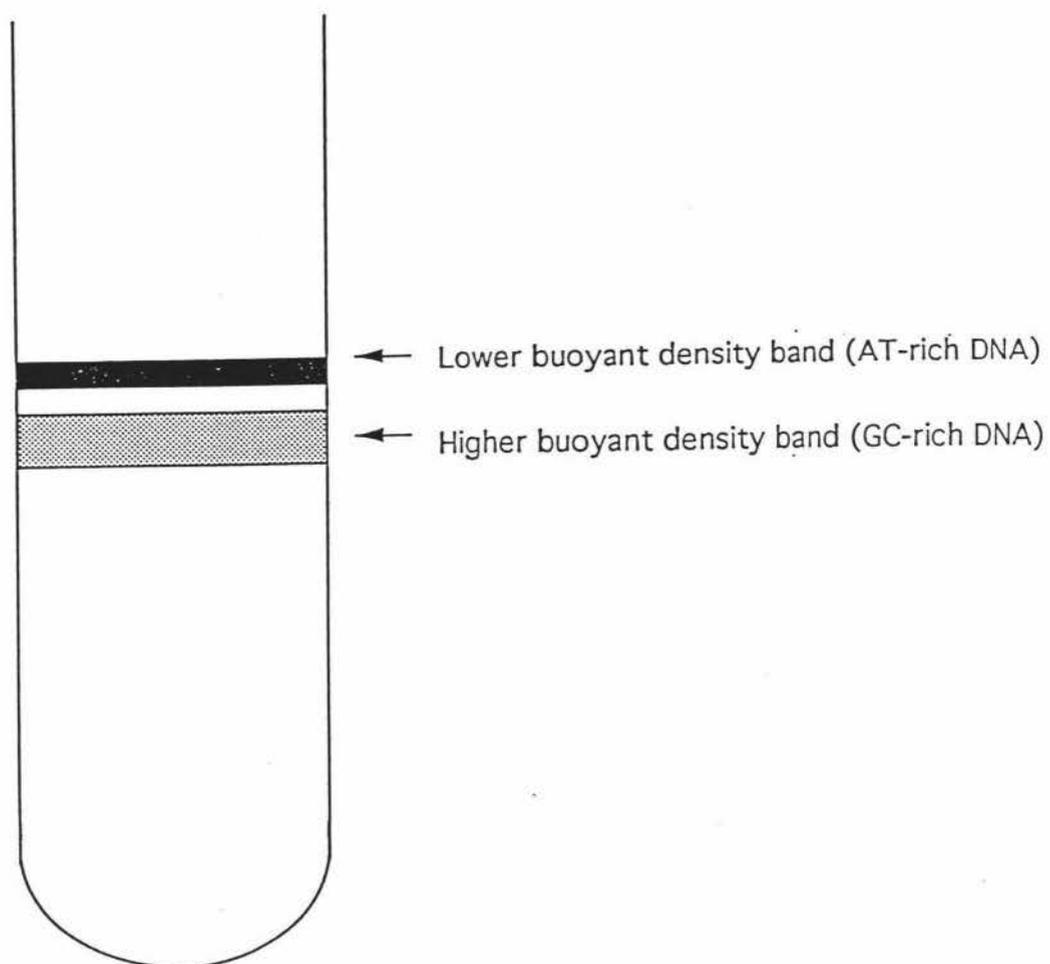


Fig. 5: Diagram of the result obtained when total genomic DNA was centrifuged with CsCl in the presence of Hoescht dye 33258. The DNA separated into a lower buoyant density (AT-rich) band and a higher buoyant density, more diffuse, (GC-rich) band.

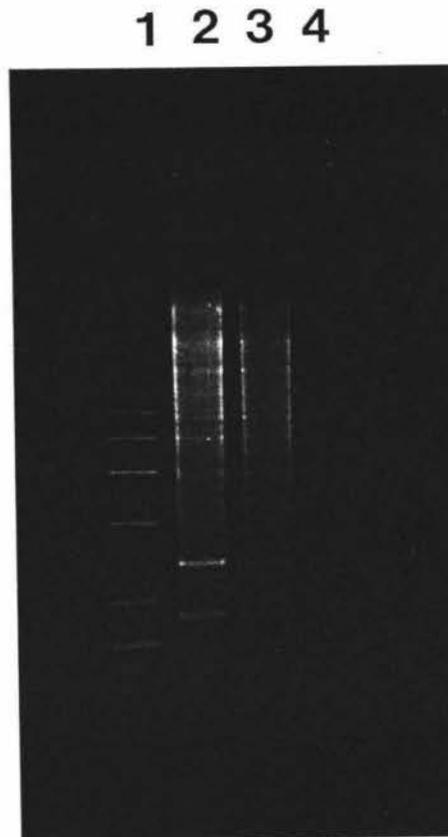


Fig. 6: Bam HI digestion of total genomic DNA (lane 2), AT-rich DNA (lane 3) and GC-rich fraction DNA (lane 4) from *G. intestinalis* strain Hast 76. Lane 1 contains the BRL 1 kb standard.

Note that the 3 prominent bands seen in the total DNA digest (lane 2) are diminished in intensity in the AT-rich band but appear in the digest of the GC-rich DNA with an almost complete absence of other bands.

Fig. 7: Bam HI digestion of the GC-rich fraction of DNA from each of ten New Zealand isolates of *G. intestinalis*: NP 77 (lane 1); Ham 84 (lane 2); Wang 3 (lane 3); Ham 7 (lane 4); Hast 68 (lane 5); Hast 11 (lane 6); Whang 8 (lane 7); Whang 48 (lane 8); Hast 86 (lane 9); Hast 76 (lane 10). The outer left hand lane contains the 1 kb DNA ladder (BRL).

Note that no differences were detected between the isolates.

Fig. 8: Sma I digestion of the GC-rich fraction of DNA from each of ten New Zealand isolates of *G. intestinalis*. The order of the isolates is identical to that listed above (see Fig. 7).

Note that no differences were detected between the isolates.

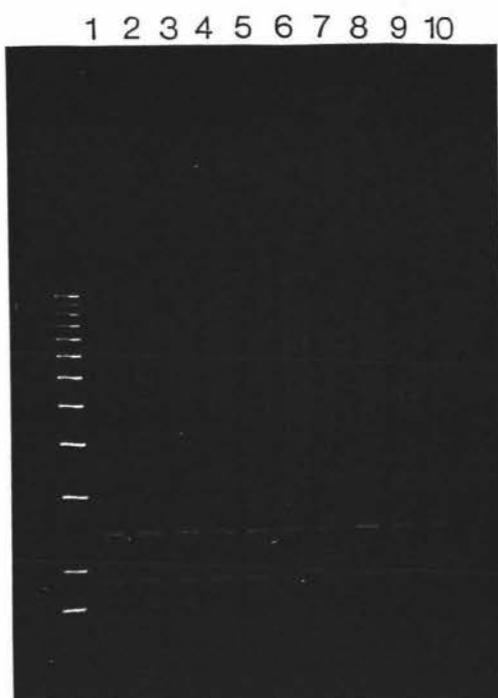


Fig. 7

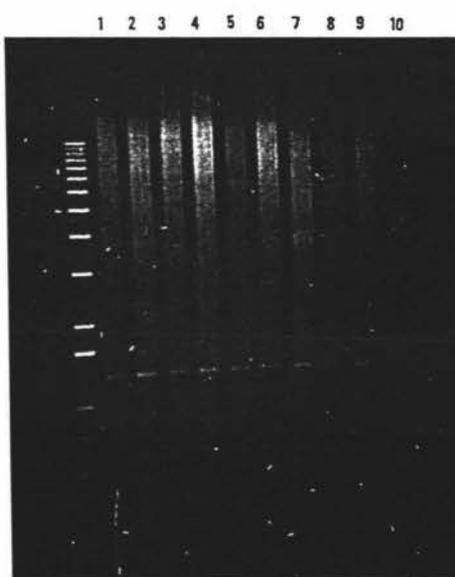


Fig. 8

Fig. 9: Apa I digestion of the GC-rich fraction of DNA from each of ten New Zealand isolates of *G. intestinalis*: NP 77 (lane 1); Ham 84 (lane 2); Wang 3 (lane 3); Ham 7 (lane 4); Hast 68 (lane 5); Hast 11 (lane 6); Whang 8 (lane 7); Whang 48 (lane 8); Hast 86 (lane 9); Hast 76 (lane 10). The outer lane contains the 1 kb DNA standard (BRL).

Note that no differences were detected between the isolates.

Fig. 10: Hinf I digestion of the GC-rich fraction from each of ten New Zealand isolates of *G. intestinalis*. The order of the isolates is identical to that listed above (see Fig. 9).

Note that no differences were detected between the isolates.

1 2 3 4 5 6 7 8 9 10

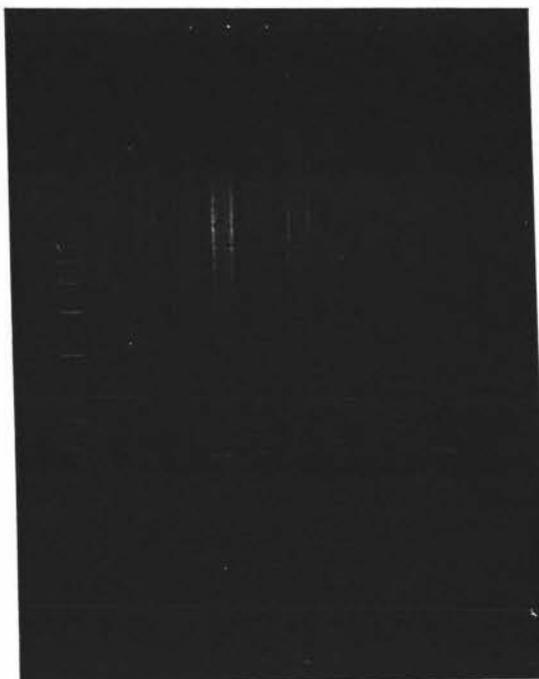


Fig. 9

1 2 3 4 5 6 7 8 9 10

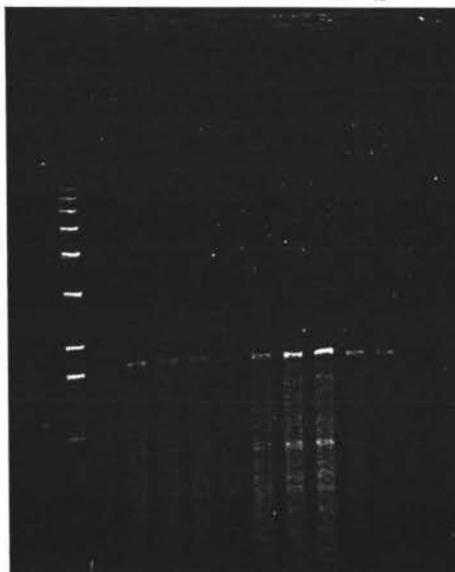


Fig. 10

Fig. 11: Bgl II digestion of the GC-rich fraction from each of ten New Zealand isolates of *G. intestinalis*: NP 77 (lane 1); Ham 84 (lane 2); Wang 3 (lane 3); Ham 7 (lane 4); Hast 68 (lane 5); Hast 11 (lane 6); Whang 8 (lane 7); Whang 48 (lane 8); Hast 86 (lane 9); Hast 76 (lane 10). The outer right hand lane contains the 1 kb DNA standard (BRL).

Note that no differences were detected between the isolates.

Fig. 12: Hae III digestion of the GC-rich fraction of DNA from each of ten New Zealand isolates of *G. intestinalis*. The order of the isolates is identical to that listed above (see Fig. 11).

Note that a smear was produced which implies that Hae III recognises many sites in the rDNA repeat. No differences were detected between the isolates.

1 2 3 4 5 6 7 8 9 10



Fig. 11

1 2 3 4 5 6 7 8 9 10

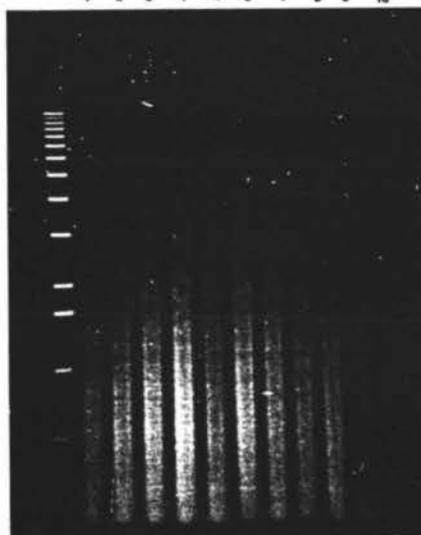


Fig. 12

Fig. 13: Fok I digestion of the GC-rich fraction of DNA from each of ten New Zealand strains of *G. intestinalis*: NP 77 (lane 1); Ham 84 (lane 2); Wang 3 (lane 3); Ham 7 (lane 4); Hast 68 (lane 5); Hast 11 (lane 6); Whang 8 (lane 7); Whang 48 (lane 8); Hast 86 (lane 9); Hast 76 (lane 10). The far right hand lane contains the 1 kb DNA standard (BRL).

Note that the differences observed between the strains appear to be that of band intensity rather than that of the presence or absence of a band.

Fig. 14: Kpn I digestion of the GC-rich fraction of DNA from ten New Zealand strains of *G. intestinalis*. The order of the strains is identical to that listed above (Fig. 13).

Note that strains Ham 84, Wang 3, Ham 7, Hast 11, Whang 48 and Hast 86 (lanes 2-6, 8 and 9) all possess a band (approximately 5.6-5.7 kb) not found in NP 77, Whang 8 or Hast 76 (lanes 1, 7 and 10).

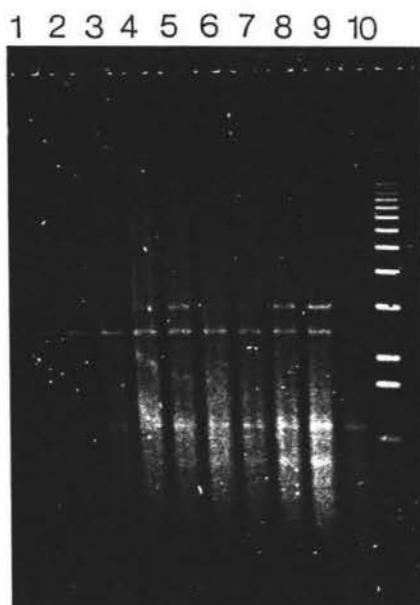


Fig. 13



Fig. 14

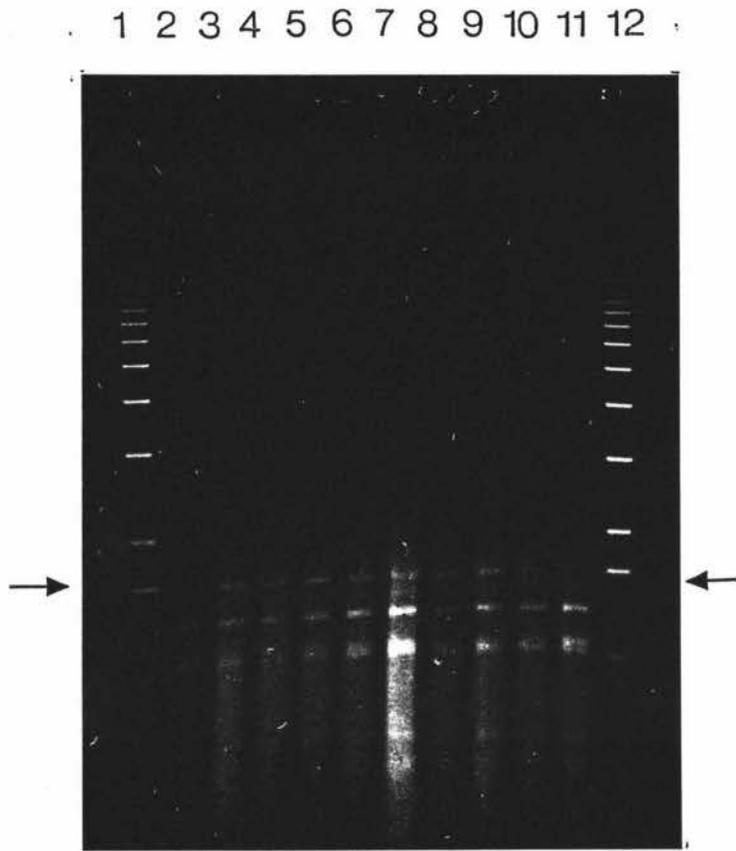


Fig. 15: Taq I digestion of the GC-rich fraction of DNA from each of ten New Zealand isolates of *G. intestinalis*: NP 77 (lane 2); Ham 84 (lane 3); Wang 3 (lane 4); Ham 7 (lane 5); Hast 68 (lane 6); Hast 11 (lane 7); Whang 8 (lane 8); Whang 48 (lane 9); Hast 86 (lane 10); Hast 76 (lane 11).

Note that two banding patterns appear. Those in lanes 3-10 are identical but lanes 2 and 11, which are identical, nevertheless differ from the other lanes in the loss of a 1.64 kb band (as shown). Lanes 1 and 12 contain the 1 kb standard (BRL).

TABLE 2: Observed and expected band size from restriction enzyme digestion of the *G. intestinalis* gene repeat contained within the GC-rich fraction of DNA.

NAME OF ENZYME USED IN DIGESTION	EXPECTED BAND SIZE (kilobases)	OBSERVED BAND SIZE (kilobases)
Apa I	1.44, 1.14, 0.5, 0.44, 0.36, 0.3	1.4, 1.15, 0.54, 0.48
Bam HI	2.4, 1.85, 1.3	2.5, 1.9, 1.15
Bgl II	5.56	5.56
Fok I	2.4, 1.1, 0.78, 0.5, 0.36, 0.32	3.0, 2.4, 1.1, 0.8
Hae III	0.85, 0.84, 0.74, 0.56, 0.54, 0.52, 0.46, 0.44, 0.38, 0.31	A smear was observed as Hae III has many recognition sites in the rRNA gene repeat.
Hinf I	1.7, 0.83, 0.62, 0.56, 0.45	1.75, 1.45, 0.94, 0.74, 0.66
Kpn I	5.56	approx. 5.7, 5.56
Sma I	1.3, 0.6, 0.5, 0.38	1.3, 0.65, 0.55
Taq I	1.3, 1.08, 1.03, 0.62, 0.5, 0.48	1.6, 1.3, 1.1, 1.05, 0.7, 0.62

NOTE: Bands smaller than 0.5 kb were difficult to detect in gel photos.

4.4 DISCUSSION

Restriction Fragment Length Polymorphism (RFLP) formation is due base changes resulting in the gain or loss of a restriction site. Such polymorphisms are detected when the DNA is digested with an enzyme recognising the new restriction site and the fragments separated by electrophoresis.

In this chapter, the total genomic DNA from each of ten New Zealand strains of *G. intestinalis* was digested with restriction enzymes Bam HI, Eco RI, Alu I, Apa I and Sph I and the products of digestion were electrophoresed. The large number of fragments produced resulted in poor resolution of bands on the gel (Fig. 4). However, the large size of the *Giardia* genome (8×10^7 bp) implies that multiple recognition sites will be present for any restriction enzyme so this result is not surprising.

This result is consistent with that of other workers (50, 89, 111) who found that the examination of ethidium bromide-stained gels usually did not show differences between DNA from *Giardia* strains. While the large number of bands do not allow detailed conclusions, their consistent conclusions were that the digests from all strains were very similar and that this similarity in banding patterns indicated a general lack of DNA-sequence divergence although the geographic locality and hosts of the strains were diverse.

Although many bands were generated, certain bands were more evident than others. These bands represent repetitive sequences which are GC-rich (92) and become more visible when most of the nonrepetitive genomic DNA is removed. This is achieved by ultracentrifugation of this DNA with Caesium chloride (CsCl) containing Hoescht dye 33258 (Fig. 5). The Hoescht dye preferentially binds to AT-rich DNA sequences and decreases their buoyant density (64) so that the combination of dye and centrifugation resulted in the separation of two bands, the lower of which was GC-rich DNA containing repetitive sequences (92).

Restriction Fragment Length Polymorphisms (RFLP's) are most easily detected in a

repetitive sequence where a large number of copies of this variation are present. A new restriction site (possibly unique to one strain) created by a base mutation allows digestion with an enzyme recognising this site to demonstrate this polymorphism.

This approach has been shown to be useful for distinguishing various isolates of *Candida albicans* (77) so it should be useful for distinguishing strains of *Giardia* because *G. intestinalis* ribosomal RNA (rRNA) genes are organised in tandem arrays of a 5.56 kb repeat containing both constant and variable regions (15, 32, 67, 92). This arrangement is illustrated in Fig.16 and the complete nucleotide sequence (Fig. 17) and restriction map of this tandem repeat are also known (51, 114). Furthermore, as mentioned previously, DNA isolated from *G. intestinalis* can be separated into two distinct populations with different buoyant densities by centrifugation through a CsCl gradient containing Hoescht dye 33258. The lower buoyant density AT-rich (total genomic DNA minus GC-rich fraction) comprises approximately 90% of the total DNA and is composed of nonrepetitive DNA encoding such structural genes as alpha and beta tubulin (92). The higher buoyant density band is extremely GC-rich and comprises approximately 10% of the total DNA. This GC-rich band is comprised of a 5.56 kb sequence, reiterated in tandem, which, based on results from restriction banding patterns, Southern blots and hybridisation of rRNA probes, indicates that this sequence contains the tandemly repeated DNA sequence which encodes mainly for ribosomal RNAs (92).

Ribosomal RNA (rRNA) genes have been shown to be very useful in the classification of many organisms because they are highly conserved (115). Thus, although we did not have the strain of *G. intestinalis* used to obtain the rRNA gene sequence (51), we could expect our strains to display banding patterns similar to those expected from the restriction map available. However, although conserved coding regions are present within rRNA genes, variable regions are also present and may differ in sequence between strains. It was restriction enzymes that had sites of recognition within these variable regions that were then used to demonstrate differences between strains of *G. intestinalis*. However, it is important to note that this study was not to show that bands produced from enzyme digests matched the

restriction map available from one strain of *G. intestinalis*, although this helped to demonstrate that we were, in fact, examining the rRNA repeat, but rather to find if the fragment lengths produced would distinguish strains.

Following the initial use of total genomic DNA digests, we examined a subcomponent of the total genomic DNA, namely the GC-rich fraction. Electrophoresed products from Bam HI digestion of total genomic DNA, AT-rich DNA and GC-rich DNA (Fig. 6) showed that the GC-rich fraction produced fewer bands as compared to that of the AT-rich and total genomic DNA so greater resolution was achieved. AT-rich DNA and total genomic DNA both displayed smearing due to an excessive number of bands present. This is because Bam HI has a large number of recognition sites in the *Giardia* genome. However, three bands (of sizes 2.4 kb, 1.85 kb, and 1.3 kb) were more evident than the others in the total genomic digest. These corresponded to the three bands present (2.4 kb, 1.85 kb and 1.3 kb) in the GC-rich fraction digest. Each of these bands represents the fragment sizes we anticipated from our knowledge of the sequence and restriction map of the *G. intestinalis* rRNA repeat (see Table 2).

These results correspond to those of Ortega-Pierres *et al* (92) where the banding patterns obtained with Bam HI digestion were consistent with the rRNA gene map proposed by Boothryd *et al* (15) for the repeated unit. Furthermore, Ortega-Pierres *et al* demonstrated that probes of large and small subunit rRNA obtained from *G. intestinalis* hybridised to the three bands produced by Bam HI digestion of GC-rich fraction DNA but failed to hybridise to any of the bands produced by Bam HI digestion of AT-rich DNA. The hybridisation patterns produced were similar, if not identical, to the restriction banding pattern produced by Bam HI digested GC-rich DNA. This indicated that the GC-rich DNA population contains the tandemly repeated DNA sequence which encodes mainly for rRNAs (92).

From this, it can be assumed that our results demonstrate that not only does the GC-rich fraction DNA contain the rRNA gene repeat but that the reduction in the number of bands produced, hence a reduction in smearing, enhances any ribosomal fragments

present.

The next step was to find if any restriction enzyme was able to distinguish a strain by digestion of the GC-rich fraction of DNA. The GC-rich fraction of DNA from each of ten New Zealand strains of *G. intestinalis* was digested with restriction enzymes Apa I, Fok I, Hae III, Hinf I, Sma I and Taq I that were all known to possess recognition sites within the variable region of the rRNA gene repeat. In addition, restriction enzymes Kpn I, Bgl II and Bam HI with recognition sites anywhere within the 5.56 kb rRNA repeat were used.

Although no differences were detected in the banding patterns of the ten strains using Bam HI, Sma I, Apa I, Hinf I, Bgl II or Hae III (Figs. 7-12), the size of the bands observed correlated to the size of bands expected from our knowledge of the restriction map of the *G. intestinalis* rRNA repeat (Table 2). However, restriction enzymes Taq I, Kpn I and Fok I revealed distinctive differences between the strains (Figs. 13-15).

Digestion with Taq I (Fig. 15) demonstrated that strains NP 77 and Hast 76 (lanes 1 and 10) possess the fragment sizes we predicted from our knowledge of the rRNA gene repeat restriction map. However, strains Ham 84, Wang 3, Ham 7, Hast 68, Hast 11, Whang 8, Whang 48 and Hast 86 (lanes 2-9) all possess a 1.6 kb fragment larger than the other fragments produced and that we had not anticipated from our knowledge of the rRNA genes restriction map (Table 2).

Digestion of GC-rich DNA with Kpn I (Fig. 14) shows that strains Ham 84, Wang 3, Ham 7, Hast 68, Hast 11, Whang 48 and Hast 86 (lanes 2-6,8 and 9) possess a band just greater than the 5.56 kb rRNA gene repeat that is not found in strains NP77, Whang 8 or Hast 76.

Digestion of the GC-rich fraction of the ten strains of *G. intestinalis* with Fok I (Fig. 13) demonstrates that strains Ham 84, Wang 3, Ham 7, Hast 68, Whang 48 and Hast 86 (lanes 2-5,8 and 9) possess a 3 kb band that is 500 bp larger than the largest band

we had anticipated (Table 2). It is arguable that this band is also possessed by strains NP 77, Hast 11, Whang 8 and Hast 76 (lanes 1,6,7 and 10) as the differences first observed appear to be that of band intensity rather than that of the presence or absence of a band. Hast 76 also appears to possess a band just greater than 2 kb in size but again the same explanation of band intensity rather than a difference in banding pattern could also be applied to this situation as on closer inspection of the gel it appears that NP 77, Ham 84 and Wang 3 (lanes 1-3) may also possess this band. It is therefore fair to assume that this band may also be present in the other six strains.

The differences detected between strains of *G. intestinalis* using restriction enzyme digestion of the GC-rich fraction of their DNA gives some hope that distinguishing between strains of *Giardia* using this approach is possible. However, this procedure is labourious and requires large amounts of DNA that can only be obtained from the *in vitro* culture of *Giardia* strains which in many, indeed most, cases is not yet possible. We are therefore unable, as yet, to use this technique to differentiate between strains of *Giardia* which infect animals from those that infect humans.

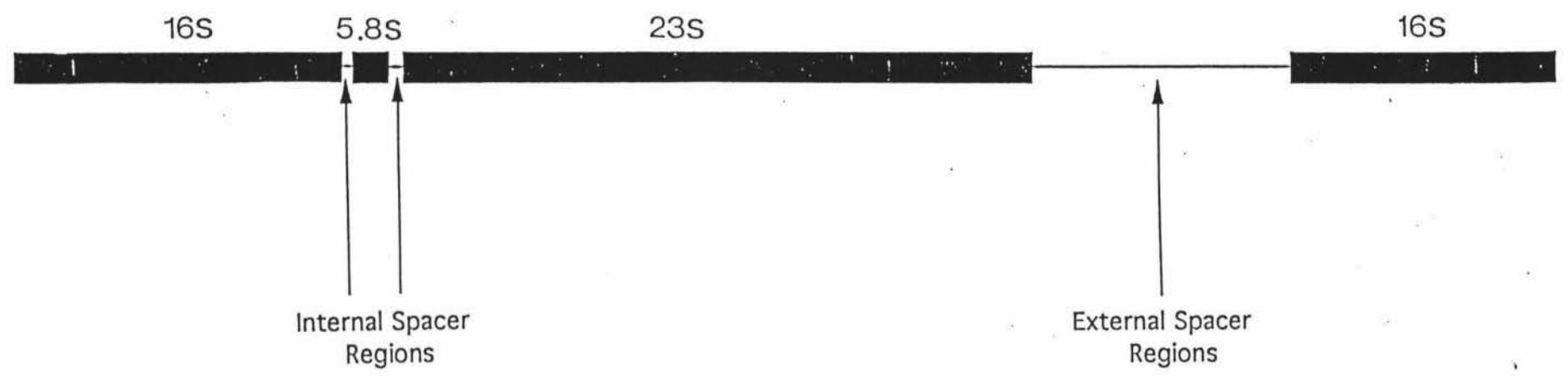
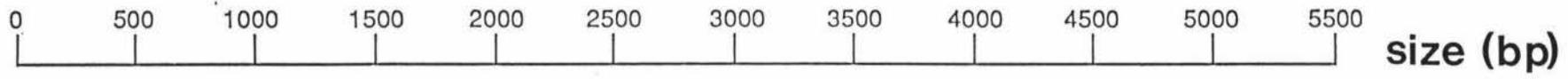


Fig. 16: Organisation of the *G. intestinalis* rRNA genes as a 5.56 kb repeating unit showing the presence of both constant and variable regions.

1 CGGCGGCGA CGGTTGAAAC AGGATGATCC CGTCGAGACC GCGGCGCGG CAGGCGCTG CCAAGACCCG CTCTGTCAIT MAAGGCGAA GCGCGGGGC TAGAAGCGA
111 TCAGACACCA CGGTATTCGC GCGCGTAAAC GGTGCGCGC CGCGGCGCG GCGCGCGCC CCAGGAAAC CGGAGGCTC CGGCTCTGG GGGAGTATG
221 CGCGCAAGGC TGAACCTTGA AGGATTGAC GGAAGGATAC CACGAGACGT GAGATCTGG GCTCAATCTG ACTCAACCGG GCACTTACAG CAGGCGCGA GCGCGGAGG
331 ACCGACAGCC GCGCGCGCTT TCGGATCGC GCGGCGCGT GCTCCAGCC GCTGCGCGA CGCGTCTCT CCAITGCGAC AACGAGCGAG ACCCGCGCG
441 CGCGCGCGC GCGCGCGCG GCGCGAGCG GAGGACGCG GCGGATAGC AGGTCTGTGA TGCCCTCAGA CGCGCTGCG GCGACGCGG CTACACTGGC GCGCGAGCG
551 GCGCGCGCGC AGGACGCGC GAGCGCGCG CGTGGCGCG ACTCGCGCGT GGAACGCGC CGCGCACAG GAATGTCTT TAGGCGCGC CCGCCACCGC GCGCGGAGG
661 CGTCCCTGC CTTGTACAC ACCGCGCGT GCTCTACCG ACTGGCGCG GCGCGAGCG GCGAAGCGG GCGAGCGCG GCGCTGGAG GAGGCGAAG
771 TCGTAAACAG GTAATCCGTA GGTGAAGCTG CGATGGAATC CCTCGCGCGC CGCGCGCGT GCGCGCGCG CCGCGTGGC CCGCGAAGCG CCGCGCGCG GATGCTCGG
881 CCGCGCGCG GACGAAAGC GCGCGGAGC GCGAGACCG GTGCGGACCG CGCGCGCGG AGAAGCACCG ACCCTCGAAC GCGCGCGCG CCGCGCGCG CCGCTCGAG
991 CGCGCGCGT GCGCGCGCG GCGCGCGAG GAGCGCGCG GCGCGCTCC CGCGCGCTG GCGCGCGCG GCGCGCGCG AACTTAAAGCA TATCAGTACG
1101 CCGCGGAGCA GAAACCAACC GCGATTCGCC GTAGCGCGCA GCGAGCGCG AGTAGCGCG CCGGAAAGCG CGCTGTGCG GCGAGCGCA GCGCGCGCG GAGGCGCGC
1211 GAGCGCGCG CCGGAGAGG TCGAAGCGC GTACGCGCG GCGCGCGCGT GCGCGCGCG TAGCGTGTCT TGAGCGTGA GCGCGAAGCG AGCGCGCGC CTTCCAAAGC
1321 TAAATAGCG CCGGAGCGA TAGCGGACCA AGTAGCGCG GCAAGCGGT AAAGGACCG CTGCGCGCG CTAAGAAAGC CTGAACCGG CCGCGCGCG GCGCGCGCG
1431 CCGCTCTGA AACAGGACC GAGGAGCGC GCGCGCGCG GAGCGGAGG GAGCGCGCG GCGCGAGCG GCGCGAGAGC CCGCGCGCG GCGCGCGCG TCGCGGAGT
1541 CGCGCGCGA GCGCGCGCG GTGCGCGCG AAGCGCGTGA TCTATCGCG GCGAGGCGCA GCGCGCGCG AAGCGTGTG GAGCGCGCG GCGCGCGCG CCGGAGTCA
1651 CCGCTCTGA GCGCGCGA GCGCGGAAAG ACTCATGAA CCGCGTGAAT CTGTGTGCG TCGGAAATGT CTCCAGGAC AGCGCGCGC CCGGAGTTC GCGCGTGA
1761 GCGCTGCGC GCGCGGAGCG GCGCGCTGC CCGCGCGCG CCGCGCGCGT CCGCGCGCG GCGCGCGCG GCGCGCGCG TCGCGCGCG GCGCGGATG GCGCGGAGC
1871 GCGCGCGCT CTGTAAGCA GCGCGCGCG GCGCGGACCA TCGCGAGCG GCGCGAGGT GCGCGCGCG GCGCGCGCG ACGCGTGGC CCGGCTCGA CAGTGAAG
1981 GCGCGCGAG AAGTGGTAT CCGCGCGCG GTGTGAAGA ACCGACGCG GCGCGCGCG GCGCGCGCG AAGGAGCGG CCGCGCGCG GCGCGCGCG GCGCGCGCG
2091 GCGCGCGCG GTAGGAGCG GCGAGGCGC GCGCGCGCG GCGCGCGCG GCGCGCGCG GCGCGCGCG CTGTGCGA TCTCGCGAG AGTAGCGCGT ACTCGCGCG
2201 CCGGAGGACT GAGGAGCGA CCGGTTCCG GCGCGCTGA TGTGCGCGC GGTGACTCG GCGTAAAGCG CCGGTAAGA CCGGAAAGCG GCGTGGCGC CCGTCAAG
2311 GCGGAGCGC GAGACTCGC GCGGCGCGC CCGCGCGCG CCGCGCGCG GCGCGCGCG GCGCGCGCG ACCGCGCGC GCGCGCGCG GCGCGCGCG GCGCGCGCG
2421 CCGCGGAGC CTTGCGCG AGAGGCGCG CCGCGCGCG ACCCGCGCG TCGCGCGCG CCGCTGAAA GCGCGCGCG GCGCGCGCG CCGCGTACCG ACCGAGCGA
2531 GACTCGCGG TCAGGAGCT CTAGCGCGG AGCGAGCGG GCGCGCGCG GCGCGCGCG CCGCGCGCG AAGGAGTGC TCTGAGCGC GCGCGCGCG
2641 GAAGTGAAC GCGCGCGCG ATCCGAGCT TTTACTAGA ACACAGCGT GCGAGGCGC GCGCGCGCG TCGCGCGCG TGAATTCGCG CCGAGTCCAC GACCGTCA
2751 GTGAAGGAT CCGCGGAGC CCGTAAAC GCGCGGAGTA ACTATGACT TGTAAAGTA GCGAAATGCC TCGTGGCGA ATTTCCGAG TCGATGAAT GACCAAGAG
2861 GATCCACTG TCGGAGCGC GCGCTCGGA GCTTCCAGC TCGGGAACCG GCGAGGCGC GCGCGCGCG CAAGAAGA CTTTTGACT TGACTCCAGC CCGCGCGCG
2971 GCGCGCGCG GCGCGCGCG GCGCGCGCG GCGCGCGCG CCGTGAACA CCGTACCGC CCGCGCGCG GCGCGCGCG GCGCGCGCG GCGCGCGCG GCGCGCGCG
3081 TCGCGTGGC GCGCGCGCG TGTACAGCG GACCGAGCG GCGCGCGCG AGTACGCGA CCGCGCGCG ACGAGAGCG CACAAGCGC CCGGAGCGC
3191 GCGCGCGCG GCGCGCGCG CCGCGAAAG CCGCGTACC GATCTTCCG CCGCGCGCG GCGCGCGCG AGGTGCGA AAGTTTACA CAGGATAAC TGCTGTGTG
3301 CCGCGGAGC CCGCGCGCG CCGCGTTTT TGTCTCTCG ATGTGCGCT TCGCGCGCG CCGCGCGCG GCGCGCGCG GCGCGCGCG GCGCGCGCG GCGCGCGCG
3411 GTGAGCTGG TTTAGACCG GTGAGACA GTTAGTTTA CCTACTGCG CCGCGCGCG GCGCGCGCG GCGCGAGTA AGAAGAAAG CCGCGCGCG CCGCGCGCG
3521 GCGGTTGCG GCGCGCGCG GCGCGCGCG CCGCGCGCG GCGCGTGGC GTGAGCGCT CTAAGCGCG ACCCGCGCT GCGCGCGCG CCGCGCGCG CCGCGCGCG
3631 GTGCGCGCT CCGGAGCGC CCGCGCGCG GAGACACC CCGCGCGCG CTCTGTAGC GCGGAGAGC CTGGATCGC CTGAGGAGC CCGTGCAG GCGCGCGCG
3741 GCGCGCGCG CCCACTTCT CTGGGCGCG GCGCGCGCG CAGACAGCA GAGCGCGAA AGAGAAGATT GAGGAGTGC AAGGAGTGC CAGCGCGCG
3851 TCGACGCGCA CCGCGCGCG TGCGTTGCG AGAGAGACCG CGTGTGCGC AGGCGCGCG CCGGAGAGCG CCGCGCGCG GCGCGCGCG TCCAGACAG
3961 GAGCGCGCG GCGAGGCGCA GCGCGTCCAG CAGCGCACCG AACGACCGCG ACCTGCGCG ACGGCGCGT GCGAGGCGG GTGAGGAG CCGCGCGCG
4071 GCGCGGAGC CCGCGAAGCA CCGCGGAGC GCGCAGAGCA GCGGATCCCG CCGCGAGTA AACACTCGG TCTCTCTCG GCGTCTCG GCGCGCGCG CCGCGTGA
4181 CCGAGCGCA CCGACAGCAC CCGCGGCTGC CCGAGTGGT CCGAGGCGCG TCGCGCGCG CTCTCGGAC CCGCGCGCG CCGCGCGCG CCGCGCACG AGTGCCTTC
4291 CGTGGCAGC AATCATATAG GTTAGAGAG GACCGTATAT AGCCACCGCT GCTGAGATCC CCGCGAAG CCGTTTGGG GCGTCTCG GCGAGAGCG CCGAGAGCG
4401 CCGGATGCAC AGTCAGCGT CAATTGATC GTTTTGCAGT CAAGGGCGCT CCGCGCGCG GGTGACGGA GAACAGGCG CCGGATCT CCGAGCGCA GCGGAGCGC
4511 GCGACAGCG TCGCACAGG AGGTTTGC GTGATATGCA GTCTGCGAG GTCCCGCGC GCTCCCGCG GACCGCGCG GACCGCGCG CCGCGCGCG GCGCGCGCG
4621 AAGGATTTT AAAAAAATG GTGCAAGGG GACTCCAGCG GTGTGTGGT GCGCGCGCG CTGACCGAC TATATAGAG AAGGTTCTT ATCTCGGAG GCGCGCGCG
4731 TTGAGGGGG GCGAGGCGCG CCAAGGAAAT CCAAAAAAGT GTGGTGCAG GCGAGCGCG GCGCGCGCG TCGCGCGCG ACTATAGGG GCGCGCGCG
4841 CCGGCAATG GCGAGGAGG GCGCGCGCG GTGCGAGCG GCGCGCGCG CCGAGGAGG CCGCGCGCG GCGCGCGCG CCGCGCGCG TCGGTCGAT CTTGCGGAG
4951 CCGGAGCGT TCGCAAGCA CGAAGCATG CATGCCGCT CCGCGCGCG GCGCGGAGC GCTCAGGACA CCGGTTGCG CCGCGCGCG GCGCGCGCG GCGCGCGCG
5061 CGTGGCAAC CCGCGCGCG GAGGTGCGC CAAGGCGCG GCGCGCGCG GAGGAGCG GAGGAGCG CCGCGCGCG GCGCGCGCG GCGCGCGCG GCGCGCGCG
5171 GCGCGCGCG GCGGAGCGC CGAGCGCTG CCGAAGATCA GCGTTCGAT CCGGAGAGCG GCGCGCGCG ACGCGCGCG CATCCAAAG GCGAGAGG CCGCGAGCT
5281 CCGCAATGC CCGCGCGCG GCGAGGAGG GCGAGCGCG GCGCGCGCG GCGCGCGCG GCGCGCGCG GCGCGCGCG CAAAGTCTG TCGCAGCAG CCGCGAATT
5391 CCGCTCGCG GAGCGTCCG CCGCGTCTG GAGTTGAAA CCGCGTGTG TCGCGCGCG CCGCGAGAG GAAAGCGAG CCGTCCAGC AGCGCGGTG GACCGCGCG
5501 GTGGACCGC GCGCGCGCG GCGCGCGCG GCGAGCGCG AGGAGAGCG GCGCGCGCG CCGCGCGCG CCGTCCAGC AGCGCGGTG GACCGCGCG

Fig. 17: The complete nucleotide sequence of the *G. intestinalis* rRNA gene repeat (Uproft et al, 1990).

**CHAPTER 5: CONSTRUCTION OF A GENE LIBRARY FROM
GIARDIA INTESTINALIS STRAIN NP 77**

5.1 INTRODUCTION

Restriction enzyme digests of total genomic DNA from ten strains of *G. intestinalis* resulted in the generation of many fragments so the resolution of individual bands was poor. Enhanced resolution of bands and hence differentiation of strains was achieved using the GC-rich DNA fraction. This approach, however, is extremely labourious and requires a large amount of DNA. To extend this technique and reveal more subtle differences, Southern blots of digests of total genomic DNA and hybridisation with selected probes may be used to identify and to visualize a distinct set of restricted fragments. This, however, clearly requires the availability and selection of appropriate probes, the development of which would be greatly facilitated by the availability of a gene library.

This chapter is concerned with the production and evaluation of a gene library from *G. intestinalis* strain NP 77.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of high yield trophozoite cultures

Materials

- 1) TY1-S33 medium preincubated to 37°C - see appendix
- 2) Phosphate Buffered Saline (PBS) - see appendix
- 3) 50 ml Falcon™ centrifuge tubes
- 4) Tris-EDTA (TE) Buffer (pH 7.5) - see appendix
- 5) *G. intestinalis* strain NP 77 trophozoite culture. This culture was isolated from human faeces and obtained from the Department of Microbiology and Genetics, Massey University.

Method

The method described in section 4.2.1 was used.

5.2.2 Extraction of high molecular weight DNA

Several approaches were examined in an attempt to find a method that would produce intact, high molecular weight DNA.

- a) **Ionas 1989 (55a)**

Materials

The materials described in section 4.2.2 were used.

Method

Trophozoites suspensions (as described in section 4.2.1) were either freshly prepared or stored at -70°C until use. Frozen trophozoite preparations were thawed in a waterbath before use. (Note: although trophozoite pellets were resuspended in 1 ml of TE buffer, the volume of TE buffer was increased to 15ml upon subsequent adaptations of this method).

0.1 ml SDS (10%) and 0.1 ml Pronase type XIV (10 mg/ml) were added per 1 ml of trophozoite suspension and incubated at 50°C overnight to lyse the cells and digest their proteins. 0.2 ml RNase was added per 1 ml of lysate and incubated for 60 min to digest the RNA. 5M Sodium perchlorate was then added to give a final concentration of 1 M and the lysate incubated at 50°C for a further 60 min.

Phenol/Chloroform/isoamyl alcohol mixed with one tenth of its volume of STE buffer was used as follows to deproteinate the lysate. The upper (aqueous) layer containing the DNA was removed using a 5 ml sterile autopipette fitted with a sterile tip, the point of which was removed, placed into another centrifuge tube and re-extracted twice more with the phenol/chloroform/isoamyl alcohol solution or until the white precipitate was no longer visible at the interface. The DNA was then placed into dialysis tubing and dialysed at 4°C against 4L (total of 16L) of TE buffer with three changes over a 48 hour period.

The DNA was concentrated by adding 1/10th of the volume of 3M sodium acetate, mixed gently, followed by 2.5 volumes of cold (-20°C) 95% ethanol. After gentle mixing this was placed at -20°C for at least 2h or 4°C overnight. The DNA was pelleted the next day following centrifugation at 15000g for 30 min in an SS34 rotor at 4°C . The supernatant was discarded and an equal volume of 70% ethanol was added to wash the pellet. The DNA was centrifuged again at 15000g for a further 30 min. After discarding the

supernatant, the DNA pellet was dried under vacuum for 20 min in a dessicator and finally resuspended in 500 μ l of TE buffer. The DNA was then transferred to an eppendorf tube and stored at 4°C.

b) Nash *et al*, 1985 (89)

Materials

1) Extraction buffer

50 mM Tris-HCl (pH 8.0)

100 mM NaCl

50 mM EDTA

This solution was autoclaved and stored at room temperature.

2) Extraction buffer + 1% SDS

Extraction Buffer	100	ml
-------------------	-----	----

SDS	1.0	g
-----	-----	---

This solution was stored at room temperature.

3) Proteinase K 20 mg/ml

Proteinase K	20	mg
--------------	----	----

Sterile Water	1	ml
---------------	---	----

This was stored as 50 μ l aliquots, each containing 1 mg, at -20°C.

4) Ethidium bromide 10 mg/ml - see appendix.

5) TE Dialysis buffer (pH 7.5)

50 mM Tris-HCl

10 mM EDTA

This solution was autoclaved and stored at room temperature.

6) 3M Sodium acetate - see appendix.

7) 95% ethanol - see appendix.

8) 70% ethanol - see appendix.

9) TE buffer - see appendix.

Method

Pelleted preparations of trophozoites (as described in section 4.2.1), frozen in 0.5-1 ml of TE buffer, were thawed into an equal volume of extraction buffer. An equal volume of extraction buffer with 1% SDS was then added followed immediately by 1 mg of proteinase K. The mixture was incubated at 37°C for 2 hr, and extraction buffer was added to the lysate to a total volume of 16 ml. This was mixed with 23 ml of extraction buffer saturated with caesium chloride and 1 ml of ethidium bromide and was centrifuged for 18 hr at 216 000g in a Beckman VTI 50 rotor. A single band of DNA was removed by aspiration and the ethidium bromide extracted by partitioning five times with isopropanol.

The CsCl was removed by dialysis against TE buffer (3 hr with three changes of dialysate).

The DNA was precipitated with 2.5 volumes of 95% ethanol after the solution was first adjusted to 0.3M sodium acetate and left at -20°C overnight. The precipitate was recovered by centrifugation at 17 300g for 30 min at 4°C and was washed with 70% ethanol at room temperature. The precipitate was dried under vacuum for 20 min, resuspended in 250µl of TE buffer, and stored in an eppendorf tube at 4°C.

c) Strauss, 1990 (110)

Materials

1) Digestion buffer

5M NaCl	0.20	ml
1M Tris-HCl (pH8)	0.10	ml
0.2M Tris-EDTA	1.25	ml
Distilled Water to	10.00	ml

After autoclaving, the following were added immediately before use:

10% SDS (see 5.2.2 (a))	0.6	ml
Proteinase K (see 5.2.2 (b))	31.0	mg

2) Phenol/Chloroform/Isoamyl alcohol - see appendix.

3) STE Buffer - see appendix.

4) 4M Lithium chloride

Lithium Chloride (LiCl)	3.39	g
Distilled water to	200	ml

This was filter sterilized and stored at room temperature.

5) 95% ethanol - see appendix.

6) 70% ethanol - see appendix.

Method

Pelleted preparations (as described in section 4.2.1), were resuspended in 5 ml of digestion buffer and incubated, with shaking, in a 50°C water bath

overnight in tightly capped tubes to lyse cells and digest their proteins.

An equal volume of phenol/chloroform/isoamyl alcohol, with one tenth of its volume of STE buffer bubbled through, was added to the lysate to extract the nucleic acids and the samples centrifuged for 10 min at 1700g in the Heraeus Christ® centrifuge. The samples were thoroughly extracted three times in this way or until there was no white material remaining at the interphase. The top (aqueous) layer containing the DNA was removed to a new tube and then dialysed for 48 hr against TE buffer (four changes of 4 L) at 4°C.

After dialysis, the DNA was precipitated by adding one tenth of the volume of 4 M LiCl, mixed and 2.5 volumes of 95% ethanol. This was left at -20°C overnight and then centrifuged for 30 min at 17500g (4°C) the following day. The precipitate was washed with 70% ethanol, dried for 20 min under vacuum and resuspended in 150µl of TE buffer. The DNA solution was shaken at 65°C for several hours to facilitate solubilization and stored at 4°C until required.

d) Sambrook, Fritsch and Maniatis, 1989 (101)

Method

This method was based on that of Blin and Stafford (14).

Materials

1) Lysis buffer

0.5 M EDTA (pH 8.0)

100 µg/ml Proteinase K

0.5% sarcosyl

This was freshly prepared before use.

2) Phenol/Chloroform/Isoamyl Alcohol - see appendix.

3) STE buffer - see appendix.

4) Dialysis buffer

50 mM Tris-HCl (pH 8.0)

10 mM EDTA

10 mM NaCl

Distilled water to 4L

This solution was prepared freshly as required.

5) 3M Sodium acetate - see appendix.

6) 95% ethanol - see appendix.

7) 70% ethanol - see appendix.

8) TE buffer - see appendix

Method

Pelleted preparations of trophozoites (as described in section 4.2.2) were resuspended in 1 ml of ice-cold TE buffer. 10 volumes of lysis buffer were added and the suspension of lysed cells incubated at 50°C for 3 hr in a shaking waterbath.

The DNA was gently extracted three times with an equal volume of phenol/chloroform/isoamyl alcohol (see section 4.2.2) and centrifuged for 10 min at 2500g. If necessary, the interface was re-extracted with additional phenol solution and TE buffer ("backwashing"). The two top (aqueous) phases were pooled. The sample was dialysed against 4L of dialysis buffer (3

changes over 48 hr) at 4°C.

After dialysis, the DNA was precipitated (as described in sections 5.2.2 (a) and (b) and resuspended in TE buffer to a final volume of 150 µl. This was stored at 4°C.

e) **van Keulen, 1992** (personal communication)

Materials

1) Lysis solution

50 mM Tris-HCl (pH 8.0)

0.1 M EDTA

0.1 M NaCl

Distilled water to 10.0 ml

After autoclaving, the following were added to this solution immediately prior to use:

10.0% sarcosyl	0.1 ml
----------------	--------

Proteinase K	1.0 mg
--------------	--------

2) 10% Sarcosyl

Sarcosyl	1.0 g
----------	-------

Distilled Water to	10.0 ml
--------------------	---------

This was stored at room temperature without autoclaving.

3) Proteinase K (20 mg/ml)

Proteinase K	20 mg
--------------	-------

Sterile Water	1 ml
---------------	------

This was stored as 50 µl aliquots (containing 1 mg) at 20°C.

- 4) 3M Sodium acetate - see appendix.
- 5) Isopropanol
- 6) Sterile Water
- 7) Phenol/Chloroform/isoamyl alcohol - see appendix.
- 8) STE buffer - see appendix.
- 9) 95% ethanol - see appendix.
- 10) 70% ethanol - see appendix.
- 11) TE buffer - see appendix.
- 12) 2% Cetyltrimethyl ammonium bromide (CTAB)

CTAB (Sigma)	2.0	g
Distilled Water to	200	ml

This was stored at room temperature without autoclaving.

- 13) High salt buffer

2.4 M NaCl
10 mM EDTA

- 14) Chloroform

Method

Fresh preparations of pelleted trophozoites (see section 4.2.2) were resuspended in 5 ml of lysis solution and incubated for 30-45 min at 65°C.

An equal volume of phenol/chloroform/isoamyl alcohol, with one tenth of its volume of STE buffer bubbled through, was added to the lysate followed by centrifugation for 10 min at 2500g. The top (aqueous) layer was removed and the DNA precipitated by the addition of one tenth of the volume of 3M sodium acetate, gently mixed, and 0.6 volumes of isopropanol. Most of the nucleic acids were then pelleted by centrifugation for 10 min at 2500g. Any nucleic acids that failed to pellet were collected by their attachment to a sterile glass rod, removed as a spool of DNA and added to the DNA pellet.

The DNA pellet was resuspended in 600 μ l of sterile water using an autopipette with a sterile tip, the end of which had been removed, and divided over 6 eppendorf tubes (100 μ l in each). An equal volume of phenol/chloroform/isoamyl alcohol, with one tenth of its volume of STE buffer bubbled through, was added to each tube, gently mixed, and centrifuged for 2 min in a microfuge. This step was repeated, after which the top (aqueous) layer was removed and the DNA precipitated by the addition of one tenth of the total volume of 3M Sodium acetate and 2.5 volumes of 95% ethanol and left at -20°C for 2 hr. The DNA was pelleted by centrifugation in a microfuge for 15 min at 4°C. The DNA pellet was washed in 70% ethanol and recentrifuged for 15 min in a microfuge at room temperature.

Each DNA pellet was resuspended in 700 μ l of sterile water and 500 μ l of 2% CTAB solution added. This was left at room temperature for 10 min and centrifuged for 10 min in a microfuge. 200 μ l of high salt buffer was added to each tube and the pellet resuspended with an autopipette fitted with a sterile tip, the end of which had been removed and left overnight to facilitate suspension (as high molecular weight DNA does not easily resuspend in salt solutions).

When the DNA was in solution, or close to it, the solution was diluted 1:1 with sterile water and an equal volume of chloroform added. This was

centrifuged for 5 min in a microfuge and the top (aqueous) layer, containing DNA, removed. The aqueous layer was twice extracted with phenol/chloroform/isoamyl alcohol followed by two ethanol precipitations. (Note that these techniques have been previously described in this method). The DNA was finally resuspended in TE buffer to a total volume of 300 μ l.

Note: If the amount of cells was small ($< 10^7$) the CTAB precipitation step was omitted as most of the carbohydrates were removed by isopropanol precipitation.

5.2.3 Examination of the state of the DNA

The DNA extracted from all five methods previously described (5.2.ii a-e) was examined by running 5 μ l samples of DNA (with 5 μ l bromophenol blue dye) through a 0.4% agarose gel at 50V for 18-24 hr at 4°C (101). BRL high molecular weight markersTM were run in each outer lane to check that the DNA was intact and undegraded (i.e. DNA size \gg 25 kb).

5.2.4 Determination of DNA concentration and purity.

A. Spectrophometric Method

As described in section 4.2.5. However, the presence of RNA gave an overestimation of the amount of DNA present so DNA concentration was determined using a combination of concentration standards and fluorometric methods.

B. Concentration Standards

5 μ l of standard amounts of phage Lambda DNA were run through a 0.4% agarose gel (101) at 50V overnight, alongside 10 μ l, 5 μ l and 1 μ l samples of

G.intestinalis NP 77 DNA, to give final concentrations of 5.0 ng, 10.0 ng, 20 ng, 50 ng, 100 ng and 200 ng of Lambda DNA respectively. The concentration of the sample DNA was calculated by comparison of intensity, after staining with ethidium bromide, to the standards.

C. Fluorometric Method

Materials

1) 10x TNE Buffer

Trizma base	12.1	g
EDTA	3.70	g
NaCl	58.4	g
Distilled water	800	ml
HCl conc to pH 7.4		
Distilled water to	1000	ml

This solution was filtered and stored at 4°C.

2) Hoescht stock solution (1 mg/ml)

Hoescht 33258	10.0	mg
Distilled water	10.0	ml

This was stored at 4°C protected from the light. The stock was good for at least 6 months.

3) Calf thymus DNA stock solution (1 mg/ml in 10 mM Tris-HCl; 50 mM EDTA, pH 8). This was prepared by the supplier and stored at 4°C.

4) Working dye solution B (for measuring DNA samples between 100 ng/ml and 2000 ng/ml final concentration).

Hoescht stock solution	100.0	µl
------------------------	-------	----

10x TNE	10.0 ml
Milli-Q water	90.0 ml

This solution was prepared fresh daily and stored at room temperature.

Method

The TKO 100 mini fluorometer™ (Hoefer Scientific instruments) is designed to detect relative fluorescence at 460 nm, making it ideal for DNA assays based on the binding of bis benzimidazole (Hoescht 33258).

The fluorescence enhancement of Hoescht 33258 dye is highly specific for DNA, binding preferentially to A-T rich regions and binds twice as well to dsDNA as to ssDNA. Consequently, the presence of RNA in the sample does not interfere with the quantitation of DNA as RNA enhancement is usually well below 1% of that produced by the same concentration by weight of DNA. This makes the fluorometric method ideal for estimating the DNA content of *G.intestinalis* samples containing RNA.

The TKO 100 was turned on at least 15 min before use. Calf thymus DNA was used as the reference standard as it is approximately 40% G-C and 60% A-T in nucleotide content which is comparable with *Giardia* DNA. The 10x TNE Buffer stock solution was diluted to give a 1x TNE working solution containing Hoescht 33258 dye (working dye solution B). The TKO 105 glass fluorometry cuvette was filled with 2ml of working dye solution B. The sides of the cuvette were wiped clean and the cuvette placed in the sample chamber - always in the same orientation ("G" imprint to the front).

The machine was adjusted to zero with the scale knob at 50% sensitivity (i.e. 5 clockwise turns of the knob from the fully counter clockwise position). 2 µl of calf thymus DNA (1 mg/ml) was delivered into the 2ml of dye solution in the cuvette and mixed by pipetting in and out without introducing bubbles

into the solution. The cuvette chamber was closed and the "scale" knob adjusted until the display read "1000" indicating 1000 ng/ml. This was repeated twice more until the reference standard read "1000" reproducibly. The cuvette was emptied and drained thoroughly between samples.

DNA samples of *G.intestinalis* strain Hast 11 were measured in the same manner in units of ng/ml. Each sample was blanked by adjusting the 'zero' control knob each time 2ml of working dye solution B was loaded. The 'scale' control knob was not adjusted.

$$\text{e.g. } 2 \mu\text{l DNA added} = 2 \mu\text{l DNA}/2\text{ml} = 1 \mu\text{l DNA/ml.}$$

So if the scale read '250' this means the concentration of DNA in the sample was 250ng/ml for 1 μl of sample therefore the total DNA concentration was 250 $\mu\text{g/ml}$.

5.2.5 Establishing Conditions for partial digestion of high molecular weight DNA.

Materials

1) Sau 3A restriction enzyme (Boehringer Mannheim) with volume activity of 5 units/ μl .

2) Sau 3A incubation buffer (10x concentrated).

Tris acetate 33 mM/L

Mg-acetate 10 mM/L

K-acetate 66 mM/L

dithiothreitol 0.5 mM/L

pH 7.9 (at 37°C)

(Boehringer Mannheim Incubation buffer A)

This was stored at -20°C in accordance with the manufacturer's instructions.

3) Sterile Water

4) 0.4% agarose gel

TBE buffer (see appendix)	200.0	ml
Agarose (Ultra Pure)	0.8	g

This was heated in a microwave for 5 min on Med-High to melt the agarose and cooled to 50°C before pouring.

5) TBE buffer - see appendix

6) Ethidium bromide (10 mg/ml) - see appendix.

Agarose gels were stained in the following ethidium bromide solution:

Ethidium bromide (10 mg/ml)	25	µl
Water	500	ml

7) BRL-High Molecular Weight Standard™

This was stored at 4°C and used in accordance with the manufacturers instructions.

8) High molecular weight *G. intestinalis* DNA (180 µg/ml)

9) Bromophenol blue dye - see appendix.

10) Reaction buffer

A 9:1 ratio of sterile water to 10x incubation buffer, respectively, was

prepared freshly and stored on ice.

Method

The method described in Sambrook *et al* (110) was used with some modifications.

A mixture containing 10 μg of *G. intestinalis* DNA and 15 μl of restriction enzyme incubation buffer in a final volume of 150 μl was prepared and mixed well by inverting the tube several times. 30 μl of this mixture was dispensed into an eppendorf tube (tube 1). 15 μl was dispensed into tubes labelled 2-8 and the remainder dispensed into tube 9. All tubes were chilled on ice.

2 μl of restriction enzyme (containing 10 units of activity) was diluted to 0.0625 units/ μl by the addition of 158 μl of reaction buffer. 2 μl of this dilution (containing 0.125 units) was added to tube 1 and mixed well. The concentration of the enzyme was thus 0.0625 units/ μg DNA. 15 μl of this reaction mixture was then transferred to tube 2. The enzyme concentration was now diluted to 0.0313 units/ μg DNA. This tube was mixed well and the twofold serial dilution continued through to tube 8 (nothing was added to tube 9).

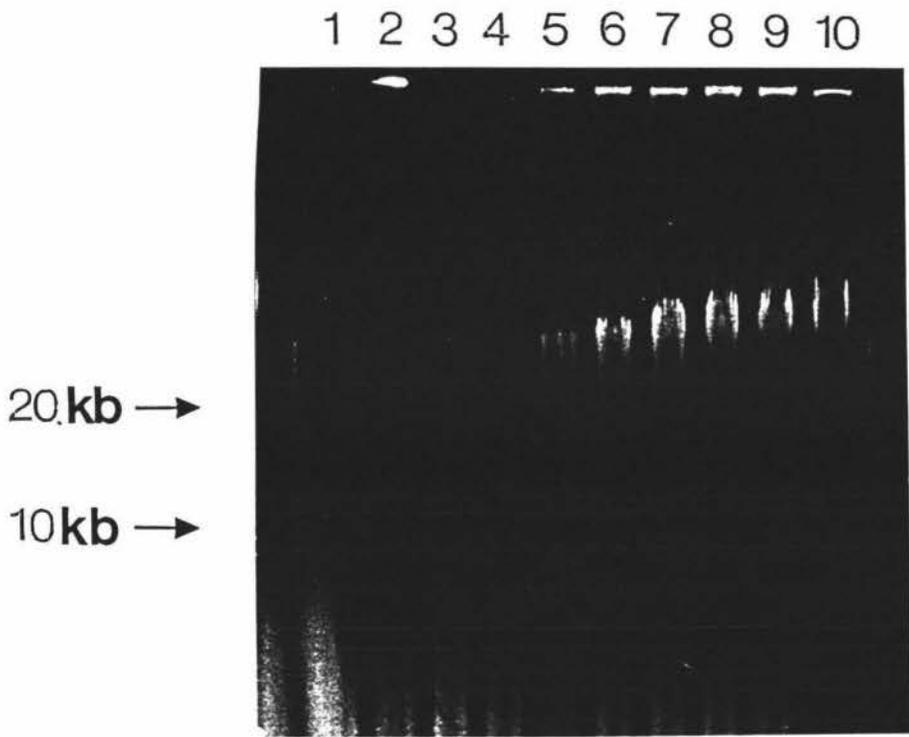
Tubes 1-8 were incubated in a 37°C waterbath for 1 hr. The reaction was stopped by heating the tubes at 65°C for 10 min. 3 μl of bromophenol blue dye was added to each tube (1-9), mixed, and the samples analysed by electrophoresis through a 0.4% agarose gel. BRL High Molecular weight standardsTM, with accurate markers in the 10-48 kb range, were run on the two outside lanes. Electrophoresis was carried out very slowly (50V; 18 hr; 4°C) until the bromophenol blue was just about to migrate off the gel. The gel was stained in ethidium bromide for 10 min, destained for 1 hr, and photographed under shortwave UV light without overexposing the film.

Using the photograph and the markers, the amount of enzyme required to produce the maximum intensity of fluorescence in the 15-20 kb region of the gel was ascertained. This was accomplished by blocking off all the DNA of desired size from that of the undesired size. The various lanes of the gel were then compared to estimate the degree of digestion that produced the maximum amount of DNA of the desired size. As the intensity of fluorescence is related to the mass distribution of the DNA, to obtain the maximum number of molecules in this size range, half the amount of enzyme that produced the maximum amount of fluorescence was used in the large-scale partial digest. This corresponded to enzyme concentrations 3.9×10^{-3} and 9.8×10^{-4} units/ μg DNA (see Fig. 18).

Fig. 18: Pilot-scale partial Sau 3A I digestion of intact genomic DNA from *G. intestinalis* strain NP 77.

Lane 1: 6.25×10^{-2} enzyme units/microgram DNA
Lane 2: 3.13×10^{-2} enzyme units/microgram DNA
Lane 3: 1.56×10^{-2} enzyme units/microgram DNA
Lane 4: 7.80×10^{-3} enzyme units/microgram DNA
Lane 5: 3.90×10^{-3} enzyme units/microgram DNA
Lane 6: 1.95×10^{-3} enzyme units/microgram DNA
Lane 7: 9.80×10^{-4} enzyme units/microgram DNA
Lane 8: 4.90×10^{-4} enzyme units/microgram DNA
Lanes 9 & 10: Undigested genomic DNA

Note that lanes 4, 5 and 6 produced the greatest amount of fluorescence in the 10-20 kb region of the gel hence half of each of these enzyme concentrations were used in the large-scale partial Sau 3A I digestions (Fig. 20).



5.2.6 Large Scale preparation of partially digested DNA.

Materials

The materials used in this section were the same as those used in section 5.2.iii except for the following:

1) RNase (1 mg/ml)

RNase	10 mg
RNase buffer (see appendix)	10 ml

This was preincubated at 100°C for 15 min to destroy any DNases and stored as 1 ml aliquots at -20°C.

2) Phenol/Chloroform/isoamyl alcohol - see appendix.

3) STE buffer - see appendix.

4) 3M sodium acetate - see appendix.

5) 95% ethanol - see appendix.

6) 70% ethanol - see appendix.

7) TE Buffer - see appendix.

Method

Using optimised conditions (see 5.2.5), digestions using 50 µg of high molecular weight, eukaryotic DNA was carried out using identical enzyme concentration, time, temperature and DNA concentration to those used for the pilot reaction (refer to section 5.2.5). The reaction was stopped by heating

the digest at 65°C for 10 min.

An aliquot of the DNA (1µg=15µl of the digest) was analysed by electrophoresis through a 0.4% agarose gel to check that the size distribution of the digestion products was correct. If the size distribution did not correspond to that observed in lanes 6 and 7 (corresponding to 3.9×10^{-3} [tube 5], 1.95×10^{-3} [tube 6] and 9.8×10^{-4} [tube 7] units of enzyme/µg DNA) of the pilot scale digest then the same concentration of enzyme was again added to the 50µg DNA digest and reincubated for 1 hr at 37°C.

After reincubation, a further 1µg aliquot of DNA was analysed by electrophoresis through a 0.4% agarose gel (as described above). When the size distribution of the products from the large-scale digest corresponded to that observed in the pilot-scale digest, 7µl of RNase (1 mg/ml stock) was added to each large scale digest and these were incubated for 15 min at 37°C.

The DNA was gently extracted with 2 volumes of phenol/chloroform/isoamyl alcohol (with one tenth of its volume of STE buffer bubbled through it) and centrifuged for 3 min in a bench microfuge. The top (aqueous) phase was removed and 1 volume of chloroform added. This mixture was pulsed for 2 seconds in the microfuge. The DNA was precipitated following the addition of one tenth of the volume of 3M sodium acetate and 2 volumes of cold 95% ethanol. The DNA was left to precipitate overnight at 4°C.

The next day the DNA was pelleted by centrifuging at 4°C for 10 min in a microfuge. The 95% ethanol was drained off and an equal volume of 70% ethanol added to wash salt and proteins from the pellet. This was centrifuged for 10 min at room temperature in a microfuge. After removing the 70% ethanol, the pellet was dried for 20 min under vacuum and resuspended in 45µl of TE buffer.

A 1µl (1µg) sample of digested DNA electrophoresed through a 0.4% agarose

gel at 4°C overnight to check that there was no RNA and that the DNA remained in the same condition (i.e. 15-20 kb sized fragments) without further shearing.

Note: After examining the gel photo it was decided to proceed only with tubes 5 and 6 (Fig. 19). The DNA concentrations were measured using a fluorometer as follows (refer to section 5.2.iii(c)):

Tube (5): DNA concentration = $300\mu\text{g/ml} = 0.3\mu\text{g/ml}$

Tube (6): DNA concentration = $300\mu\text{g/ml} = 0.3\mu\text{g/ml}$

The DNA was stored at 4°C until further use.

5.2.7 Partial fill-in reaction for genomic DNA.

Size fractionation of genomic DNA is not necessary when using LambdaGEM-11 Xho I Half-Site Arms®(Promega). To construct a gene library using this method, it is only necessary to partially digest the genomic DNA with Sau 3A I and then fill-in the first two nucleotides of this site using Klenow and the incubation buffer supplied by the manufacturer.

The procedure outlined in the Promega Technical Bulletin No. 057 was used in this section (97).

Materials

1) Sterile water

2) Phenol saturated with TE buffer (pH 8.0)

Liquid phenol was mixed with one tenth of its volume of TE buffer and stored at 4°C in the dark.

- 3) TE buffer (pH 8.0) - see appendix
- 4) Chloroform
- 5) 20mM dATP: stored at -20°C
- 6) 20mM dGTP: stored at -20°C
- 7) Klenow: stored at -20°C
- 8) 10x React 4 Incubation Buffer (BRL): stored at -20°C
- 9) 3M sodium acetate - see appendix.
- 10) 95% ethanol - see appendix.

Method

7µg of partially digested DNA underwent partial end-filling in eppendorf tubes as follows:

	TUBE 5	TUBE 6
DNA (7 µg)	24.1 µl	23.3 µl
10x REACT 4	5.0 µl	5.0 µl
20 mM dATP	2.5 µl	2.5 µl
20 mM dGTP	2.5 µl	2.5 µl
KLENOW	4.0 µl	4.0 µl
WATER	11.9 µl	12.7 µl
TOTAL VOLUME	50 µl	50 µl

These tubes were incubated for 30 min at 37°C. An extra 50µl of water was added after incubation to bring the total volume to 100µl.

1 volume of phenol and one volume of chloroform was added and the mixture centrifuged for 5 minutes in a microfuge. The top (aqueous) layer was removed and 50µl of water added to the lower phenol layer and centrifuged for a further 5 min ("backwashing"). This process was repeated.

The aqueous layers were pooled (a total volume of 200µl) and 2 volumes of chloroform added. This was centrifuged for 1 min after which the top (aqueous) layer was removed and 100µl of water added to the lower chloroform layer and centrifuged for an additional 1 min ("backwashing").

The total volume of the pooled aqueous layers was now 300µl. 30µl of 3M Sodium acetate was added to this and mixed carefully. 2.5 volumes of 95% ethanol was added and the mixtures left overnight at -20°C to precipitate the DNA.

The next day, the DNA was pelleted after centrifugation for 10 min at 4°C in a microfuge followed by a 70% ethanol wash for 15 min at room temperature in a bench microfuge. Each pellet was dried, after decanting the 70% ethanol, for 25 min under vacuum, resuspended in 15µl of TE buffer and stored at 4°C until further use.

5.2.8 Ligation of insert to vector arms

Materials

1) Ligase 10x Buffer (New England Biolabs).

This was stored at -20°C

2) Sterile water

3) Lambda GEM-11 Xho I Half Site arms® (0.5µg)

This was stored at -20°C and used according to the manufacturers instructions.

4) Positive control insert (pTI 11 = 16 kb plasmid; Promega).

These were stored at -20°C and used according to the manufacturers instructions.

5) DNA ligase (New England Biolabs; 6.5 Weiss units/µl)

This was stored at -20°C and used according to the manufacturers instructions.

Method

The techniques outlined in Promega Technical Bulletin No. 057 were used (97).

Ligations were performed for DNA from both Tubes 5 and 6 as follows:

Tube A = negative control (arms only)

B = 1:1 (vector:insert ratio)

C = 4:1 (vector:insert ratio)

D = positive control (pTI 11)

Note: The concentration of DNA in tubes 5 and 6 was 0.3 µg/µl.

	A	B	C	D
ARMS (0.5 µg/µl)	2 µl	2 µl	2 µl	2 µl
INSERT (0.3 µg/µl)	-	2 µl	0.5 µl	2 µl
10x LIGASE BUFFER	1 µl	1 µl	1 µl	1 µl
WATER	6 µl	4 µl	5.5 µl	4 µl
DNA LIGASE (NEB)	1 µl	1 µl	1 µl	1 µl
TOTAL VOLUME	10 µl	10 µl	10 µl	10 µl

These reactions were mixed gently and incubated overnight at 4°C.

5.2.9 Small-scale packaging of ligated DNA

Packaging *in vitro* refers to the use of a phage-infected *E. coli* cell extract to supply the mixture of proteins and precursors required for encapsidating Lambda DNA. The following procedure uses Promega's Packagene® *in vitro* packaging system.

Materials

- 1) Packaging extracts (Promega Packagene®)

These were stored at -80°C and used according to the manufacturers instructions.

2) Chloroform

3) SM Buffer

NaCl	5.8 g
Magnesium sulphate	2.0 g
1M Tris (pH 7.5)	50.0 ml
Gelatin	20.0 g
Water to	1000.0 ml

This was autoclaved in 50ml aliquots and stored at room temperature.

4) LB (Luria-Bertani) medium

Bacto-Tryptone	10 g
Bacto-Yeast extract	5 g
NaCl	5 g
5M NaOH to pH 7.5	
Distilled water to	1 L

This was stored at room temperature after autoclaving.

5) LB plates

LB media (pH 7.5)	1 L
Davis agar	15 g

This was autoclaved and cooled to 50°C before plates were poured. Plates could be stored for up to one week at room temperature or 3 weeks at 4°C .

6) 1M Magnesium Sulphate (MgSO₄)

Magnesium sulphate	24.65	g
Distilled Water	100.0	ml

This was stored at room temperature after autoclaving.

7) 20% Maltose

Maltose	20	g
Distilled Water	100	ml

This was stored at room temperature after autoclaving.

8) TB Top Agar

Bacto-Tryptone	10	g
NaCl	5	g
Distilled Water to	1	L

This was divided into 50ml aliquots. 0.4 g of agar was added to each aliquot and these were stored at room temperature after autoclaving.

9) LB + Tet (15µg/ml) plates

LB agar (sterilized and cooled to 50°C)	1.0	L
Tetracycline (10 mg/ml)	1.5	ml

Plates poured could be stored for up to 3 weeks at 4°C.

10) Tetracycline stock solution 10mg/ml

Tetracycline	100	mg
Methanol	10	ml

This solution was stored at -20°C.

Method

The technique outlined in Promega's Technical Bulletin No. 057 was used (97).

a) Packaging of the library

Packagene® extracts were thawed on ice. (Note: the volume of the Packagene® extract is 50µl). 25µl of each extract was removed immediately after thawing and transferred to labelled eppendorf tubes.

2.5µl of each ligation (5A-D and 6A-D) was added to the appropriately labelled tube containing Packagene extract and mixed by gently tapping the bottom of the tube several times. These were incubated at 22°C for 3 hr.

After incubation, 222.5µl of SM buffer was added to each tube (250µg DNA/250µl = 1µg/µl DNA), mixed, and 25µl of chloroform added and mixed by gentle inversion. Packaged phage could be stored at 4°C for seven days with no drop in titre, or up to 3 weeks, although the titre may drop several fold.

b) Titre of the Library

It is necessary to titre the library to find the concentration of viable phage as only phage containing an insert can form plaques when grown on *E.coli* strain KW 251.

Two days before ligation and packaging were performed, a single colony of *E. coli* strain KW 251 bacteria was streaked on to a LB + Tet plate and incubated overnight at 37°C. The next day a single colony from this freshly streaked plate was used to inoculate 50ml of LB medium supplemented with 0.2% maltose and 10mM MgSO₄. This was shaken overnight at 37°C and

stored immediately on ice the following day.

10^{-2} , 10^{-3} and 10^{-4} dilutions of each tube of packaged phage (refer to (a) of this section) were made in SM buffer. 100 μ l of the diluted phage was added to 100 μ l of the overnight KW 251 culture and the phage allowed to absorb to the cells by incubation at 37°C for 30 min.

1ml of 1M MgSO₄ was added to 100 ml of TB top agar that had been melted and cooled to 45°C. 3ml of molten (45°C) TB top agar was added to each tube, mixed by quickly vortexing and immediately poured onto LB plates prewarmed to 37°C. The top agar was allowed to harden and the inverted plates incubated at 37°C overnight.

The next day the number of plaques was counted and the titre of the phage calculated using the following formula:

$$\frac{(\text{number of plaques}) \times (\text{dilution factor})}{(\text{volume plated} = 0.1 \text{ ml})} = \text{pfu/ml}$$

The packaging efficiency of the arms was also calculated as follows:

$$\frac{(\text{pfu/ml})}{(\text{concentration of vector DNA packaged})} = \text{recombinants}/\mu\text{g } \lambda \text{ DNA}$$

Titres from the various plates were compared to determine the optimal ratios of vector arms and genomic DNA insert for ligation.

The expected packaging efficiency for the LambdaGEM-11 Half-Sites Arms® positive control was 2×10^6 - 2×10^7 pfu/ μ g and that of the negative control

(background nonrecombinant Half-site Arms) was ≤ 100 pfu/ μg .

5.2.10 Large Scale Packaging of Ligated DNA

Materials

The same materials as those described in section 5.2.9 (trial packaging) were used.

Method

The same method was used as described in section 5.2.9.

The optimal ratios of vector arms and genomic DNA insert for ligation were found to be those of ligations 6C and 6D (refer to section 5.2.8). 2.5 μl of ligated DNA from tubes 6C and 6D were added to each of 25 μl of Packagene Extract® (as a scaled-up reaction). These reactions were named 6C1 and 6D1 respectively. 5 μl of ligated DNA from tube 6D was added to 50 μl of Packagene Extract as a larger reaction and was named 6D2. Reactions were mixed by gently tapping the bottom of the tube several times. Conditions and times of incubation were identical to those outlined in section 5.2.9. After incubation 222.5 μl of SM buffer was added to each of packaging reactions 6C and 6D1. 445 μl of SM buffer was added to packaging reaction 6D2. 25 μl of chloroform was then added to all of the tubes to prevent bacterial growth during storage. The packaged phage were stored at 4°C.

Titration of each of the packaged phage reactions were performed by adding 100 μl of diluted phage (10^{-2} , 10^{-3} and 10^{-4} dilutions in SM buffer) to 100 μl of an overnight culture of *E. coli* strain KW251 cells(see section 5.2.9 b) and the phage allowed to absorb to cells by incubation at 37°C for 30 min.

1 ml of 1M MgSO_4 was added to 100 ml of TB top agar that had been melted and cooled to 45°C. 3 ml of molten top agar (45°) was added to each

tube, mixed by quickly vortexing and immediately poured on to LB plates prewarmed to 37°C. The top agar was allowed to harden and the inverted plates were incubated at 37°C overnight. The next day the number of plaques were counted and the titre of phage and packaging efficiency of the arms were calculated as described in section 5.2.9.

5.2.11 Amplification of the Library

Materials

- 1) SM Buffer - see section 5.2.9.
- 2) 1M Tris-HCl - see appendix.
- 3) LB (Luria-Bertani) plates - see section 5.2.9.
- 4) TB Top Agar - see section 5.2.9.
- 5) 1M MgSO₄ - see section 5.2.9.
- 6) 20% Maltose - see section 5.2.9.
- 7) LB (Luria Bertani) medium - see section 5.2.9.
- 8) LB + Tetracycline (TC) (15µ/ml) plates - see section 5.2.9.
- 9) Tetracycline (Tc) 10 mg/ml stock solution - see section 5.2.9.

Method

The library of recombinant phage was amplified by growing a plate stock

directly from the packaging mixture as described by Sambrook *et al* (101).

An overnight culture of *E. coli* strain KW251 was prepared by inoculating a single colony growing on a LB + Tet plate into 50ml of LB medium supplemented with 0.5ml 20% Maltose and 0.5ml 1M MgSO₄.7H₂O and the flask incubated, with shaking, overnight at 37°C. The next morning the culture was removed and stored on ice until use.

Appropriate dilutions of the library from packaging mixtures 6Cl and 6D2 were made in SM buffer to give confluent lysis (approximately 10⁵ pfu when plated). 100µl of phage dilution was added to 100µl of *E. coli* KW251 and incubated at 37°C for 30 min. 5 tubes were prepared from each packaging mixture. 3ml molten top agar was added, the mixture briefly vortexed, and immediately poured on to LB plates prewarmed to 37°C. The plates were inverted and incubated for 6 hr at 37°C.

Plaques were not allowed to grow so large that they touched. This short period of growth minimized the chances for infection of bacteria with two different recombinants, thereby reducing the possibility of recombination between repetitive sequences carried by different recombinants with consequent "scrambling" of the library. It also minimized the opportunity for changes in the bacteriophage population that may occur because of differences in the rate of growth of different recombinants.

5ml of SM buffer was added to each plate and the plates were incubated overnight at 4°C. The bacteriophage suspension was removed from each plate with a sterile pasteur pipette and transferred to a sterile SS34 tube. Each plate was rinsed with a further 1ml of SM buffer and the suspensions pooled. Chloroform was added to the phage suspensions to give a final concentration of 1%. Cellular and agar debris were removed by centrifugation at 4000g for 4 min. The supernatant containing the phage was removed, chloroform added to a final concentration of 0.3%, and stored in a glass bottle at 4°C.

The amplified library was titred as described in section 5.2.9. Further steps involving concentration of phage were omitted as the titre of the phage was very high (10^{10} pfu/ml).

5.2.12 Storage of the Library

Materials

- 1) Dimethyl sulphoxide (DMSO)
- 2) 1ml sterile Nunc™ cryopreservation tubes

Method

The amplified and original packaged libraries were stored as 0.5ml aliquots in 1ml Nunc™ tubes with DMSO added to a final concentration of 7%. Tubes were wrapped in paper towels and placed in polystyrene container so that they froze slowly overnight at -80°C . The following day the tubes were removed to a labelled freezer box and stored at -80°C until required.

5.2.13 Demonstration of the representation of the library.

Material

- 1) LB-agarose plates
- | | | |
|---------------------|------|---|
| Bacto-tryptone | 10.0 | g |
| Bacto-yeast extract | 5.0 | g |
| NaCl | 5.0 | g |
| Agarose | 15.0 | g |
| NaOH to pH 7.5 | | |

Distilled water to 1.0 L

This was autoclaved and cooled to 45°C before plates were poured. Plates were stored inverted at room temperature for one week or 4°C for one month.

2) LB Agar - see section 5.2.9

3) 20% Maltose - see section 5.2.9

4) 1M MgSO₄.7H₂O - see section 5.2.9

5) TB top Agarose

Bacto-tryptone	10.0	g
NaCl	5.0	g
Agarose	0.8	g
Distilled water to	100.0	ml

This was autoclaved in 50ml aliquots and stored at room temperature. The agarose was melted in a microwave before use and cooled to 45°C before adding 0.5ml of 1M MgSO₄.

6) SM buffer - see section 5.2.9

7) Phage buffer 200ml

20 mM Tris-HCl (pH 7.5)
100mM NaCl
10mM MgSO₄.7H₂O
Distilled water to 200ml

This solution was autoclaved and stored at room temperature.

8) RNase 1mg/ml

RNase A	10 mg
RNase buffer (see appendix)	10 ml

This was preincubated at 100°C for 15 min and stored as 1 ml aliquots at -20°C.

9) DNase (1 mg/ml)

1mg/ml stock solution of DNase I in 0.15M NaCl and 50% glycerol. This was divided into small aliquots and stored at -20°C.

10) 3M Sodium acetate - see appendix.

11) 95% ethanol - see appendix.

12) 70% ethanol - see appendix.

13) TE buffer - refer to appendix.

14) Restriction enzyme Hind III and its 10X reaction buffer were used according to the manufacturers instructions and stored at -20°C.

15) Polyethylene glycol (PEG) solution

PEG 8000	80.0 g
NaCl	46.8 g
SM buffer	400.0 ml

This was autoclaved and stored at room temperature until use.

16) 10% Sodium dodecyl sulphate (SDS)

SDS	10.0 g
Distilled Water	100.0 ml

This was stored at room temperature without autoclaving.

17) 0.25 M EDTA

EDTA	0.93	g
Distilled Water to	10.00	ml

This was autoclaved and stored at room temperature.

18) TE-saturated phenol

10 ml of TE buffer was added to 100 ml of liquid phenol to neutralise/reduce the acidity of the phenol. This was stored at 4°C in the dark until use.

19) Chloroform

This was stored at room temperature in the dark.

20) TBE buffer - see appendix.

21) Bromophenol blue dye - see appendix.

22) 0.7% Agarose

Agarose (Ultra Pure)	2.1	g
TBE buffer	300.0	ml

The agarose was melted by heating in a microwave for 5-6 min on med-high and cooled to 50°C before pouring the gel.

23) Ethidium bromide (10 mg/ml) - see appendix.

Method

A combination of the methods outlined by Current Protocols (29) and Sambrook *et al* (101), with modifications, was used.

An overnight culture of *E. coli* KW251 in LB medium supplemented with 2% maltose and 10mM MgSO₄ was prepared (see section 5.2.9 (b)).

The phage library was diluted in SM buffer to give 10 PFU (Plaque Forming Units) when plated. 100µl of phage dilution was added to 100µl of KW251 cells and incubated at 37°C for 30 min. This was prepared in duplicate. 3ml of molten agarose (45°C) was added to each tube, quickly vortexed, and poured on to prewarmed (37°C) LB agarose plates. Agarose was used in both the LB plates and top agar as agar contains inhibitors of restriction endonucleases which may affect results. The plates were inverted and incubated at 37°C for 6 hours.

12 individual, well separated plaques were picked from each plate using sterile autopipette tips, the ends of which had been removed, and each plaque placed in an eppendorf tube containing 800µl of SM buffer and 50µl of chloroform. These were left overnight at 4°C for the phage to diffuse out of the agar into the SM buffer. An overnight culture of KW251 cells was also prepared (as described above).

The following day 50µl of each phage suspension was diluted with 50µl of SM buffer. 100µl of diluted phage was added to 100µl of an overnight culture of KW251 cells and incubated at 37°C for 30 min. 3 ml of molten Top agarose (45°C) was added to each tube. The tubes were vortexed, and the suspension poured on to LB agarose plates prewarmed to 37°C. Plates were incubated at 37°C for 6 hours. 5 ml of SM buffer was added to each plate and these were incubated overnight at 4°C.

The following day, the lysate was removed from each plate and centrifuged at 5K for 10 min at 4°C to pellet cellular debris. The supernatant containing the phage, was decanted into a 40ml centrifuge tube. RNase and DNase were added to give a final concentration of 1µg/ml, respectively, and incubated at 37°C for 30 min. 5 ml of PEG solution was added to each phage suspension, mixed well and the tubes were incubated on ice for one hour. The phage suspension was centrifuged at 5K for 30 min at 4°C to pellet the phage, the supernatant discarded and the tube drained. Each phage pellet was resuspended in 0.5ml of SM buffer and transferred to an eppendorf tube.

5 µl of 10% SDS and 10µl of 0.25M EDTA was added to each tube and incubated for 15 min at 68°C. The tubes were vortexed briefly and an equal volume of phenol (TE saturated) was added. The tubes were vortexed for 10 sec, left standing for 5 min for the phases to separate, vortexed for a further 10 sec and left standing for a further 5 min. The top (aqueous) layer containing the phage DNA was removed to a fresh eppendorf tube. 500µl of TE-saturated phenol and 500 µl of chloroform were added to each fresh tube, vortexed for 10 min, and left standing for 5 min. The top layer, containing the DNA, was removed to a fresh eppendorf tube where an equal volume of chloroform was added and the tube centrifuged for 5 min. The top layer was removed and the DNA ethanol precipitated by the addition of one tenth of the volume of sodium acetate, the solution mixed well, and 2 volumes of cold 95% ethanol. The tubes were left overnight at -20°C to precipitate the DNA.

The next day the DNA was pelleted following centrifugation for 15 min at 4°C in an Eppendorf machine. The supernatant was discarded and the pellet washed with 1 ml of 70% ethanol by centrifugation at room temperature for 10 min in an Eppendorf machine. The ethanol was discarded and the pellet dried under vacuum. Each pellet was resuspended in 50µl of TE buffer and the DNA concentration of each was determined with a fluorometer (refer to section 5.2.3 (c)).

1 µg of DNA from each of the 12 single recombinant clones was digested with Hind III, following the manufacturers instructions, in a total volume of 100 µl and incubated at 37°C for 2-3 hours. Complete digestion of the DNA from each clone was tested by running a 15µl sample of each digest with 3µl of bromophenol blue dye on a 0.8% agarose gel at 100V for 2 hours. The gel was stained for 10 min with ethidium bromide, destained for 1 hour and photographed under shortwave UV light.

A further 1.5 units of Hind III was added to each tube where the DNA of the clone appeared to be undigested or only partially digested and the digests were reincubated at 37°C for a further 2-3 hours.

The remainder of each digest was ethanol precipitated following the addition of one tenth of the volume of 3M sodium acetate and 2.5 volumes of cold (95%) ethanol and incubation at -20°C for 2 hours. The DNA was pelleted following centrifugation for 15 min at 4°C in an eppendorf machine and the pellet washed with 70% ethanol at room temperature (see section 5.2.ii). After drying each DNA pellet in the Speedvac™, the DNA digests from each clone were resuspended in 30 µl of sterile water and 5 µl of bromophenol blue dye. The fragments produced from digestion of the DNA from each clone were separated by agarose gel electrophoresis (0.7% agarose; 30 V at room temperature overnight).

5.2.14 Determination of the size of inserts

Materials

- 1) Lambda DNA digested with Hind III (molecular standard).

Method

The method described in section 4.2.8 was used.

5.3 RESULTS

Several methods were used to try to produce intact, high molecular weight DNA from *G. intestinalis* trophozoites (55a, 89, 101, 110). All the methods originally examined produced DNA that was sheared, and hence could not be used for the construction of a genomic library. A typical result is illustrated in Fig. 19. Subsequently, the method of van Keulen (personal communication) was used and produced intact, high molecular weight (> 55 kb) DNA in sufficient quantities for library construction (Fig. 19).

Intact, DNA from *G. intestinalis* strain NP 77 was partially digested with Sau 3AI to produce fragment sizes of 10-20 kb (Fig. 20). These were then end-filled and ligated into the DNA of a Lambda vector. DNA was packaged *in vitro* and the resulting phage was titred by plating on *E. coli* strain KW251. Under these conditions, only phage containing insert DNA were able to form plaques. Preliminary packaging experiments were conducted on a small-scale with positive and negative controls to confirm that the expected result was achieved (Table 3). Identical conditions were used in a large-scale reaction (Table 4) but as the results of both negative and positive controls were satisfactory in the small-scale reaction it was considered wasteful to repeat them. The resulting library was amplified, titred (Table 4) and stored at -80°C until further use. 12 single plaques were selected and each clone was amplified and its DNA extracted. The DNA from each was digested with Hind III. The results are shown (Fig. 21). Although the clones were digested with a number of different enzymes known to recognise sites in the arms of the vector, only partial digestion of each clone was achieved. Digests of twelve new clones produced similar results. Nevertheless, the average insert size could still be calculated (Table 5).

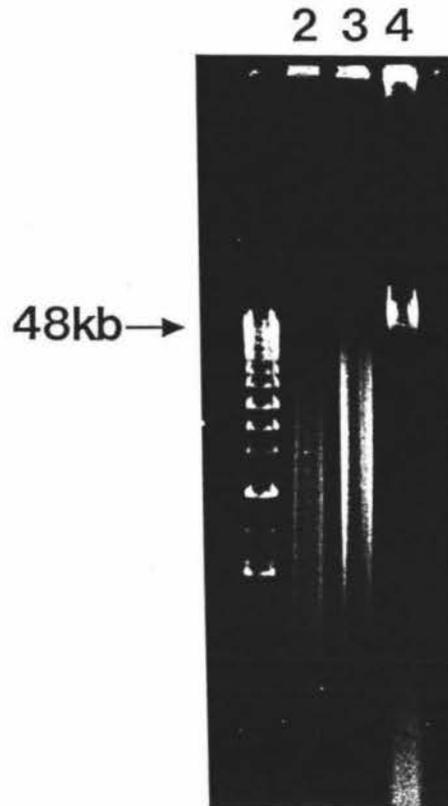


Fig. 19: Total genomic DNA extracted from *G. intestinalis* trophozoites using the methods of Ionas (lane 2), Strauss (lane 3), and van Keulen (lane 4). Lane 1 contains the High Molecular Weight DNA markers (BRL).

Note that only the method of van Keulen (lane 5) produced intact genomic DNA with a molecular weight of greater than 48 kb.

Fig. 20: Large-scale partial Sau 3A.I digestion of intact genomic DNA from *G. intestinalis* strain NP 77.

Lane 3: 3.90×10^{-3} enzyme units/microgram DNA

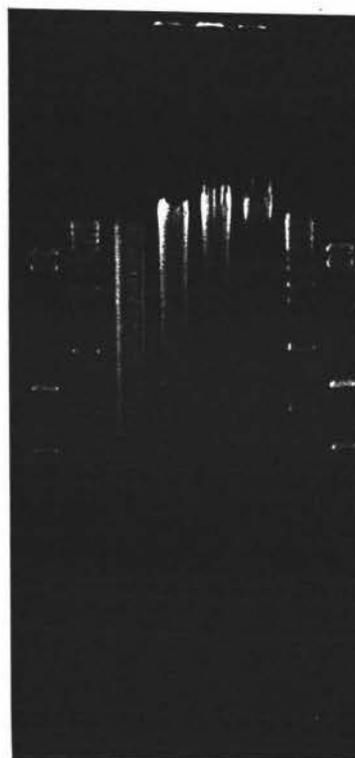
Lane 4: 1.95×10^{-3} enzyme units/microgram DNA

Lane 5: 9.80×10^{-4} enzyme units/microgram DNA

Lane 6: Intact genomic DNA (undigested control)

Note that the DNA represented in lanes 3 and 4 contained the highest number of 10-20 kb fragments, hence these digests were used in the ligation reactions and were referred to as 5 and 6, respectively.

3 4 5 6



← 20kb

← 10 kb

TABLE 3: Titre of the small-scale library.

TUBE NUMBER	PHAGE TITRE (PFU/ml)	NUMBER OF RECOMBINANTS/ $\mu\text{g } \lambda$ DNA
A = Negative control	0	0
E = Positive control	9.16×10^7	9.16×10^7
5C	1.89×10^5	1.89×10^5
6C	4.50×10^5	4.50×10^5
5D	4.90×10^5	4.90×10^5
6D	6.60×10^5	6.60×10^5

NOTE: C and D refer to the molar ratio of arms:vector DNA used in the ligation reaction (C = 1:1; D = 4:1)

TABLE 4: Titre of large-scale and amplified libraries.

TUBE NUMBER	PHAGE TITRE (PFU/ml)	NUMBER OF RECOMBINANTS/ μg λ DNA
6C1	1.73×10^6	1.73×10^6
6D1	9.30×10^5	9.30×10^5
6D2	8.90×10^5	8.90×10^5
6C1 Amplified	6.60×10^{10}	6.60×10^{10}
6D2 Amplified	3.60×10^{10}	3.60×10^{10}

Note: 6C1 and 6D1 refer to packaging reactions of ligated DNA from tubes 6C and 6D, respectively, in which only 25ul of Packagene ExtractTM was used. 6D2 refers to the large-scale packaging of ligated DNA from tube 6D using 50ul of Packagene ExtractTM.

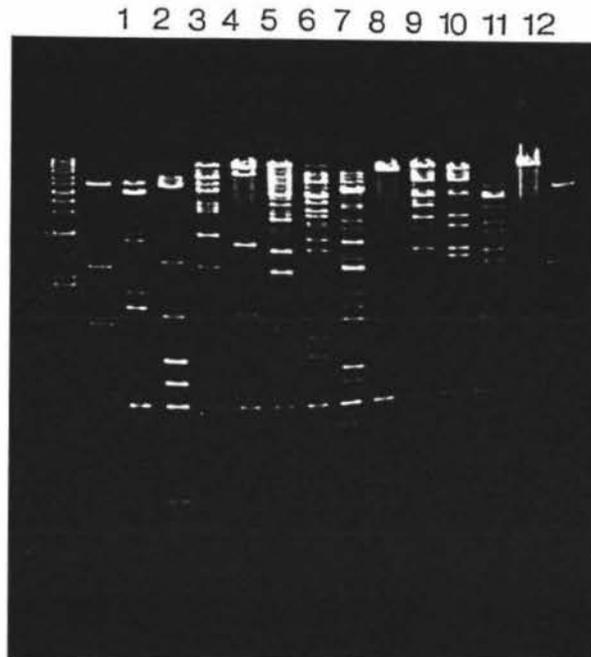


Fig. 21: Hind III digestion of 12 clones from the *G. intestinalis* strain NP 77 gene library. The outer left and right hand lanes contain the High Molecular Weight DNA (BRL) and Lambda Hind III DNA markers, respectively.

Note that the banding pattern of each clone is different and that the common bands present in each digest are those of the vector arms.

TABLE 5: Average size of inserts present in the gene library.

CLONE NUMBER	SIZE OF INSERT (kilobases)
1	13.3 kb
2	19.6 kb
3	18.5 kb
4	11.8 kb
5	18.5 kb
6	19.0 kb
7	14.1 kb
9	10.5 kb
10	20.8 kb
11	17.9 kb
AVERAGE SIZE OF INSERT	16.4 kb

Note: The average insert size was calculated from the insert size of the ten best clone digests (29, 101).

5.4 DISCUSSION

Five methods were used in an effort to produce intact, high molecular weight DNA from *G. intestinalis* trophozoites. Intact DNA is required for the construction of genomic libraries. The DNA is partially digested with *Sau* 3AI in the initial steps of library construction, but if the DNA is not intact before digestion then, on comparison of digests with the undigested control DNA, it is difficult to distinguish between DNA that is sheared from DNA partially digested by *Sau* 3AI. More importantly, sheared DNA will not ligate with the vector DNA. The methods of Ionas (1988), Strauss (1990), Sambrook et al (1989) and Nash et al (1985) all produced sheared DNA (refer Fig. 18). The method of van Keulen *et al* (115) which involved the combination of two methods (101, 119) was also used but as sheared DNA was produced the result was not included in this thesis. However, as van Keulen *et al* were obviously able to produce intact DNA to construct a library (115), Harry van Keulen was personally approached for a detailed description of the method he had used. This detailed method is outlined in the materials and methods section of this chapter and repeatedly produced intact DNA (Fig. 18).

All methods of isolating DNA shared three basic steps: cell lysis using a detergent (eg. SDS or sarkosyl); removal of protein by phenol/chloroform extraction; and concentration of the DNA by ethanol precipitation. Comparison of these methods suggested several critical parameters for the generation of high molecular weight DNA (> 55 kb). Firstly, it is important to minimise the activity of endogenous nucleases either by freezing cells as soon as they are harvested or by extracting DNA immediately from fresh trophozoites and by the inclusion of EDTA in the lysis buffer. Dilution of the lysate and initial extraction steps (eg. phenol/chloroform extraction) also reduced nuclease activity and aided separation of the organic phase from the aqueous phase. Shearing forces were minimised by gentle (but thorough) mixing during extraction steps. Cost effectiveness was also an important consideration as ultracentrifugation using caesium chloride was a time-consuming and costly process. The powerful proteolytic activity of proteinase K removed many of the cellular proteins. However, the most important factor in producing high yields of

intact DNA proved to be the inclusion of an isopropanol precipitation step (van Keulen's method) after a primary single phenol/chloroform step. This step removes much of the polysaccharides present in the cell lysate.

Giardia strains contain high levels of polysaccharide and we concluded that the action of polysaccharides and proteins binding to the DNA present in the solution results in the loss of much of the intact DNA in the thick layer of white material formed at the interphase during phenol/chloroform extraction (119). Thus precipitation of the nucleic acids by the isopropanol, without precipitating polysaccharides and proteins, increased the yield of intact DNA. The other advantage of including this step was that since the DNA could be dissolved in a small volume the remaining steps could be performed in Eppendorf tubes thus reducing the amount of phenol/chloroform and other solutions required. Traces of polysaccharide still remaining could be removed by precipitating the DNA with cetyltrimethylammonium bromide (CTAB). However, CTAB precipitation was not required for our DNA extractions as most of the polysaccharide was removed by the isopropanol precipitation step. Finally, the precipitation of DNA with 3M sodium acetate and cold 95% ethanol followed by a 70% ethanol wash removed any remaining organic solvents, salt and cellular proteins so that the intact DNA produced was relatively pure and free from inhibitors and was readily digested by *Sau 3A I*.

The intact DNA from *G. intestinalis* strain NP 77 was used to produce a gene library. A high titre (10^6 PFU/ml) of recombinant plaques (Table 4) indicated efficient packaging of the recombinant phage and consequently suggests that an appropriate library was produced. However, it is possible that the apparent library could represent merely a subfraction of the *G. intestinalis* genome and if so, the cloned DNA fragments could frequently be the same whereas in an adequate library of a large genome there would be great heterogeneity of the packaged DNA. Consequently, it was necessary to demonstrate that the library created is representative of the entire *Giardia* genome. It is not practical to show that each region of the *Giardia* genome is present in the library but a variety of methods can be used to show that the library is representative.

One method involves the screening of duplicate plaque lifts from a single plating of 10,000 to 50,000 clones with an actin hybridization probe. Each duplicating signal on the film is scored as a positive. Actin is used as the probe of choice with which to evaluate the library because it is small in size (approximately 1 kb), its sequence is highly conserved across species and probes are widely available (29). It was calculated that for a library constructed from *G. intestinalis*, which has a genome size of 8×10^4 kb and with insert sizes of 10-20 kb, that the number of positive clones will be approximately 10-20 if about 35,000 clones are screened (see calculations below) and the library is representative. However, this method involves the preliminary use of southern blots of genomic DNA to establish the optimal conditions of hybridization for the actin probe which is extremely laborious. An alternative method recommended for evaluating libraries is to take a calculated number of phage and show the inserts from each are different (29). This latter approach was used to evaluate the gene library produced for *G. intestinalis* strain NP 77.

Twelve single recombinant clones were selected, amplified and their DNA extracted. DNA from each of the clones was digested with Hind III to show that each contained a different insert (Fig. 21). At least 10 of the 12 recombinant clones selected were required to have inserts visible by ethidium bromide fluorescence, with an average size of 10-20 kb (29). Since this was achieved with our library, we conclude that it is likely to be representative of the *G. intestinalis* strain NP 77 genome.

Bearing in mind the size of the *G. intestinalis* genome (8×10^7 bp), the question arises as to whether or not enough clones are present in the library to include all genomic sequences. The number of clones required for such a library can be calculated by a formula derived by Clarke and Carbon (1976; 24b).

$$N = \frac{\ln(1-P)}{\ln\left(1-\frac{1}{n}\right)}$$

where: N = number of independent recombinants;
 P = probability of including any DNA sequence in a random library of N;
 and: n = size of the genome relative to a single cloned fragment (insert).

Given that the size of the *Giardia* genome is 8×10^4 kb and the average size of the insert is 16.4 kb (Table 5):

$$n = \frac{8 \times 10^4}{1.64 \times 10^1} = 4.88 \times 10^3$$

So, for a 99.9% probability that we have created a complete library:

$$N = \frac{\ln(1-0.999)}{\ln\left(1-\frac{1}{4.88 \times 10^3}\right)} = 3.37 \times 10^4$$

Hence 3.37×10^4 clones are required for an adequate library and so this number of clones should be used for any screening of the library for clones of interest.

To check that this number of clones was present in our library (note: the number of recombinant phage/ μ g of Lambda DNA calculated for reaction mixture 6D1 was used) the following calculations were made:

The number of recombinant phage/ μg Lambda DNA = 9.30×10^5 (Table 4). However, only 0.25 μg of Lambda DNA was used in this packaging reaction (refer to section 5.2.10). Therefore, the number of recombinant phage/ μg Lambda DNA is 2.33×10^5 in the total volume of the packaged phage (250 μl) ie. 9.3×10^2 recombinant phage/ μl .

As the minimum requirement for 99.9% probability that a complete library has been created is 3.37×10^4 recombinants (clones), then for the 250 μl total volume of tube 6D1:

$$\frac{\text{no. recombinant phage/microgram } \Lambda \text{ DNA}}{\text{no. clones for a complete library}} = \frac{2.30 \times 10^5}{3.37 \times 10^4} = 6.82$$

This means that our library was 6.82 times greater than that required for a minimum library and contained an adequate number of clones for a representative library.

CHAPTER 6: GENERAL DISCUSSION

Giardia intestinalis infects both humans and animals yet there is no strong evidence to suggest that animals represent a major source of infection for humans. Furthermore, when humans are infected with *Giardia* the clinical outcome may vary widely, thus many cases are asymptomatic whereas others develop severe and chronic diarrhoea. These and other considerations raise the question as to whether or not the strains of *Giardia* which infect humans vary in virulence and whether any or all of the strains which infect humans are derived from animals.

Such questions and detailed epidemiological studies could be addressed if methods were developed to distinguish individual strains or groups of strains of *Giardia*. This thesis represents the application of the techniques of molecular genetics to the problem of how *G. intestinalis* strains may be distinguished.

The concept that digests of total genomic DNA, followed by electrophoresis of the fragments produced, can give banding patterns which allow individual strains of microorganisms to be distinguished has been well established in practice (1, 20, 31, 37, 50, 77, 89). The simplicity of this technique led us to attempt it with *Giardia*.

A difficulty arose because the genome is large and gave rise to a large number of restriction products. The actual number of fragments produced depends on the total genomic length, the size of the recognition site (ie. a restriction enzyme recognising a 4 bp sequence is likely to produce more fragments than a 6 bp cutter) and the match or mismatch of the GC content of the genome relative to the restriction enzyme recognition sequence. Nevertheless, without a complete knowledge of the gene sequence, accurate predictions of the number of fragments cannot be made.

In the hope of finding a restriction enzyme that would digest total genomic DNA to produce a relatively small number of fragments, restriction enzymes Bam HI, Alu I,

Apa I and Sph I were used. It was found (Fig. 4), however, that these produced a large number of bands with resultant poor resolution so it was concluded that this approach was not a practical approach to distinguishing strains.

During these experiments it was observed that some bands were more dense than others and consequently probably represented repetitive sequences (92) corresponding to ribosomal DNA (15, 51, 60, 67, 68, 77, 90, 92, 108, 115). Hence we concluded that there may be some potential, in the context of strain recognition, in examining this portion of the DNA on its own and to use it to compare isolates. This approach was technically possible as Ortega-Pierres *et al* (92) has shown that total genomic DNA could be separated, by centrifugation in a CsCl gradient containing Hoescht dye 33258, into two distinct bands: an AT-rich band (total genomic DNA minus the GC-rich fraction) and a GC-rich band. The GC-rich band encoded mainly for ribosomal RNA genes which are present as a 5.56 kb tandemly repeated sequence (15).

Our results confirmed that *Giardia* DNA could be separated into two bands using the method described by Ortega-Pierres *et al* (92) and in subsequent experiments the bands produced were separated and individually cleaved by restriction endonucleases to find if the fragment lengths produced would distinguish the strains. Bam HI digestion of total genomic DNA, AT-rich DNA and GC-rich DNA showed that, as expected, the GC-rich fraction produced many fewer bands than that of the AT-rich or the total genomic DNA digests. This gave greater resolution of the banding patterns (Fig. 6). The dense bands in the total genomic digest corresponded to the three bands present in the GC-rich digest (2.4 kb, 1.85 kb and 1.3 kb in size). These fragments represented the rRNA genes present in *Giardia* as tandemly repeated sequences and corresponded to both the results of Ortega-Pierres *et al* (92) and the rRNA gene map proposed by Boothryd *et al* (15).

The GC-rich fraction of DNA from each of the ten strains was digested with restriction enzymes to see if strains could be distinguished. This is more likely to be achieved using enzymes which are known to cleave the DNA in the non-conserved region of the rRNA genes. Restriction enzymes Apa I, Fok I, Hae III, Hinf I, Sma

I and Taq I that are known to possess recognition sites within the variable region of the rRNA gene repeat were used as well as Kpn I, Bgl II and Bam HI with recognition sites anywhere within the rRNA gene repeat.

No differences were detected in the banding patterns of the strains using Bam HI, Sma I, Apa I, Hinf I, Bgl II or Hae III (Figs. 7-12). However, differences between the strains were observed with Taq I, Kpn I and Fok I (Figs. 13-15). These differences were not great and in most instances appeared to be differences of band intensity rather than the presence or absence of a band. Nevertheless, the fact that the differences were detected between strains demonstrated the potential usefulness of this approach in distinguishing strains of *Giardia*. However, this technique is laborious and requires the production of large amounts of DNA and its subsequent separation into two bands by CsCl centrifugation. The production of DNA is also laborious and requires the large-scale *in vitro* culture of *Giardia*. A further difficulty arises because the *in vitro* culture of most *Giardia* strains is not yet possible. Consequently, it was concluded that this technique does not represent a practical method to distinguish strains of *Giardia* or to compare these strains which infect humans with those that infect animals.

Thus two major problems arise with genetic approaches to strain differentiation: the laboriousness of the procedures involved and the ability to produce large quantities of DNA that in turn requires *in vitro* culture of *Giardia* which cannot usually be achieved. Both of these problems could be overcome if we could develop a probe representing a length of DNA which varies in different strains by possessing different endonuclease sites. Such a probe could also be used on relatively small quantities of DNA from *Giardia* strains which could not be cultured long term *in vitro* because such strains could be used to infect neonatal mice and subsequently purified as trophozoites from the intestine following attachment *in vitro* and extensive washing. Experiments in this lab have shown that strains recovered from mice give identical banding patterns to the inoculated strain (data not shown).

Subsequently, the identification of such a probe could be applied to all strains of

Giardia whether or not they can be cultivated *in vitro*. Furthermore, total genomic digests could be probed without DNA purification and fractionation with CsCl. These considerations led us to the conclusion that the construction of a gene library was an obvious and perhaps an essential preliminary to using this approach. The remainder of this thesis is concerned with the production of such a library.

The first task in the production of a gene library involves the production of intact, high molecular weight DNA because sheared DNA will not ligate with the vector DNA. This task proved to be a major problem.

Five methods were used in an effort to produce intact DNA from *G. intestinalis* trophozoites (55a, 89, 101, 110). The shortcomings of these methods have already been discussed. Van Keulen (personal communication) was able to produce high molecular weight DNA. A comparison of the method of van Keulen with the four other methods suggested that several important parameters are required for the production of intact DNA: (1) the lysates should be incubated at 50-60°C to minimize the activity of endogenous nucleases, (2) the lysate should be diluted to aid separation of the phases after phenol/chloroform extraction steps, (3) gentle mixing during extraction steps, (4) the use of proteinase K to aid the removal of most of the cellular proteins.

The above steps were common to all or most methods, however, van Keulen included the use of isopropanol to precipitate the DNA (which was then redissolved) after a primary single phenol/chloroform extraction. This removed much of the polysaccharides in the cell lysate since it remained in the supernatant and was discarded. This modification consistently achieved production of a good yield of high molecular weight DNA following extraction from *G. intestinalis* isolates.

DNA from *G. intestinalis* strain NP 77 was used to construct a gene library in Lambda phage (Promega). It was then amplified and stored at -70°C. While the high titre of the unamplified library (10^6 PFU/ml) indicated efficient packaging of the recombinant phage and consequently suggested that an appropriate library had been

produced, it was desirable to demonstrate that the library was representative of the *Giardia* genome and did not represent merely a subfraction of the genome.

To demonstrate this, twelve recombinant clones were selected, amplified and their DNA was extracted and digested with Hind III. The results (Fig. 21) showed that the banding patterns observed for each clone were different so it was concluded that the twelve inserts were all different. At least ten of the twelve recombinant clones selected were required to have inserts visible by ethidium bromide fluorescence with an average size of 10-20 kb (29). Since this was achieved with our library, with a calculated average insert size of 16.4 kb (Table 5), we concluded that our library was likely to be representative of the entire *G. intestinalis* NP 77 genome.

To find if an adequate number of clones were present to represent all the DNA sequences of *G. intestinalis* (ie. to form a complete library), the formula of Clarke and Carbon (24b) was applied using the average insert size calculated earlier. The result of this calculation showed that our library was 6.82 times greater in size than that required for a minimum library. Consequently, it was concluded that our library contained an adequate number of clones.

While the production of a library does not in itself achieve the original objective of developing a method (using molecular genetics) to distinguish strains of *G. intestinalis*, nevertheless, it should help to facilitate this aim. For example, this library could be used to select a sequence which, while present in all *G. intestinalis* strains, is sufficiently variable between strains to such an extent that such differences can be detected by restriction enzyme digestion. Such a sequence, once identified, could be used to probe total genomic digests of *Giardia* strains from a range of hosts. Ultimately, this technique could serve both as a useful epidemiological tool in tracing the source of infection within a community and in answering the question of whether or not *Giardia* cysts present in water are infective for humans.

It may also be relevant to note that a library of *G. intestinalis* DNA will almost certainly be put to a variety of uses such as the isolation of clones containing genes

of interest for amplification and subsequent sequencing, promoter analysis, transcription and expression; and the identification of midrepetitive clones for species and strain specific probes used in probing PCR products.

We conclude that this library will facilitate the aim of distinguishing *Giardia* isolates but will also serve many other purposes and represents a valuable resource for the *Giardia* Research group in this, and perhaps other, departments.

APPENDIX**Media and reagents commonly used in this thesis.**(i) TY1-S-33 Growth Medium

Trypticase Soy Broth	20.0 g
Yeast Extract (Difco No.0127-01)	10.0 g
Glucose	10.0 g
NaCl	2.0 g
K ₂ HP0 ₄	1.0 g
KH ₂ P0 ₄	0.6 g
L-cysteine Monohydrochloride	1.5 g
Ferric Ammonium Citrate	0.023 g
L-Ascorbic Acid	0.2 g
Bile Bacteriological	0.8 g
Benzyl Penicillen	0.06 g
Gentamycin Sulphate	0.05 g
Vancomycin	0.02 g
NCTC 135 (Sigma No. N3262)	0.94 g
* Bovine Serum	100.0 ml
Distilled Water to	1 L

Once all the ingredients were added, the volume was made up to one litre with distilled water and the medium mixed using a magnetic stirrer. After mixing, the medium was adjusted to pH 7.0 with 5M NaOH and clarified by positive pressure filtration through six pieces of Whatman TM filter paper followed by a non-sterile 0.45µm pore membrane to remove large particles that would clog the 0.2µm membrane.

Sterilization was achieved by filtering through a sterile supor TM 0.2µm pore membrane that had been sterilized by autoclaving at 121°C for 15 min within the filter unit. Sterile medium was stored at 4°C until use or for a maximum length of 10-14 days.

*Note: Bovine serum was prepared in the laboratory by centrifugation of bovine blood obtained from the AFFCO Meat Works (Feilding) and stored at -20°C until use.

(ii) Phosphate Buffered Saline

NaCl	8.5 g
Na ₂ HPO ₄ .12H ₂ O	2.7 g
NaH ₂ HPO ₄ .2H ₂ O	0.39 g
1M HCl or NaOH to pH 7.2	
Distilled Water to	1000.0 ml

This solution was autoclaved at 121°C for 15 min and stored at 4°C until use.

(iii) 10x Tris-Borate Buffer (10x TBB)

Trizma Base	108.0 g
EDTA (disodium salt)	9.3 g
Boric Acid	55.0 g
Distilled Water to	1.0 L

This was stored at room temperature and diluted 1/10 in distilled water to produce 1xTBB for use in gel electrophoresis.

(iv) RNase Buffer

10mM Tris-HCl (pH 7.5)

15mM NaCl

This was autoclaved and stored at room temperature until use.

(v) 1.0M Tris-HCl (pH 7.5)

Trizma Base	121.1 g
5M HCl to pH 7.5	
Distilled Water to	1000 ml

This solution was autoclaved and stored at room temperature.

(vi) 0.2M EDTA (pH 7.2)

Ethylamine diamine tetraacetic acid (EDTA)	7.44 g
5M NaOH (to pH 7.2)	
Distilled Water to	1000.0 ml

This solution was autoclaved and stored at room temperature.

(vii) Saline Tris-EDTA (STE) Buffer (x10)

5.0 M NaCl	20.0 ml
1.0M Tris-HCl (pH 7.5)	50.0 ml
0.2 M EDTA (pH 7.2)	5.0 ml
Distilled Water to	100.0 ml

This was stored at room temperature after autoclaving.

(viii) Tris-EDTA (TE) solution

1.0 M Tris-HCl (pH 7.5)	1.0 ml
-------------------------	--------

0.2 M EDTA (pH 7.2)	5.0 ml
Distilled Water to	10.0 ml

This was autoclaved and stored at room temperature.

(ix) Phenol/Chloroform/Isoamyl alcohol

A 25:24:1 ratio, respectively, was prepared and stored at room temperature in the dark for up to 10 days.

(x) 3M Sodium acetate

Sodium acetate	49.2 g
Distilled Water to	200.0 ml

This solution was autoclaved and stored at room temperature.

(xi) 95% Ethanol

Absolute ethanol	95.0 ml
Distilled Water	5.0 ml

This was stored at -20°C.

(xii) 70% Ethanol

Absolute ethanol	70.0 ml
Distilled Water	30.0 ml

This was stored at room temperature.

(xiii) Bromophenol Blue Dye (10x concentration)

Bromophenol blue	0.05 g
Glycerol	40.00 ml
Distilled Water to	50.00 ml

This solution was stored at room temperature.

(xiv) Ethidium bromide

Ethidium bromide	100.0 mg
Distilled Water to	10.0 ml

This was stored in the dark (i.e. wrapped in tin foil) at room temperature.

BIBLIOGRAPHY

1. **Adam, R.D. (1991)**
The biology of *Giardia* spp.
Microbiological Reviews; 55 (4): 706-732
2. **Adam, R.D., Nash, T.E. and Wellems, T.E. (1988)**
The *Giardia lamblia* trophozoite contains sets of closely related chromosomes.
Nucleic Acids Research; 16 (10): 4555-4567
3. **Adam, R.D., Nash, T.E. and Wellems, T.E. (1991)**
Telomeric location of *Giardia* rDNA genes.
Molecular and Cellular Biology; 11 (6): 3326-3330
4. **Aggarwal A. and Nash T.E. (1987)**
Comparison of two antigenically distinct *Giardia lamblia* isolates in gerbils.
Am. J. trop. Med. Hyg.; 36: 325-332
5. **Akin E.W. and Jakubowski W. (1986)**
Drinking water transmission of giardiasis in the United States.
Wat. Sci. Tech.; 18: 219-226
6. **Ament M.E. and Rubin C.E. (1972)**
Relation of giardiasis to abnormal intestinal structure and function in gastrointestinal immunodeficiency syndromes.
Gastroenterology; 62: 216-226
7. **Andrews, R.H., Adams, M., Boreham, P.F.L., Mayrhofer, G. and Meloni, B.P. (1989)**

Giardia intestinalis: electrophoretic evidence for a species complex.
Int. Jou. Parasit.; 19: (2): 183-190

8. **Belkhiri, A. and Dick, M.W. (1988)**
Comparative studies on the DNA of *Pythium* species and possibly related taxa.
Jou. Gen. Microbiol.; 134: 2673-2683
9. **Belosevic M., Faubert G.M., MacLean J.D., Law C., and Croll N.A. (1983)**
Giardia lamblia infections in Mongolian gerbils: an animal model.
J. Infect. Dis.; 147: 222-226
10. **Bemrick W.J. (1984)**
Some perspectives on the transmission of giardiasis.
In: *Giardia and giardiasis*. S.L. Erlandsen and E.A Meyer (editors). Plenum Press. New York. pp. 379-400
11. **Bemrick W.J. and Erlandsen S.L. (1988)**
Giardiasis - is it really a zoonosis?
Parasitol. Today.; 4: 69-71
12. **Bertram, M.A., Meyer, E.A., Lile, J.D. and Morse, S.A. (1983)**
A comparison of isoenzymes of five axenic *Giardia* isolates.
The Journal of Parasitology; 69, (5): 793-801
13. **Binz, N., Thompson, R.C.A., Lyubery, A.J. and Hobbs, R.P. (1992)**
Comparative studies on the growth dynamics of two genetically distinct isolates of *Giardia duodenalis* *in vitro*.
International Journal for Parasitology; 22 (2): 195-202

14. **Blin, N. and Stafford, D.W. (1976)**
A general method for isolation of high molecular weight DNA from eukaryotes.
Nucleic Acids Research; 3 (9): 2303-2308
15. **Boothroyd, J.C., Wang, A., Campbell, D.A. and Wang, C.C. (1987)**
An unusually compact ribosomal DNA repeat in the protozoan.
Giardia lamblia Nucleic Acids Research; 15 (10): 4065-4084
16. **Boreham, P.F.L., Upcroft, J.A. and Upcroft, P. (1990)**
Changing approaches to the study of *Giardia* epidemiology: 1681-2000
Int. Jou. Parasit.; 20 (4): 479-487
17. **Boreham P.F.L., Phillips R.E. and Shepherd R.W. (1987)**
Heterogeneity in the responses of clones of *Giardia intestinalis* to anti-giardial drugs.
Trans. R. Soc. Trop. Med. Hyg.; 81: 406-407
18. **Boucher S.E., and Gillan F.D. (1990)**
Excystation of *in vitro*-derived *Giardia lamblia* cysts.
Infection and Immunity; 58(11): 3516-3522
19. **Brown, T.J., Ionas, G., Miller, S.J., Tonks, M.C. and Kelly, P.J. (1992)**
New Zealand strains of *Giardia intestinalis* from humans: first isolations, culture and growth characteristics.
NZJ Med. Lab. Science; 46 (1): 7-10
20. **Butcher, P.D. and Farthing, M.J.G. (1989)**
DNA probes for the faecal diagnosis of *Giardia lamblia* infections in man.
Biochemical Society Transactions; 17: 363-364
21. **Campbell S.R., van Keulen H., Erlandsen S.L., Senturia J.B. and Jarroll**

- E.L. (1990)
Giardia spp. comparison of electrophoretic karyotypes.
Exp. Parasitol.; 71: 470-482
22. Casson, L.W., Sorber, C.A., Sykora, J.L., Gavaghan, P.D., Shapiro, M.A. and Jakubowski, W. (1990)
Giardia in waste water - effect of treatment
Res. J. Water Pollution Control Fed.; 62 (5): 670-675
23. Cavalier-Smith, T.
Eukaryotes with no mitochondria (1987)
Nature; 326: 332-333
24. Cedillo-Rivera R., Enciso-Moreno J.A., Martinez-Palomo A. and Ortega-Pierres G. (1989)
Giardia lamblia: isoenzyme analysis of 19 axenic strains isolated from symptomatic and asymptomatic patients in Mexico.
Trans. R. Soc. Trop. Med. Hyg.; 83: 644-646
- 24b. Clarke L. and Carbon J. (1976)
A colony bank containing synthetic Col EI hybrid plasmids representative of the entire *E. coli* genome.
Cell; 9: 91-99
25. Craun G.F (1979)
Waterborne outbreaks of giardiasis.
In: Waterborne transmission of giardiasis. U.S.E.P.A. Office of Research and Development, Cincinnati. -check pages.
- 26a. Craun G.F. (1984)
Waterborne outbreaks from *Giardia*.
In: Giardia and giardiasis. S.L. Erlandsen and E.A. Meyer (editors). Plenum

Press, New York.

- 26b. **Craun G.F. (1986)**
Waterborne giardiasis in the United States 1965-1984.
Lancet ii: 513-514
27. **Crossley R. and Holberton D. (1985)**
Assembly of 2.5 nm filaments from giardin, a protein associated with cytoskeletal microtubules in *Giardia*.
J. Cell. Sci.; 78: 205-231
28. **Crossley R. and Holberton D. (1983)**
Characterisation of proteins from the cytoskeleton of *Giardia lamblia*.
J. Cell. Sci.; 59: 81-103
29. **Current Protocols (1990)**
Construction of recombinant DNA libraries: support protocol.
Current Protocols in Molecular Biology. John Wiley and Sons, Inc.
Supplement 18, Unit 5.8.5.
30. **Davies, R.B., Kukutaki, K. and Hibler, C.P. (1985)**
Cross transmission of *Giardia*. In: *Giardia lamblia* in water supplies - detection, occurrence and removal.
American Water Works Association, Denver, Colorado, USA. pp. 103-108
31. **De Jonckheere, J.F., Majewska, A.C., and Kasprzak, W. (1990)**
Giardia isolates from primates and rodents display the same molecular polymorphism as human isolates.
Molecular and Biochemical Parasitology; 39: 23-28
32. **Edlind, T.D. and Chakraborty, P.R. (1987)**
Unusual ribosomal RNA of the intestinal parasite *Giardia lamblia*.

Nucleic Acids Research; 15 (19): 7889-7901

33. **Erlandsen S.L. and Bemrick W.J. (1987)**
SEM evidence for a new species, *Giardia psittaci*.
J. Parasitol.; 73: 623-629
34. **Erlandsen S.L., Bemrick W.J., Wells C.L., Feely D.E., Knudson L., Campbell S.R., van Keulen H. and Jarroll E.L. (1990)**
Axenic culture and characterization of *Giardia ardeae* from the Great Blue Heron (*Ardea herodias*).
J. Parasitol.; 76: 717-724
35. **Erlandsen S.L., Sherlock L.A., Januschka M., Schupp D.G., Schaefer F.W. (III), Jakubowski W., and Bemrick W.J. (1988)**
Cross-transmission of *Giardia* spp: inoculation of beavers and muskrats with cysts of human, beaver, mouse and muskrat origin.
Appl. Environ. Microbiol.; 54: 2777-2785
36. **Ey P.L., Andrews R.H. and Mayrhofer G. (1993)**
Differentiation of major genotypes of *Giardia intestinalis* by PCR analysis of a gene encoding a trophozoite surface antigen.
Parasitology; 106: 1-10
37. **Ey P.L., Khanna K., Andrews R.H., Manning P.A. and Mayrhofer G. (1992)**
Distinct genetic groups of *Giardia intestinalis* distinguished by Restriction Fragment Length Polymorphisms.
J. Gen. Microbiol.; 138: 2629-2637
38. **Farthing, M.J.G. (1992)**
New Perspectives in giardiasis
J. Med. Microbiol.; 37: 1-2

39. Feely D.E. (1988)
Morphology of the cyst of *Giardia microti* using light and electron microscopy.
J. Protozool.; 35: 52-54
40. Feely D.E (1986)
A simplified method for *in vitro* excystation of *Giardia muris*.
J. Parasitol.; 72: 474-475
41. Feely D.E., Holberton D.V. and Erlandsen S.L. (1990)
The biology of *Giardia*.
In: Giardiasis. E.A Meyer (editor). Elsevier Science Publishers, Amsterdam, The Netherlands. pp. 11-49
42. Feely D.E., Gardner M.D. and Hardin E.L. (1991)
Excystation of *Giardia muris* induced by a phosphate-bicarbonate medium: Localization of acid phosphatase.
J. Parasitol.; 77(3): 441-448
43. Filice F.P. (1952)
Studies on the cytology and life history of a *Giardia* from the laboratory rat.
Univ. Calif. Publ. Zool.; 57: 53-146
44. Giardia Task Group (1985)
Review of giardiasis in water supply.
In: Giardia lamblia in water supplies - detection, occurrence and removal
American Water Works Association. Denver, Colorado, USA. pp. 3-19
45. Gillin F.D. and Reiner D.S. (1982)
Attachment of the flagellate *Giardia lamblia*: Role of reducing agents, serum, temperature and ionic composition.
Mol. Cell. Biol.; 2(4): 369-377

46. Gupta S.R., Rao C.K., Biswas H., Krishnaswami A.K., Wattal B.L. and Raghaven N.G.S. (1972)
Role of the house-fly in the transmission of intestinal parasitic cysts/ova.
Indian. J. Med. Res.; 60: 1120-1125
47. Gusella, J.F., Keys, C., Varsanyi-Breiner, A., Kao, F., Jones, C., Puck, T.T., and Housman, D. (1980)
Isolation and localization of DNA segments from specific human chromosomes.
Proc. Natl. Acad. Sci. USA.; 77 (5): 2829-2833
48. Hardman, N. (1986)
Structure and function of repetitive DNA in eukaryotes.
Biochem J.; 234: 1-11
49. Harley S.V. (1988)
The importance of non-waterborne modes of transmission of giardiasis, a case study.
In: Advances in current Giardia research. ed. P.M Wallis and B.R. Hammond, University of Calgary Press, Calgary.
50. Hay, D.C. and others (1990)
Characterisation of *Giardia* species of canine and human origin using RFLPs.
The Veterinary Record; March 17: p. 274
51. Healey, A., Mitchell, R., Upcroft, J.A., Boreham, P.F.L., and Upcroft, P. (1990)
Complete nucleotide sequence of the ribosomal RNA tandem repeat unit from *Giardia intestinalis*.
Nuclei Acids Research; 18 (13): 4006
52. Hegner R.W. (1926)

The biology of host-parasite relationships among protozoa living in man.
Q. Rev. Biol.; 1: 393-418

53. **Hibler, C.P. and Hancock, C.M. (1989)**
Waterborne giardiasis. In: Drinking Water Microbiology (edited by McFetter, G.). Springer/Verlag pp. 271-293
54. **Hoff J.C. (1988)**
Disinfection and the control of waterborne giardiasis.
In: Controlling waterborne giardiasis. G.S. Logsdon (editor). American Society of Civil Engineers, New York.
55. **Islam A. (1990)**
Giardiasis in developing countries.
In: Giardiasis. E.A. Meyer (editor). Elsevier Science Publishers, Amsterdam, The Netherlands. pp. 235-266.
- 55b. **Ionas G. (1989)**
A study of the heterogeneity of *Mycoplasma ovipneumoniae* isolates by the examination of their proteins and deoxyribonucleic acid.
PhD. Thesis. Massey University, New Zealand.
56. **Jakubowski, W. (1990)**
The control of *Giardia* in water supplies.
In: Giardiasis (edited by E.A. Meyer). Elsevier Science Publishers B.V. (Biomedical Division). pp. 335-353
57. **Janoff E.N and Smith P.D. (1990)**
The role of immunity in *Giardia* infections.
In: Giardiasis. E.A. Meyer (editor). Elsevier Science Publishers, Amsterdam, The Netherlands. pp. 215-233

58. Jarroll E.L., Manning P., Lindmark D.G., Coggins J.R. and Erlandsen S.L. (1989)
Giardia cyst wall-specific carbohydrate: evidence for the presence of galactosamine.
Mol. Biochem. Parasitol.; 32: 121-132
59. Jarroll E.L., Bingham A.K., and Meyer E.A. (1980)
Giardia cyst destruction: effectiveness of six small-quantity water disinfection methods.
Am. J. Trop. Med. Hyg.; 29: 8-11
60. Jelinek, W.R. and Schmid, C.W. (1982)
Repetitive sequences in eukaryotic DNA and their expression.
Ann. Rev. Biochem.; 51: 813-844
61. Jokipii L. and Jokipii A.M.M. (1974)
Giardiasis in travellers: a prospective study.
J. Infect. Dis.; 130: 295-299
62. Jokipii L. and Jokipii A.M.M. (1990)
Nitroimidazole treatment of giardiasis.
In: Giardiasis. E.A Meyer (editor). Elsevier Science Publishers, Amsterdam, The Netherlands. pp. 315-323
63. Kabnick K.S. and Peattie D.A (1990)
In situ analyses reveal that the two nuclei of *Giardia lamblia* are equivalent.
J. Cell Sci.; 95: 353-360
64. Karlovsky, P. and de Cock, A.W.A.M. (1991)
Buoyant density of DNA - Hoescht 33258
(Bisbenzimidazole) complexes in CsCl gradients:

Hoescht 33258 binds to single AT base pairs.

Analytical biochemistry; 194: 192-197

65. **Kirkpatrick C.E and Farrell J.P. (1984)**
Feline giardiasis: observations on natural and induced infections.
Am. J. Vet. Res.; 45: 2182-2188
66. **Kirner, J.C., Littler, J.D. and Angelo, L.A. (1985)**
A waterborne outbreak of giardiasis in Camas, Wash.
In: Giardia lamblia in water supplies - detection, occurrence and removal.
American Water Works Association, Denver, Colorado, USA, pp. 75-80
67. **Le Blancq, S.M., Kase, R.S., and Van der Ploeg, L.H.T. (1991)**
Analysis of a *Giardia lamblia* RNA encoding telomere with [TAGGG]_n as
the telomere repeat.
Nuc. Acids. Res.; 19 (20): 5790
68. **Le Blanq, S.M., Korman, S.H. and Van der Ploeg L.H.T. (1991)**
Frequent rearrangements of rRNA - encoding chromosomes in *Giardia
lamblia*.
Nucleic Acids Research; 19 (6): 4405-4412
69. **Lehmann, D.L. and Wallis, P.M. (1988)**
Differentiation of *Giardia duodenalis* from *Giardia muris* by immobilization
in various sera.
In: Advances in Giardia Research (edited by P.M. Wallis and B.R.
Hammond), University of Calgary Press, Calgary. pp. 169-172
70. **Levine N.D. (1979)**
Giardia lamblia; classifications, structure, identification.
In: Waterborne Transmission of Giardiasis. EPA-600/9-79-001.

71. **Lin, S.D. (1985)**
Giardia lamblia and water supply.
In: *Giardia lamblia* in water supplies - detection, occurrence and removal.
American Water Works Association, Denver, Colorado, USA. pp. 75-80
72. **Lindmark, D.G. and Miller, J.J. (1988)**
Enzyme activities of *Giardia lamblia* and *Giardia muris* trophozoites and cysts.
In: Advances in *Giardia* Research (edited by P.M. Wallis and B.R. Hammond), University of Calgary Press, Calgary. pp. 187-189
73. **Lindridge E. (1992)**
SDS-PAGE comparison of the total proteins of *Giardia intestinalis* and *Giardia muris*.
BSc.(Hons.) dissertation. Massey University, Palmerston North, New Zealand.
74. **Logsdon G.S., Symons J.M. and Hoye R.L. (1979)**
Water filtration techniques of removal of cysts and cyst methods.
In: Waterborne Transmission of Giardiasis. W. Jakubowski and J.C Hoff (editors). U.S. Environmental Protection Agency, Ohio. pp. 240-256
75. **Logsdon G.S. (1988)**
Controlling Waterborne Giardiasis. A.S.C.E., New York.
76. **McIntyre P., Boreham P.F.L., Phillips R.E. and shepherd R.W. (1986)**
Chemotherapy in giardiasis: clinical responses and *in vitro* drug sensitivity of human isolates in axenic culture.
J. Pediatr.; 108: 1105-1010
77. **Magee, B.B., D'Souza, T.M. and Magee, P.T. (1987)**
Strain and species identification by Restriction Fragment Length Polymorphisms in the ribosomal DNA repeat of *Candida* species.

Jou. Bact.; 169 (4): 1639-1643

78. Mahbubani M.H., Bej A.K., Perlin M.H., Schaefer F.W (III), Jakubowski W. and Atlas R.M (1992)
Differentiation of *Giardia duodenalis* from other *Giardia* spp. by using Polymerase Chain Reaction and gene probes.
Jou. Clin. Microbiol.; 30: 74-78
79. Marino M.R. (1993)
Giardia in New Zealand animals: prevalence, viability in the environment and preservation.
MSc. Thesis. Massey University, Palmerston North, New Zealand.
80. Meloni B.P., Lymbery A.J. and Thompson R.C.A. (1988)
Isoenzyme electrophoresis of 30 isolates of *Giardia* from humans and felines.
Am. J. Trop. Med. Hyg.; 38: 65-73
81. Meloni B.P. and Thompson R.C.A. (1987)
Comparative studies on the axenic *in vitro* cultivation of *Giardia* of human and canine origin: evidence for intraspecific variation.
Trans. Royal Soc. Trop. Med. Hyg.; 81: 637-640
82. Meyer E.A (1976)
Giardia lamblia: isolation and axenic cultivation.
Exp. Parasitol.; 39: 101-105
83. Meyer E.A (1990)
Introduction.
In: Giardiasis. E.A. Meyer (editor). Elsevier Science Publishers, Amsterdam, The Netherlands. pp. 1-9
84. Meyer E.A. and Radulescu S. (1979)

Giardia and giardiasis.

Adv. Parasitol.; 17: 1-14

85. **Miller S.J. (1988)**
Giardia intestinalis: aerobic metabolism and physiology of *in vitro* growth.
MSc. thesis. Massey Univesity, Palmerston North, New Zealand.
86. **Montanez, C., Cervantes, L., Ovando, C. and Ortega-Pierres, G. (1988)**
Properties of *Giardia lamblia* RNAs.
In: Advances in Giardia Research (edited by P.M. Wallis and B.R. Hammond), University of Calgary Press, Calgary. pp. 181-185.
87. **Nash T.E., Herrington D.A., Losonsky G.A. and Levine M.M. (1987)**
Experimental human infections with *Giardia lamblia*.
J. Infect. Dis.; 156: 974-984
88. **Nash T.E. and Keister D.B. (1985)**
Differences in excretory-secretory products and surface antigens among 19 isolates of *Giardia*.
J. Infect. Dis.; 152: 1166-1171
89. **Nash, T.E., McCutchan, T., Keister, D., Dame, J.B., Conrad, J.D. and Gillin, F.D. (1985)**
Restriction-endonuclease analysis of DNA from 15 *Giardia* isolates obtained from humans and animals.
The Journal of Infectious Diseases; 152 (1): 64-73
90. **Noller, H.F.**
Structure of ribosomal RNA.
Ann. Rev. Biochem.; 53: 119-162
91. **Ortega-Barria E., Ward H.D., Evans J.E. and Pereira M.E.A. (1990)**

N-acetyl-D-glucosamine is present in cysts and trophozoites of *Giardia lamblia* and serves as a receptor for wheatgerm agglutinin.

Mol. Biochem. Parasitol.; 43: 151-166

92. **Ortega-Pierres, G., Alonso, R., Cervantes, L. and Montanez, C. (1990)**
 Characterization of two DNA populations of *Giardia lamblia*.
Molecular Microbiology; 4 (11): 1985-1991
93. **Owen R.L. (1984)**
 Direct faecal-oral transmission of giardiasis.
 In: *Giardia and giardiasis; biology, pathogenesis and epidemiology.*
94. **Peattie D.A., Alonso R.A., Hein A. and Caulfield J.P. (1989)**
 Ultrastructure localization of giardins to the edges of disc microribbons of *Giardia lamblia* and the nucleotide and deduced protein sequence of the alpha giardin.
J. Cell Biol.; 109: 2323-2335
95. **Perlmutter D.H., Leitchner A.M., Goldman H. and Winter H.S. (1985)**
 Chronic diarrhoea associated with hypogammaglobulinaemia and enteropathy in infants and children.
Dig. Dis. Sci.; 30: 1149-1155
96. **Phillips S.C., Mildvan D., Williams D.C., Gelb A.M. and White M.C.(1981)**
 Sexual transmission of enteric protozoa and helminths in a venereal disease clinic population.
N. Engl. J. Med.; 305: 603-606
97. **Promega**
 Instructions for use of products B1940, B1950, B1960, B1970. (B1970 = LambdaGEMR-11 Xho 1 Half-site arms).

Promega Technical Bulletin No. 057; Madison, Wisconsin, USA.

98. **Promega (1991)**
Protocols and Applications Guide.
Mad. Wisconsin. USA.

99. **Rendtorff R.C and Holt C.J. (1954)**
The experimental transmission of human intestinal protozoan parasites II.
Giardia lamblia cysts given in capsules.
Am. J. Hyg.; 59: 209-220

100. **Rossoff J.D., Sanders C.A., Sonnad S.S., DeLay P.R., Hadley W.K.,
Vincenzi F.F., Yajko D.M. and O'Hanley P.D. (1989)**
Stool diagnosis of giardiasis using a commercially available enzyme
immunoassay to detect *Giardia*-specific antigen 65 (GSA 65).
J. Clin. Microbiol.; 27: 1997-2002

101. **Sambrook J., Fritsch E.F. and Maniatis T. (1989)**
Molecular Cloning: A Laboratory Manual (second edition). Cold Spring
Harbour Laboratory Press, New York (p. 6.9-6.13; 9.14-9.30)

102. **Sauch J.F. (1985)**
Use of immunofluorescence and phase-contrast microscopy for detection and
identification of *Giardia* cysts in water samples.
Appl. Environ. Microbiol.; 50(6): 1434-1438

103. **Sauch J.F. (1988)**
A new method for excystation of *Giardia*.
In: Advances in Giardia Research. ed. P.M. Wallis and B.R Hammond,
University of Calgary Press, Calgary. pp. 261-264

104. Schaefer, F.W. and Rice, E.W. (1985)
Giardia methodology for water supply analysis.
In: *Giardia lamblia* in water supplies - detection, occurrence and removal.
American Water Works Association, Denver, Colorado, USA. pp. 75-80
105. Schmerin M.J., Jones T.C. and Klein H. (1978)
Giardiasis: association with homosexuality.
Ann. Intern. Med.; 88: 801-803
106. Schupp D.G., Jauaschka M.M., Sherlock L.A.F., Stihls H.H., Meyer E.A.,
Bemrick W.J. and Erlandsen S.L. (1988).
Production of viable *Giardia* cysts *in vitro*: determination by fluorogenic dye
staining, excystation and animal infectivity in the mouse and Mongolian
gerbil.
Gastroenterology. 95(1): 1-10
107. Sheffield H.G. and Bjorrratn B. (1977)
Ultrastructure of the cyst of *Giardia lamblia*.
Am. J. Trop. Med. Hyg.; 26: 23-30
108. Sogin, M.L., Elwood, H.J. and Gunderson, J.H. (1986)
Evolutionary diversity of eukaryotic small-subunit rRNA genes.
Proc. Natl. Acad. Sci. USA; 83: 1383-1387
109. Strandén, A.M., Eckert, J. and Kohler, P. (1990)
Electrophoretic characterization of *Giardia* isolated from humans, cattle,
sheep and a dog in Switzerland.
J. Parasitol.; 76 (5): 660-668
110. Strauss W.M. (1990)
Preparation of genomic DNA from mammalian tissue.
In: Current Protocols in Molecular Biology. John Wiley and Sons, Inc..

Supplement 13, Unit 2.2. pp.2.2.1-2.2.3

111. Uji, A., Wallis, P.M. and Wenman, W.M. (1988)
Comparison of *Giardia* isolates by DNA - DNA hybridization.
In: Advances in Giardia Research (edited by P.M. Wallis and B.R. Hammond), University of Calgary Press, Calgary.
112. Upcroft J.A., Boreham P.F.L. and Upcroft P. (1989)
Geographic variation in *Giardia* karyotypes.
Int. Jou. Parasit.; 19: 519-527
113. Upcroft, J.A., Capon, A.G., Dharmkrong-At A., Healey, A., Boreham, P.F.L. and Upcroft, P. (1987)
Giardia intestinalis antigens expressed in *Escherichia coli*.
Mol. Biochem. Parasitol; 10: 305-318
114. Upcroft, P., Mitchell, R. and Boreham, P.F.L. (1990)
DNA fingerprinting of the intestinal parasite *Giardia duodenalis* with the M13 phage genome.
Int. Jou. Parasitol.; 20 (3): 319-323
115. van Keulen, H., Campbell, S.R., Erlandsen, S.L. and Jarroll, E.L. (1991)
Cloning and restriction enzyme mapping of ribosomal DNA of *Giardia duodenalis*, *Giardia ardeae* and *Giardia muris*.
Molecular and Biochemical Parasitology; 46: 275-284
116. Ward, L.J.H. and Jarvis, A.W. (1992)
Rapid removal of caesium chloride from DNA obtained from ultracentrifuge gradients.
BioTechniques; 12:76

117. Weishahn G.P., Jarroll E.J., Lindmark D.G., Meyer E.A. and Hallick L.M. (1984)
Giardia lamblia: autoradiographic analysis of nuclear replication.
Exp. Parasitol.; 58: 94-100
118. Weiss, J.B., van Keulen, H. and Nash, T.E. (1992)
Classification of subgroups of *Giardia lamblia* based upon ribosomal RNA gene sequence using the polymerase chain reaction.
Molecular and Biochemical Parasitology; 54: 73-86
119. Yap, K.W. and Thompson, R.C.A. (1987)
CTAB precipitation of cestode DNA.
Parasitology Today; 3 (7): 220-222