The molecular epidemiology of *Dientamoeba fragilis* isolates in an Australian population

by

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Certificate

This study was carried out in the Microbiology Department, St. Vincent's Hospital, Sydney, under the supervision of Professor John T. Ellis, Associate Professor John Harkness, Associate Professor Deborah Marriott, and Dr Nigel Beebe. I certify that no part of this work has been submitted to any other university or institute. I also certify that this thesis has been written by me and that all help received and all sources used have been acknowledged in this thesis.

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Damien Stark

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Abbreviations

ANGIS	Australian National Genomic Information Service
bp	base pair
BSA	bovine serum albumin
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide
ICT	Immunochromatographic
kb	kilobases
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RFLP-PCR	restriction fragment length polymorphism – PCR
rDNA	ribosomal DNA
RNA	ribonucleic acid
SAF	sodium acetate acetic acid formalin
SSU rDNA	small-subunit ribosomal DNA
SSU rRNA	small-subunit ribosomal RNA
TBE	tris borate/EDTA
Tris	tris[hydroxymethyl]aminomethane
V	volts

Abstract

Dientamoeba fragilis is a trichomonad parasite that causes human gastrointestinal disease. It has been reported from most parts of the world in both rural and cosmopolitan areas and is a 'neglected cause of diarrhoea' and dysentry with chronic infections common. Current diagnosis of dientameobiasis is by microscopic identification of the trophozoite in stool. However this method is time-consuming and relatively insensitive while PCR technology offers an attractive alternative to conventional diagnosis. A conventional PCR assay based on the small-subunit ribosomal RNA gene of *D. fragilis* for the specific detection of *D. fragilis* DNA in fresh unpreserved stool samples was developed. The *D. fragilis* PCR was positive in 29/31 samples with positive microscopy and did not cross-react with other protozoan parasites. The PCR protocol showed a specificity of 100% and a sensitivity of 93.5% and the entire procedure can be performed in one day. A prospective study was also conducted over a 30 month period, in which 6,750 faecal samples were submitted to the Department of Microbiology at St. Vincent's hospital Sydney, Australia. Trophozoites of *Dientamoeba fragilis* were detected in 60 (0.9%) patients by permanent staining and confirmation was performed by PCR. Gastrointestinal symptoms were present in all patients, with diarrhoea and abdominal pain the most common symptoms. Thirty-two percent of patients presented with chronic symptoms. The average age of infected patients was 39.8 years. No correlation was found between *D. fragilis* and *Enterobius vermicularis*, a proposed vector of transmission for *D. fragilis*. The genetic diversity of 50 *D. fragilis* isolates was examined by PCR and the PCR products were analysed for the presence of a restriction fragment length polymorphism. These results showed no variation in the small subunit rRNA gene and demonstrated a single genotype for all Australian isolates. This study indicates the potential pathogenic properties of *D. fragilis*, and the need for all laboratories to routinely test for this organism. I also developed a 5' nuclease (TaqMan) based real-time PCR assay, targeting the small-subunit ribosomal RNA gene, for the detection of *D. fragilis* in human stool specimens and compared its sensitivity and specificity to the conventional PCR and microscopic examination by a traditional modified iron-haematoxylin staining procedure. Tests were

performed using all three techniques on 200 stool specimens referred for screening on the basis of diarrhea. The real-time PCR assay exhibited 100% sensitivity and specificity compared with microscopy. The detection limit of both PCR tests was compared; real-time PCR was 100 times more sensitive than conventional PCR, with a detection limit of 0.01 trophozoites. In conclusion, all three methods for the detection of *D. fragilis* were highly specific, with real-time PCR being the most sensitive. The use of the real-time assay in a diagnostic laboratory provides a superior sensitive and specific method for the diagnosis of *D. fragilis*.

Table of Contents

Page No.

CHAPTER 1 GENERAL INTRODUCTION

1.1 Historical Aspects 2

1.2 Morphological characteristics 3

1.3 Taxonomy 5

1.4 Clinical aspects and epidemiology 9

1.5 Genetic diversity 16

1.6 Transmission 17

1.7 Pathology 22

1.8 Diagnostic techniques 24

1.9 Therapy 29

1.10 Conclusion 32

1.11 Project aims 33

1.12 Objectives 33

CHAPTER 2 DETECTION OF *DIENTAMOEBA FRAGILIS* IN FRESH STOOL SAMPLES USING PCR

2.1 Introduction 35

2.2 Materials and methods 38

2.2.1 Stool specimens 38

2.2.2 DNA extraction 38

2.2.3 Small subunit rRNA gene amplification and sequencing 39

2.2.4 *D. fragilis* PCR 40

2.3 Results 41

2.4 Discussion 50

CHAPTER 3 THE MOLECULAR EPIDEMIOLOGY, GENETIC DIVERSITY AND CLINIAL RELEVANCE OF *DIENTAMOEBA FRAGILIS* INFECTIONS

3.1 Introduction 55

3.2 Materials and methods 56

3.2.1 Stool specimens 56

3.2.2 Microbiological investigations 56

3.2.3 PCR for *D. fragilis* 57

3.2.4 Restriction fragment length polymorphism 57

3.2.5 Follow up data 57

3.2.6 Control group 57

3.2.7 Questionnaire 57

3.3 Results 58

3.4 Discussion 68

CHAPTER 4 EVALUATION OF THREE DIAGNOSTIC METHODS, INCLUDING REAL-TIME PCR, FOR THE DETECTION OF *DIENTAMOEBA FRAGILIS* IN STOOL SPECIMENS

4.1 Introduction 74

4.2 Materials and methods 76

4.2.1 Culture of enteric protozoa and extraction of genomic DNA 76

4.2.2 Stool specimens 76

4.2.3 DNA extraction from stool specimens 76

4.2.4 Cloning of small subunit ribosomal DNA from *D. fragilis* 77

4.2.5 Conventional PCR 77

4.2.6 Real-time PCR 77

4.3 Results 79

4.4 Discussion 84

CHAPTER 5 GENERAL DISCUSSION 88

CHAPTER 6 APPENDIX 99

CHAPTER 6 BIBLIOGRAPHY 115

List of Figures

		Page No.
Figure 1.1	Trophozoite of a binucleate form of *Dientamoeba fragilis*.	4
Figure 1.2	Life-cycle of *D. fragilis*	21
Figure 2.1	Nucleotide sequence of the entire SSurRNA gene of Australia *D. fragilis* isolates	44
Figure 2.2	Nucleotide sequence alignment of the SSUrRNA gene of *D. fragilis* (AY730405) vs *D. fragilis* (U37461).	47
Figure 2.3	Detection of *D. fragilis* in faeces by PCR with primers DF400 and DF1250	48
Figure 3.1	Prevalence of *D. fragilis* in different age groups.	64
Figure 3.2	Restriction endonuclease digestion of DF400/DF1250 products.	65
Figure 3.3	Sequence alignment of part of the SSUrRNA gene sequence.	67
Figure 4.1	Evaluation of sensitivity of real-time PCR using cloned DNA	82
Figure 4.2	Detection of *D. fragilis* in faeces by real-time PCR	83

List of Tables

		Page No.
Table I	World wide prevalence of *D. fragilis*.	10
Table II	Commercially available kits for the immunodetection of enteric parasites	27
Table III	Efficacy of antimicrobials in patients with *D. fragilis* infections	31
Table IV	List of specimens containing various other protozoan parasites that were used in this study.	49
Table V	Summary of results from patients with *D. fragilis* infection.	63
Table VI	Parasites found in 24 patients with *D. fragilis* infection.	64
Table VII	Comparison of real-time PCR, conventional PCR and microscopy for detection of *D. fragilis*.	81

CHAPTER 1

GENERAL INTRODUCTION

1.1 HISTORICAL ASPECTS

A microbiologist by the name of Wenyon is regarded as discovering *D. fragilis* in 1909. He observed a previously unknown protozoa in his own parasitological stool preparations. The first description in the scientific literature of *D. fragilis* is attributed to two parasitologists, M. Jepps and C. Dobell, who described the organism in human stool samples (Jepps and Dobell, 1918). As this protozoan was very different to the amoeba that were known to occur in the human bowel at that time, they suggested that it was not only a new species but also a new genus.

Jepps and Dobell (1918) described *D. fragilis* as an amoebae, measuring 8-10μm in diameter with a binucleate structure. They observed the fragile nature of the organism as it degenerated rapidly out-side the human body - hence the name *D. fragilis*. It was initially thought to be an amoeba and was placed in the family Endamoebidae. However since that time its exact phylogenic position has been a matter of contention and even after several decades still remains unclear (Delgado-Viscogliosi *et al.*, 2000).

Even though *D. fragilis* was considered to be an amoeboid organism it was not long until Dobel (1940) challenged the validity of this scientific assumption. Through many experiments he concluded that the nuclear apparatus of *D. fragilis* was flagellate like and that its method of nuclear division was not an amoeba but flagellate like (Dobell, 1940). He postulated that *D. fragilis* was a flagellate and undertook several experiments to induce the organism to express a flagellum, all of which were unsuccessful. Dobell (1940) reported similarities *between D. fragilis* and the amoeboflagellate *Histomonas meleagridis*. *H. meleagridis* is a common pathogen of many galliform and some anseriform birds and is the causal agent of a type of entero-hepatitis termed "blackhead"; a disease most commonly affecting turkeys (Gerbod *et al.*, 2001). Having reviewed all the available scientific evidence Dobell (1940) concluded that *Dientamoeba* represented a stage in the life cycle of a flagellate which somewhere in its evolutionary development had permanently lost its flagella. Dobell's hypothesis proved to be correct as later researchers verified the close relationship between *D. fragilis* and the other flagellates

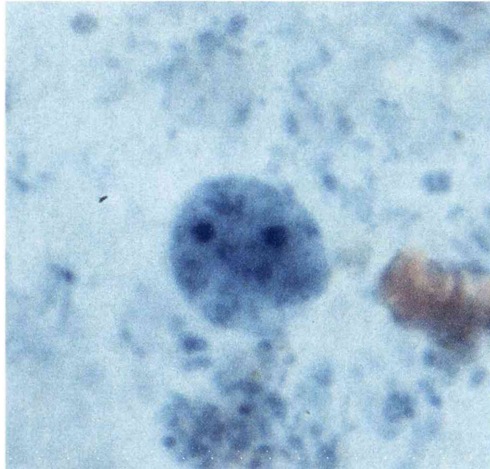
especially *H. meleagridis* (Talis, 1967; Dwyer, 1972; Camp *et al.*, 1974; Silberman *et al.*, 1996; Gerbod *et al.*, 2001).

Both Dobell and Jepps (1918) initially thought that *D. fragilis* was non-pathogenic; a conclusion which was later supported by Dobell and O'Conner (1921). However it was not long until other researchers started to question the pathogenicity of *D. fragilis*. In 1919 a year after *D. fragilis* was first described in the literature others found *D. fragilis* in two military officers with diarrhoea from the USA (Kofoid *et al.*, 1919). The following year a study found *D. fragilis* in three symptomatic children in the Philippines (Haughwout and Horrilleno, 1920) and later *D. fragilis* was implicated as a cause of diarrhoea in an adult male from England (Thomson and Robertson, 1923). Thus in the space of four years from *D. fragilis* having been discovered the controversy surrounding its pathogenicity had begun, something that continues on to this day.

1.2 MORPHOLOGICAL CHARACTERISTICS

D. fragilis is a pleomorphic trophozoite (figure 1.1) ranging in size from 4μm to 20μm with most trophozoites typically in the range of 5μm to 15μm (Wenrich, 1944; Sargeaunt and Williams, 1982). *D. fragilis* may not have a cyst stage. Although some have claimed to have seen *D. fragilis* cysts in permanently stained smears, these findings have since been dismissed as they were not substantiated by other parasitologists (Windsor and Johnson, 1999).

The trophozoites of *D. fragilis* are typically binucleate with only 30-40% uninucleate (Dobell, 1940). Dobell (1940) described multinucleated trophozoites with up to four nuclei, however only one or two are usually found. Nuclear pleomorphism is quite common with the nucleus size varying in relation to the rest of the cell (Wenrich, 1937). In stained smears the nuclear membrane is delicate and does not posses any peripheral chromatin. The karyosome contains chromatin granules that vary from four to eight, often appearing as chromatin packets (Camp *et al.*, 1974).



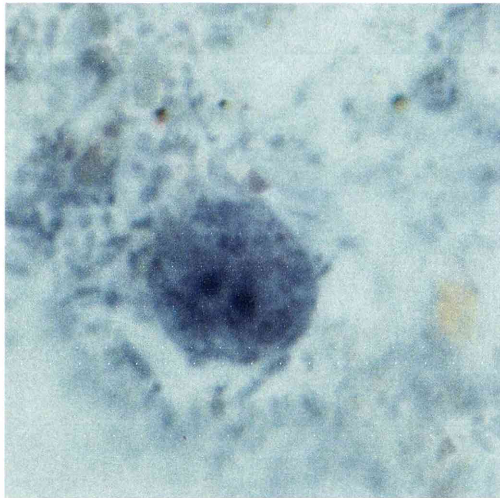


Figure 1.1: Trophozoites of a binucleate form of *Dientamoeba fragilis*.

Magnification 1000X. Modified Iron Haematoxylin stain (Fronine).

The cytoplasm of *D. fragilis* appears granular and may be vacuolated and contain food inclusions as well as ingested bacteria and yeasts (Wenrich, 1944). It has been postulated that extensive vacuolation represents a degenerative condition and is often seen in older specimens made from faecal specimens.

In freshly passed stools and from culture media the organisms may be motile by pseudopodia, however the trophozoites will become less motile when the stool reaches room temperature and no motility will be seen if the specimen is refrigerated.

1.3 TAXONOMY

Jepps and Dobell (1918) were the first to describe *D. fragilis* in the literature. In 1918 three *Entamoeba* species were known to occur in the human bowel. The non-pathogenic *Entamoeba coli* and *Entamoeba nana* (now known as *Endolimax nana*), and the pathogenic *Entamoeba histolytica*. Both authors argued that as this new organism had a binucleated form and no cyst stage that it was not only a new species but also a distinct new genus of parasite. They demonstrated that once outside the human body this organism became 'fragile' and degenerated rapidly. Hence the new name *Dientamoeba fragilis* was suggested and is still in use today. They placed this new protozoan in the family *Entamoedidae* and as it differed greatly from other *Entamoeba* species at the time it was not only considered a new species but a new genus (Jepps and Dobell, 1918).

This parasite caused considerable debate and its exact taxonomic position remained unclear with some parasitologists objecting to it being assigned to this new genus. This organism was unlike other protozoa as it possesses an amoeboid morphology which was motile by pseudopodia, yet had no demonstrated cystic stage.

Dobell would continue his research on this organism for many years to come. Using only light microscopy, staining and culture techniques Dobell recognised the close structural similarities between dividing stages of *D. fragilis* and the amoeboflagellate *H.*

meleagridis (Dobel, 1940). Notably Dobell (1940) observed that the nuclei, chromosomes and centrodesmus were similar between the two organisms. He also recognised the differences between other amoeba and *D. fragilis* such as: a large proportion of the population are binucleate forms; the nuclear structure is distinct; an extranuclear spindle is present in dividing organisms; cysts are absent from the life cycle, and the similarities with other flagellates. Having reviewed all this scientific information Dobell (1940) was the first parasitologist to conclude that *D. fragilis* was not an amoeba but a flagellate that had permanently lost its flagella. Another parasitologist studied *D. fragilis* and *H. meleagridis* and found that both organisms shared many flagellate characteristics (Wenrich, 1944).

On the basis of the above scientific data and because *D. fragilis* was shown to be significantly different from other amoeba, in 1953 *D. fragilis* was removed from *Endamoebidae* and was placed into a new family with Histomonas, *Dientamoebidae* (Grasse, 1953).

The advent of the electron microscopy provided more evidence that *D. fragilis* was indeed closely related to flagellates. Bird *et al.* (1970) published a series of electron micrographs that illustrated the fine structure of uni and binucleate trophozoites of *D. fragilis*. The demonstration of a persistent internuclear spindle of microtubules in the binucleate stage supported Dobell's assumptions. Also the well-developed parabasal filament in both uninucleated and binucleated trophozoites substantiated the close affinity with *H. meleagridis*.

Dwyer (1972a,b) used gel diffusion methods and quantitative fluorescent antibody methods to analyse the antigenic relationship between *Trichomonas*, *Histomonas*, *Dientamoeba*, and *Entamoeba*. It was evident from these qualitative gel diffusion results that *Trichomonas*, *Histomonas*, and *Dientamoeba* share some structurally identical or closely related antigens. Two years later Dwyer (1974) used immunoelectrophoresis techniques to analyse the antigenic relationship with the afore mentioned organisms. Direct and cross-absorption reaction methods were employed to ascertain quantitatively

the immunologic relationships among the several organisms. The study showed a close antigenic relationship between *Trichomonas*, *Histomonas* and *Dientamoeba*. It was also evident that *Dientamoeba* shares a relatively strong common antigenic basis with *Histomonas* and a somewhat more distant relationship with *Trichomonas*. Dwyer (1974) also showed that *Dientamoeba* was antigenically distinct from *Entamoeba histolytica* and *Entamoeba invadens*.

Camp *et al.* (1974) further refined the taxonomic position of Dientamoeba. By using electron microscopy of *D. fragilis* the fine structural observations lead to further support that *Dientamoeba* was closely related to *Histomonas*. Striking similarities were found between the two species especially with regard to the parabasal apparatus. It was also evident from the electron micrographs that many basic fine structural differences exist between *Entamoeba* species and *Dientamoeba*. On the basis of the above findings, *D. fragilis* was placed in the order *Trichomonadida* and the family *Monoceromonadidae*, subfamily *Dientamoebinae* (Camp *et al.*, 1974). In 1980 Levine *et al.* reclassified *Dientamoeba* in the order *Trichomonadida* along with *Histomonas*, *Monocercomonas* and *Trichomonas*. Parasites of the order were defined as: "Typically karyomastigonts with 4 to 6 flagella, but with only 1 flagellum in one genus and no flagella in another; karyomastigonts and akaryomastigonts in one family with permanent polymonad organisation; in mastigont(s) of typical genera one flagella recurrent, free or with proximal or entire length adherent to body surface; undulating membrane, if present associated with adherent segment of recurrent flagellum; pelta and noncontractile axostyle in each mastigont, except for one genus; hydrogensomes present; true cysts infrequent, known in very few species; all or nearly all parasitic." *Trichomonadida* and *Hypermastigida* were grouped in the superorder *Parabasabidea*.

It was not until 1996 that molecular techniques were used as an alternative to traditional phenotypic markers to determine the taxonomic position of *Dientamoeba*. Phylogenetic relationships can be established through comparison of small subunit (SSU) rRNA sequences. This molecule has been used extensively as a molecular indicator and appears to be one of the best tools available to determine taxonomic relationships (Gerbod *et al.*,

2001). Silberman *et al.* (1996) constructed molecular phylogenies based upon the complete small subunit rRNA sequences of *D. fragilis*, several trichomonad groups and a variety of other eukaryotes. *D. fragilis* SSU rRNA gene was shown to have a low G+C content relative to other trichomonads and contains approximately 100 extra nucleotides. Similarity calculations demonstrated a clear association between *D. fragilis* and other parabasalid flagellates. All phylogenic constructions showed *D. fragilis* to be closely related to trichomonads (Silberman *et al.*, 1996).

Further molecular studies of the SSU rRNA gene failed to resolve the exact position *of D. fragilis* compared to other Parabasalids (Delgado-Viscogliosi *et al.*, 2000). The phylogenic position of *H. meleagridis* was determined by analysis of the SSU rRNA gene by Gerbod *et al.* (2001). Analysis of the sequence data showed significant similarity to other parabasalids sequences. The *H. meleagridis* sequence showed a reduced G+C content and an increased chain length when compared to other trichomonads, this phenomenon is also observed in *D. fragilis*. Phylogenic analysis determined a close relationship between *H. meleagridis* and *D. fragilis*. It also showed that both organisms share a recent common ancestor, which exhibits a more complex cytoskeleton structure. Such a finding supports the hypothesis that the morphological arrangement of both species may have arisen through secondary reduction or loss of some cytoskeletal structures (Gerbod *et al.*, 2001).

Currently all the evidence supports that *D. fragilis* is a trichomonad that has permanently lost flagella and kinetosomes from all stages of its life cycle. Through microscopy, antigenic analysis and molecular characterisation of the SSU rRNA gene, *D. fragilis* has been shown to be closely related to the trichomonad *H. meleagridis*. However, its exact phylogenic position still remains to be completely resolved.

It has taken over sixty years and the advent of molecular techniques for Dobell's hypothesis that *D. fragilis* was not an amoeba but a flagellate that had permanently lost its flagella to be finally strongly supported by the application of more modern technologies.

The following classification of *D. fragilis* is currently in use;

Kingdom	Protista
Sub-Kingdom	Protozoa
Phylum	Sarcomastigophora
Sub-Phylum	Mastigophora
Class	Zoomastigophora
Order	Trichomonadida
Family	Monocercomonadidae
Genus	Dientamoeba
Species	*Dientamoeba fragilis* (Jepps and Dobell, 1918)

1.4 CLINICAL ASPECTS AND EPIDEMIOLOGY

D. fragilis has been described throughout the world, with reported cases occurring on all major continents. Worldwide prevalence of *D. fragilis* varies widely from 0.4 - 53% (Windsor and Johnson, 1999). Some of the major studies are summarised in Table I. In Australia and New Zealand the reported prevalence rate ranges from 0.4% in Western Australia (Anonymous, 1992) to 1.5% in an urban community in Brisbane (Sawangjaroen *et al.*, 1993) to 2.2% in Christchurch New Zealand (Oxner *et al.*, 1987) and 16.8% in suburban Sydney (Walker *et al.*, 1985). A longitudinal study of parasite infections in Aboriginal children from the Queensland outback found a prevalence of 5.0% for *D. fragilis* (Welch and Stuart, 1976).

Since the first description of *D. fragilis* in 1918, reports in the literature have documented this parasite in most countries throughout the world. Initially Jepps and Dobell (1918) concluded that this parasite was non-pathogenic, however researchers questioned the pathogenicity of *D. fragilis* almost immediately. A study in the Philippines in 1919 found 3 cases of *D. fragilis* in 100 symptomatic children (Haughwout and Horrilleno, 1920). The following year Jepps described 10 cases *of D. fragilis* from 971 soldiers at a war hospital (Jepps, 1921). These reports lead to an increased interest in the parasite and some

five years later *D. fragilis* had been reported throughout the world (Taliaferro and Becker, 1924). Gittings and Waltz (1927) were the first to report 2 cases of children with *D. fragilis* in which they improved clinically after treatment.

Table I. Worldwide prevalence of *D. fragilis*

Country	Number of Samples Studied	Positive (%)	Author/ Year
U.S.A	20,917	2.4%	Kean and Malloch, 1966
Israel	201,750	15.2%	Talis *et al.*, 1971
Canada	43,029	4.2%	Yang and Scholten, 1977
U.S.A	220	53%	Millet *et al.*, 1983
New Zealand	5,595	2.2%	Oxner *et al.*, 1987
Australia	260	1.5%	Sawangjaroen *et al.*, 1993
Oman	857	5.1%	Windsor *et al.*, 1998
Turkey	400	8.8%	Girginkardesler *et al.*, 2003

Wenrich *et al.* (1935) reported an incidence of 4.3% of *D. fragilis* from 1,060 university students in the USA. They found that there was a higher rate of gastrointestinal symptoms in the students infected with *D. fragilis* than those infected with *E. histolytica*, with diarrhoea and abdominal pain present in the majority of cases. The same year Hakansson (1936) described a case of *D. fragilis* in a 48-year-old physician (himself) who complained of gastrointestinal symptoms. After two weeks of recurrent symptoms he was treated with carbarsone, which lead to complete resolution of symptoms and

negative post-therapy stool samples. Hankansson then collected a group of 12 patients with *D. fragilis* infection, six of which were symptomatic, and treated them with carbarsone all of whom responded to treatment resolving their symptoms and clearing the parasite (Hankansson, 1937).

A study conducted on US Navy personnel in 1939 who were returning from military service in Asia found *D. fragilis* in 26%, 27% of whom had gastrointestinal complaints (Sapero, 1939). Hood (1940) also believed that *D. fragilis* was pathogenic and showed of their elimination of the parasite with arsenical or oxyquinoline compounds which usually cured the patients symptoms.

Wenrich (1944) reported that he had been chronically infected with *D. fragilis* on two occasions. Both infections lasted 2 years and 2 years, 2 months respectively, eventually spontaneously resolving. The infection caused frequent bouts of diarrhoea that gradually abated over time.

Knoll and Howell (1945) studied six patients with *D. fragilis*, three children and three adults who had acute and chronic gastrointestinal symptoms for up to 1.5 years. After administration of carbarsone the clinical symptoms disappeared along with the *D. fragilis*. These findings lead these researchers to propose a pathogenic role for *D. fragilis*.

During a 6-year period, 1957-1964, Kean and Malloch (1966) found *D. fragilis* in 2.4% of 20,917 stools from 14,203 patients examined in the parasitology laboratory at Cornell University Medical College, New York. One hundred pure cases, where *D. fragilis* was the only parasite found on stool examination, were followed up and clinical information gathered. Abdominal pain, diarrhoea and nausea were the most common clinical presentation. The majority were U.S. citizens who had not travelled outside the country. Treatment that eliminated the parasite was also shown to give symptomatic relief.

A relatively high incidence of *D. fragilis* was found in Israel. From 1960-1969 201,750 stools were examined, with *D. fragilis* being found in 15.2% of samples (Talis *et al.*, 1971).

A major study by Yang and Scholten (1977) found *D. fragilis* in 4.2% of 43,029 individuals who submitted stools for parasitological examination during 1970-1974 in Ontario, Canada. Infections were found to be more common in females than males with nearly half occurring in patients under 20 years old. The most common symptoms included diarrhoea, abdominal pain and loose stools (Yang and Scholten, 1977).

At the Parasitology Division of the Clinical Laboratories at the University of California, Los Angeles, stool samples from 695 children were examined for ova and parasites between 1976-1978. *D. fragilis* was recovered from 65 children, which represented an overall incidence of 9.4%. A retrospective analysis was then undertaken involving 35 children. It was shown that 91% of the children had gastrointestinal symptoms including abdominal pain, diarrhoea, and anorexia. The diarrhoeal history of the children varied from frequent and daily bowel movements to episodes of intermittent diarrhoea. An increased peripheral eosinophil count was also noted in 50% of children with *D. fragilis*. Observations of symptomatic recovery of patients after treatment of *D. fragilis* infection indicated a pathogenic role for this parasite in children (Spencer *et al.*, 1979).

In a retrospective analysis involving 50 patients with pure *D. fragilis* infections, gastrointestinal symptoms were present in the majority of the subjects. With abdominal pain, diarrhoea and nausea were the most common symptoms. There were 20 patients with chronic complaints, which had been present from 6 months to 18 years, with 17 patients having symptoms for over 2 years. Eosinophilia was found in 53% of these adult patients with chronic symptoms (Spencer *et al.*, 1982). In another study conducted by Spencer *et al.* (1983), from 104 paediatric patients, *D. fragilis* was detected in 21% of children. Diarrhoea and abdominal pain were common in those with *D. fragilis* infection. In research by Preiss *et al.* (1991) from Germany, in 123 paediatric patients infected with intestinal protozoa, *D. fragilis* was found in 102 cases. Acute and recurrent diarrhoea

were found to be the most common symptoms. Therapy that leads to the elimination of *D. fragilis* was shown to resolve symptoms. In a third of children with dientamoebiasis peripheral blood eosinophilia was seen.

At the Washington State Public Health Laboratory a total of 237 cases of *D. fragilis* were identified in 1985-1986. Nearly 80% of patients reported symptoms associated with infection, the most common clinical manifestation being diarrhoea or loose stools (Grendon *et al.*, 1995)

A more recent study over a six-month period detected *D. fragilis* in 4.1% of 857 stool samples submitted to the Department of Microbiology and Immunology at the Sultan Qaboos University Hospital in Oman. *D. fragilis* was the most common enteropathogen encountered. Of the patients with *D. fragilis* infection, 83% had abdominal pain and 50% had diarrhoea, the duration of which varied from one month to two years (Windsor *et al.*, 1998).

A Swedish retrospective study of 87 patients diagnosed with *D. fragilis* found the highest incidence in pre-school boys. The majority of patients had symptoms of diarrhoea, abdominal pain and flatus (Norberg *et al.*, 2003). Turner (1985) concluded that clinical data collected on *D. fragilis* infections resembled that of *Giardia intestinalis* infections, and the clinical presentation of the two parasites are very similar. A recent study in Turkey found *D. fragilis* to be as prevalent and pathogenic as *G. intestinalis*. In stool samples from 400 patients *D. fragilis* was found in 8.8% of cases with *G. intestinalis* in 8.5%. The most common symptoms were abdominal and diarrhoea in both infections (Girginkardesler *et al.*, 2003).

Higher rates of infection are often seen where personal hygiene is poor. This is seen in studies from mental institutions (Nailman *et al.*, 1980) and from disadvantaged groups (Melvin and Brooke, 1962). One study of interest involved approximately 300 members of a religious sect in the U.S.A. The group's religious and social activities were conducted in a semicommunal setting. *D. fragilis* was found in 53% of the community (Millet *et al.*,

1983). Over 81% of *D. fragilis* infected patients had gastrointestinal complaints, most commonly recurrent or chronic diarrhoea. Substandard hygiene practices were evident among this group. In accordance with cultural beliefs, toilet paper was not used after defecation, bare hands were used to wash the anal area with soapy water. Hand washing before meals was not a common practice and meals were often eaten without the aid of cutlery.

A recent Australian study by Borody *et al.* (2002) showed that *D. fragilis* may be linked to with irritable bowel syndrome (IBS). Twenty-one patients diagnosed with IBS and concurrent *D. fragilis* infection were treated with iodoquinol and doxycycline. All showed complete elimination of *D. fragilis* with marked clinical improvement seen in the majority of patients.

Higher rates of *D. fragilis* infection are not seen in the homosexual community. It has been well documented that higher rates of enteric protozoal infections have been reported among homosexual men in metropolitan areas throughout the world (Markell *et al.*, 1984; Ortega *et al.*, 1984). However this phenomenon is not apparent for *D. fragilis*. A study on the prevalence of enteric parasites in homosexual patients over a 2.5-year period found 48.5% of patients harboured one or more intestinal protozoa. *D. fragilis* however only made up 1% of the protozoa found compared to *E. histolytica*, which accounted for 26% (Peters *et al.*, 1986). Another study in the San Francisco Bay area in the U.S.A found a prevalence of 47% of potentially pathogenic enteric protozoa among male homosexual patients. *E. histolytica* was found in 36% of patients and *D. fragilis* only in 1.3% (Ortega *et al.*, 1984). These rates that are found in male homosexual patients are comparable to those found in heterosexual groups.

There has only been one study on the seroprevalence of *D. fragilis*. Chan *et al.* (1996) used an indirect immunofluorescence assay; of 189 randomly selected healthy individuals from Canada (age 6 months - 19 years), 91% were seropositive for *D. fragilis* antibodies. This study suggests *D. fragilis* infection is common in Canada, however the researchers

did not raise the issue of cross-reactivity and the 91% positive rate could be due in part to this phenomenon.

One study in Argentina suggested that the incidence of *D. fragilis* infections may be higher in immunocompromised patients (Mendez *et al.*, 1994). In all other studies conducted immunosuppression does not seem to be a contributing factor for infection with *D. fragilis*.

Cuffari *et al.* (1998) reported a case of eosinophilic colitis associated with *D. fragilis*. A female four-year old child presented with a three-year history of chronic diarrhoea. She was originally diagnosed as having an intolerance to cows milk. Despite adhering to a strict bovine protein-free diet for three months the patient complained of recurrence of severe abdominal cramps and diarrhoea. A colonoscopy was performed and biopsies were taken. Areas within the lamina propria showed eosinophilic infiltrates, and a biopsy from the descending colon showed more than 50 eosinophils per high power field. Isolated eosinophils were also observed infiltrating the glandular and surface epithelium. A diagnosis of eosinophilic colitis was made on the basis of the histopathology and stool samples for ova, cysts and parasites which were collected from the patient. *D. fragilis* trophozoites were detected in the patients stool. She was treated with iodoquinol and promptly became asymptomatic and remained so after follow-up for a number of years (Cuffari *et al.*, 1998).

Another case report of colitis associated with *D. fragilis* was described in a Burmese woman who presented with ulcerative colitis (Shein and Gelb, 1983). The patient was hospitalised and sigmoidoscopy revealed multiple punctate-apthous ulcers with mild to moderate erythematous, nonfriable, intervening mucosa. Stool cultures were negative for bacterial enteropathogens. After 1 week of hospitalisation the patient's symptoms of abdominal pain and multiple loose bowel movements continued. A repeat sigmoidoscopy was ordered and biopsies taken. The biopsies revealed shallow ulceration with evidence of acute and chronic inflammation. When aspirates from mucosal ulcerations were fixed and stained with trichrome many *D. fragilis* trophozoites were seen. The patient was

treated with diiodohydroxyquin and metronidazole and subsequently made a complete recovery. Based on the clinical, radiological, endoscopic and histologic findings the authors concluded *D. fragilis* to be the cause of this invasive colitis (Shein and Gelb, 1983).

Another case of ulcerative colitis was also documented in Canada in a nine-year-old boy where there was a close association with *D. fragilis* (Ring *et al.*, 1984). These three case reports suggest that *D. fragilis* may be a rare cause of colitis in certain individuals.

Numerous studies have shown that treatment which eliminates the organism results in clinical improvement (Robertson, 1923; Wenrich *et al.*, 1935; Hakansson, 1936, 1937; Wenrich, 1937; Mollari and Anzulorich, 1938; Wenrich, 1944; Knoll and Howell, 1945; Yoeli, 1955; Kean and Malloch, 1966; Steintz *et al.*, 1970; Chang, 1973; Yang and Scholten, 1977; Spencer *et al.*, 1982; Shein and Gelb, 1983; Oxner *et al.*, 1987; Butler, 1996; Cuffari *et al.*, 1998; Preiss *et al.*, 1990, 1991). There is overwhelming evidence in the scientific literature that *D. fragilis* is pathogenic with diarrhoea and abdominal pain been the most common symptoms of infection. Both acute and chronic infections have been documented. A number of case studies have also shown that *D. fragilis* may be rare cause of colitis (Windsor and Johnson, 1999).

1.5 GENETIC DIVERSITY

Only three studies have addressed the issue of genetic diversity of the organism. By using PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of ribosomal genes of 12 *D. fragilis* isolates the researchers found that organisms currently being reported as *D. fragilis* represent at least two significantly different genetic entities (Johnson and Clark, 2000). Johnson and Clarke (2000) estimated a sequence divergence of 2% between the two SSUrRNA genotypes of *D. fragilis*; this was later supported by research conducted in the Netherlands (Peek *et al.*, 2004) by sequencing a 558bp region of the SSUrDNA from *D. fragilis* isolates and comparing this with available sequence data. Windsor *et al.*, (2004) undertook PCR-RFLP analysis of the SSU rRNA gene of 33 *D.*

fragilis isolates from the U.K. All 33 isolates gave the same RFLP patterns indicating that *D. fragilis* displays very little variation in its SSU rDNA amongst U.K. strains. The exact extent of this genetic diversity is unknown and further study is required to resolve this issue. However many enteric protozoa exhibit extensive genetic diversity in the absence of morphological variation, and protozoa that were originally thought to be one species have subsequently been found to comprise of two or more new species (Sargeaunt, 1992). This has yet to be established for *D. fragilis* but may have important clinical and epidemiological implications.

1.6 TRANSMISSION

The mode of transmission of *D. fragilis* has yet to be determined. Unlike many other intestinal protozoa *D. fragilis* apparently has no cyst stage. The lack of a cyst stage makes it unlikely that the organism can survive in the environment for any length of time. Estimates of the survival time of trophozoites in stool specimen vary from 6 to 48 hours (Hakansson, 1936). Wenrich (1944) conducted a number of experiments and was unable to keep *D. fragilis* trophozoites alive in boiled pond water or boiled hay infusion. It was also shown that in tap water they swell and burst within minutes. Yang and Scholten (1977) failed to keep *D. fragilis* trophozoites alive in simulated gastric juice.

D. fragilis can be grown in xenic culture systems and a wide variety have been used to grow trophozoites from stool samples (Clark and Diamond, 2002). When using culture systems it was shown that positive cultures were only obtained from stools 8-11 hours old that had not been refrigerated. *D. fragilis* was not cultured from any stool greater than 12 hours old (Sawangjaroen *et al.*, 1993). Brug (1936) found that cultures of *D. fragilis* that were exposed to room temperature were adversely affected. Dobell (1940) also demonstrated the fragile nature of this organism. Given the above data it must be assumed that transmission must occur directly. Transmission via contaminated food and water would be unlikely as the organism does not to survive outside the human body for long periods of time (Dobell, 1940).

Dobell (1940) showed that the amoeboflagellate *H. meleagridis* and *D. fragilis* were morphologically similar. He also postulated that they both might have similar modes of transmission. *H. meleagridis* is a common pathogen of domestic fowl especially turkeys, and is transmitted from bird to bird via the eggs of the nematode *Heterakis gallinae*. Dobell (1940) thought that *D. fragilis* might be transmitted by the ova of helminth eggs.

Burrows and Swerdlow (1956) were the first to propose that *E. vermicularis*, the human pinworm, might be a vector for *D. fragilis*. They studied 1518 appendices histologically and found 22 harbouring *D. fragilis*, 12 of which also contained adults or eggs of *E. vermicularis*. Based on random and natural distributions of the two species they calculated that the actual incidence was 20 times the expected incidence. They also claimed to have visualised small amoeboid bodies in the eggs of pinworms that were similar to forms of *D. fr*agilis found in the lumen of formalin-fixed appendices. They were unable to find similar forms from appendices that did not harbour *D. fragilis*. The researchers tried to culture *D. fragilis* from the *E. vermicularis* eggs but were unsuccessful (Burrows and Swerdlow, 1956).

Other researchers also attempted culturing *D. fragilis* from the eggs of *E. vermicularis* but they also failed to isolate the parasite (Yang and Scholten, 1977). Several other investigators also found a higher than anticipated coincidence of *D. fragilis* and *E. vermicularis* infections (Burrows *et al.*, 1954; Priess *et al.*, 1991). Yang and Scholten (1977) found a nine times higher than expected coincidence of *D. fragilis* and *E. vermicularis* co-infection than theoretically expected. Of a total of 237 patients with *D. fragilis* 21 were tested for the presence of pinworm eggs. Three of the 21 patients were positive for *E. vermicularis* ova (Grendon *et al.*, 1995). For statistical significance a larger proportion of cases should have been tested for pinworm infection, as it is difficult to draw any conclusions from such a small sample size.

Burrows was also accidentally infected with *D. fragilis* and was simultaneously infected with *E. vermicularis* (Burrows and Swerdlow, 1956). Ockert (1972) experimentally infected himself with pinworm eggs from a boy who was co-infected with *D. fragilis* and

pinworm; he subsequently developed both enterobiasis and dientamoebiasis. Three years later Ockert (1975) infected two other human subjects with *D. fragilis* from the ova of *Enterobius vermicularis*. Ockert and Schmidt (1976) compared the isoelectric point of *D. fragilis* from culture and the suspected *D. fragilis* found in pinworm eggs. They found the electrostatic charges were either similar or identical between the two. They therefore concluded that the amoeboid bodies visualised inside the *E .vermicularis* ova were indeed *D. fragilis*. However this was a very large scientific conclusion to make on the basis of similar electrostatic charges alone.

A more recent study examined 414 histological sections of appendices parasitologically (Cerva *et al.*, 1991). The researchers found *E. vermicularis* eggs in 8.7% and *D. fragilis* in 4.8% of cases. They also found the coincidence of *D. fragilis* and *E. vermicularis* infections was 50%. Due to these above findings it was proposed by the researchers that *D. fragilis* is transmitted via *E. vermicularis* ova.

A study from Bangkok, Thailand, found on examination of nearly 100 faecal specimens containing the human roundworm *Ascaris lumbricoides* these specimens also had *D. fragilis* present in 38 samples. Within these ova they found oval bodies that they believed were *D. fragilis* (Sukanahaketu, 1977). As a chemical process was used to clear the thick bile stained shell of *A. lumbricoides* the morphology of the oval bodies did not resemble classically stained *D. fragilis*, with the nuclear morphology difficult to discern. However the author concluded that *A. lumbricoides* could be a vector for the transmission of *D. fragilis*.

It is unlikely that helminths such as *A. lumbricoides* and *Trichuris trichura* are the vectors of *D. fragilis*. *D. fragilis* has a metropolitan distribution in many parts of the developed world in areas where the incidence of *A. lumbricoides* and *T. trichiura* are low if not non-existent.

Kean and Malloch (1966) studied 100 patients with *D. fragilis* infections and found them all to be negative for *E. vermicularis*. This would argue against the theory that *E.*

vermicularis plays a significant role in the transmission of *D. fragilis*. A more recent study of 25 paediatric cases of *D. fragilis* found no infection was associated with *Enterobius vermicularis* (Cuffari *et al*., 1998). Most studies that have looked at *D. fragilis* infections have failed to adequately look for *E. vermicularis* infections. It has yet to be proven what role helminth ova play in the transmission of *D. fragilis*. Further study is required to ascertain the true mode of transmission of this organism.

Dobell (1940) was unable to infect himself or macaque monkeys with cultures of *D. fragilis* given orally, and rectally in other macaques. He also inoculated 6 chicks rectally with cultures of *D. fragilis*. One of the chicks developed a caecal infection. Cultures from this infection failed to infect 3 other chicks (Dobell, 1940).

Knoll and Howell (1945) inoculated *D. fragilis* cultures orally and rectally into kittens, however no infection or symptoms were demonstrated and no amoeba were recovered at autopsy. Kean and Malloch (1966) attempted to produce an infection with *D. fragilis* in the caecum of rats. Preliminary results indicated that *D. fragilis* does adhere to the caecal mucosa and cause damage to the underlying cells, as edema of the mucosa was evident. Other attempts to introduce *D. fragilis* infections in man and animals have met with little success and Koch's postulate has not been fulfilled for this organism.

There have only been a handful of reports of *D. fragilis* in species other than humans. Non-human primates including; macaques (Hegner and Chu, 1930; Knowles and Das Gupta, 1936), and baboons (Myers and Juntz, 1968) have been reported in the literature as having *D. fragilis* trophozoites in their stools and it has also been reported in a sheep (Noble and Noble, 1952). There have been no reports in the scientific literature regarding *D. fragilis* carriage in animals in the last 50 years. Given this coupled with the high incidence of *D. fragilis* in humans we must assume that humans are the primary host for this organism.

Clearly further study is required to elucidate the exact nature of the mode of transmission of *D. fragilis*. Due to the fragile nature of this organism and the fact that no cyst stages

have been demonstrated it would be advantageous for *D. fragilis* to be transmitted by a vector. Several studies have found a higher than anticipated coincidence of *D. fragilis* and *E. vermicularis* infections. Transmission by means of faecally contaminated food or

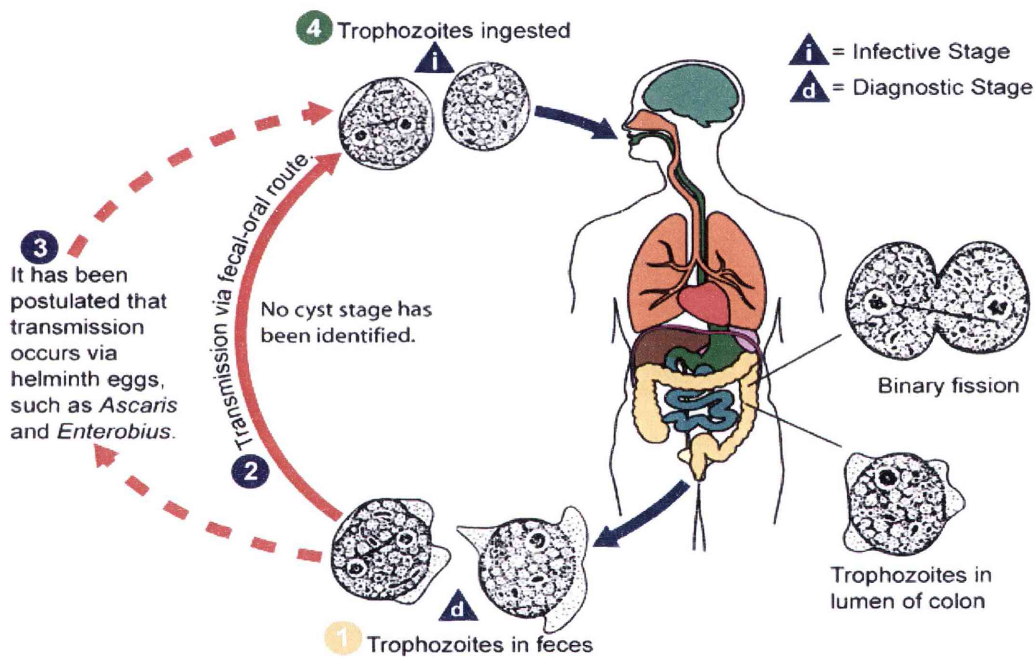


Figure 1.2 Life-cycle of *D. fragilis* (from CDC web site www.dpd.cdc.gov/dpdx/HTML/ImageLibrary?Dientamoeba_il.htm)

water seems unlikely, as there are no cyst stages and the fact that the parasite cannot survive for any length of time in the environment. However direct transmission could occur and is seen in other intestinal parasites that don't have cyst stages such as *Pentatrichomonas hominis*, which does infect humans. Higher rates of *D. fragilis* infection are also seen where personal hygiene is poor. These observations would also support direct transmission. Currently 2 modes of transmission are hypothesised for *D. fragilis*; direct transmission or via *E. vermicularis* ova (figure 1.2).

1.7 PATHOLOGY

Unfortunately there has been insufficient scientific research on the pathology of infections and as a result, information in the literature regarding the pathogenesis and pathology resulting from *D. fragilis* infection is minimal.

Burrows *et al.* (1954) was the first to report pathological findings in four appendices infected with *D. fragilis*. They found the following histopathological changes:

- Case 1 - Lymphoid hyperplasia, organised periappendicitis, fibrosis of appendix, numerous *D. fragilis* trophozoites in the lumen with ingested red blood cells.
- Case 2 - Catarrhal appendicitis, fibrosis of appendix, *D. fragilis* trophozoites with ingested red blood cells.
- Case 3 - Lymphoid hyperplasia, fibrosis of appendix, *D. fragilis* trophozoites with ingested red blood cells.
- Case 4 - Acute periappendicitis, fibrosis of appendix, *D. fragilis* trophozoites with ingested red blood cells.

As this marked fibrosis of the appendix was evident it was postulated that *D. fragilis* elaborates a low-grade irritation that induces an inflammatory response that causes the fibrosis (Burrows *et al.*, 1954). This was further documented in pathological findings from 15 appendices infected with *D. fragilis*, all of which showed marked fibrosis (Swerdlow and Burrows, 1955). A study by Cerva *et al.* (1991) found 20 *D. fragilis* trophozoites in 414 appendices that they examined histopathologically. They failed to

demonstrate any characteristic histopathology and no fibrosis was found in any of the appendices (Cerva *et al.*, 1991).

Kean and Malloch (1966) undertook experiments to produce an infection in the caecum of rats with *D. fragilis*. Preliminary results indicated that edema of the mucosa was evident, and that the trophozoites do attach to the caecal mucosa and cause damage to the cells. However no invasion of cells or ulceration was demonstrated.

D. fragilis has been shown by a number of researchers to ingest red blood cells. Dobell was the first to report this finding (Dobell, 1940). In later years two more parasitologists demonstrated the erythrophagocytic capacity of *D. fragilis* (Burrows *et al.*, 1954; Swerdlow and Burrows, 1955).

Cuffari *et al.* (1998) reported a case of eosinophilic colitis associated with *D. fragilis* in a female four-year-old child who had a history of chronic diarrhoea. Another case report of colitis associated with *D. fragilis* was described in a Burmese woman who presented ulcerative colitis. Based on the clinical, radiological, endoscopic and histologic findings the authors concluded *D. fragilis* to be the cause of this invasive colitis (Shein and Gelb, 1983). A second case of ulcerative colitis was also documented in Canada in a nine year-old boy where there was a close association with *D. fragilis* (Ring *et al.*, 1984).

Although the reports in the literature are scant, *D. fragilis* has been shown to be the cause of fibrosis, ingest red blood cells and cause an ulcerative colitis. Burrows *et al.* (1954) proposed that pathogenic protozoa should fulfil one or more of the following criteria: invade and or destroy host tissue; produce toxic by-products; or produce a localised tissue reaction. According to these authors criteria for pathogenic protozoa, *D. fragilis* was shown to induce a localised tissue reaction and invade/destroy host tissue, thus making *D. fragilis* a potentially pathogenic protozoa.

A lack of a suitable animal model has also hampered study into the clinical manifestations of *D. fragilis* infection. Macaque monkeys, chickens, kittens and rats have

all been used as animal models (Dobell, 1940; Knoll and Howell, 1945; Kean and Malloch, 1966) with little or no success.

It is evident that more research is needed to ascertain the exact pathological processes that results from *D. fragilis* infection. There are many questions that still need to be answered.

1.8 DIAGNOSTIC TECHNIQUES

It has been more than 85 years since *D. fragilis* was first described. Since this time there has been little if any advances in the techniques used to diagnose this parasite. Definitive diagnosis is still based on permanent stains of fixed faecal smears.

In wet preparations, *D. fragilis* appears as a non-specific rounded mass and the nuclear structure cannot be seen in either saline or iodine preparations (Windsor and Rafay, 1997). As trophozoites degenerate rapidly, prompt fixation of the specimen is necessary (Yang and Scholten, 1977). Successful diagnosis of *D. fragilis* is closely associated with the use of permanent stains of faecal smears.

Many different stains and fixatives have been used successfully with *D. fragilis*. Suitable fixatives include polyvinyl alcohol (Goldman and Brooke, 1953), sodium-acetate-acetic acid-formalin (Yang and Scholten, 1977), modified Schaudinn's fixative (Scholten, 1972) and merthiolate-iodine-formalin (Walker *et al.*, 1985). A wide variety of permanent stains have been used to detect *D. fragilis* the most common being iron haematoxylin and trichrome stain (Windsor and Johnson, 1999).

Parasite culture techniques have been used with success for detecting *D. fragilis*. As with fixatives and stains, a large number of culture systems have been used, including the following: Boeck and Drbohlav's (Boeck and Drbohlav, 1925), Robinson' medium (Robinson, 1968), Dobell and Laidlow's medium (Dobell and Laidlow, 1926), Cleavland-Collier's medium (Cleveland and Collier, 1930), Balamuth's medium (Balamuth, 1946)

and TYGM-9 (Diamond, 1982). Dobell was the first to grow "mono-protist" *D. fragilis* cultures in 1929 by using a diphasic medium devised by himself, an inspissated horse serum slant overlaid with diluted egg whites in Ringer's solution and supplemented with rice. He also reported that it grew best at 41°C, a temperature much higher than one would expect an intestinal protist to grow (Dobell, 1940).

Several studies have shown that culture techniques are more sensitive than permanent stains. Sawangjareon *et al.* (1993) found culture to be more sensitive than microscopy in diagnosing *D. fragilis*. A more recent study showed that culturing for *D. fragilis* using a modified Robinson medium dramatically increased the detection rate for *D. fragilis* (Windsor *et al.*, 2003).

However the cultivation of luminal parasitic protists is technically difficult, time consuming and often unrewarding (Clark and Diamond, 2002). As such, these techniques are usually restricted to specialist parasitology laboratories, and are not offered by routine laboratories. *D. fragilis* is regarded difficult to establish in long-term culture, but can often be grown for a few subcultures before dying out (Clark and Diamond, 2002). One draw back of culture systems is that specimens need to be inoculated promptly as reduced temperatures adversely affect *D. fragilis*, and trophozoites degenerate rapidly (Hakansson, 1936; Wenrich, 1944). Specimens also cannot be refrigerated as this greatly reduces recovery rates (Sawangjaroen *et al.*, 1993).

All the culture methods that have been used with *D. fragilis* are xenic culture systems. These are systems in which the parasite is grown in the presence of the bacterial flora present in the patient's stool. Attempts to grow *D. fragilis* in axenic culture systems have all failed (Chan *et al.*, 1993, 1994). The unavailability of an axenic culture system could explain why so little progress has been made with this organism. Other parasites such as *Entamoeba histolytica*, *Giardia* and *Trichomonas* have all been grown in axenic systems allowing closer study of the organisms to be undertaken.

Immunofluorescence microscopy using commercially available monoclonal antibodies and several commercial enzyme immuno-assay tests are available for the detection of antigen in stool for *Cryptosporidium parvum*, *Giardia intestinalis* and *Entamoeba histolytica*. A list of commercially available kits for the immunodetection of the pathogenic enteric parasites are shown in Table II. No such tests are commercially available for *D. fragilis*.

Chan *et al.* (1993) developed an indirect fluorescent-antibody assay to detect *D. fragilis* in preserved faecal samples. A total of 155 specimens were tested, 42 with no parasite, 9 with *D. fragilis* and 104 with various other protozoa. There were no false positive readings and no cross-reaction with the other protozoa. Two of the nine positive samples gave doubtful results. The authors concluded that this was due to the low number of trophozoites in the two samples. This method shows promise and indicates that other diagnostic tests such as enzyme immuno-assays could be developed.

Molecular biology techniques such as PCR offer a highly sensitive and specific alternative to traditional diagnostic approaches such as microscopy. Regions of ribosomal DNA are ideal targets for PCR development as they exhibit interspecific variability yet are highly conserved. Ribosomal RNA (rRNA) genes are amongst the most ubiquitous and conserved DNA sequences in nature. For this reason they have been extensively used for phylogenic analysis and as diagnostic probes (Stackebrandt *et al.*, 1992). PCR techniques have been used for the diagnosis of a wide variety of parasites from clinical samples, including; *Cryptosporidium parvum* (Limor *et al.*, 2002), *Entamoeba histolytica* (Troll *et al.*, 1997), *Entamoeba dispar* (*Troll et al.*, 1997), *Leishmania* (Aviles *et al.*, 1999), malaria (Sethabur *et al.*, 1992) and microsporidia (Wolk *et al.*, 2002).

Table II. Commercially available kits for the immunodetection of enteric parasites[a]

Organism	Kit name -Test type	Company
Cryptosporidium spp.	ProSpecT - EIA	Alexon-Trend
	Crypto-CELISA - EIA	Cellabs
	Premier - EIA	Meridian
	Cryptosporidium - DFA	Novacastra
	Cryptosporidium - EIA	Techlab
	RIM Cryptosporidium - EIA	Remel
Cryptosporidium spp. and *Giardia intestinalis*	ProSpecT - EIA	Alexon-Trend
	ColorPAC – ICT	Becton Dickinson
	Crypto'Giardia-Cel - DFA	Cellabs
	Merifluor - DFA	Meridan
C. parvum, *G. intestinalis*, *Entamoeba histolytica / dispar* group	ICT	Biosite
E. histolytica	*E. histolytica* - EIA	TechLab
	E. histolytica - EIA	Wampole
	Entamoeba-CELISA - EIA	Cellabs
E. histolytica / dispar group	ProSpecT - EIA	Alexon-Trend
	Entamoeba-CELISA - EIA	Cellabs
	E. histolytica/dispar	TechLab
	E. histolytica/dispar	Wampole
G. intestinalis	ProSpecT - EIA	Alexon-Trend
	ProSpecT - ICT	Alexon-Trend
	Giardia-CELISA - DFA	Cellabs
	Giardia-Cel - EIA	Cellabs
	Premier - DFA	Meridian
	Giardia - EIA	Novacastra
	Giardia - EIA	TechLab
	Giardia - EIA	Wampole

[a] Data from Wilson and Schantz (2000).

Only one study has reported the development of a conventional PCR by amplification of the small-subunit rRNA gene to detect *D. fragilis*; however the sensitivity and specificity of the assay was not determined in this study (Peek *et al.*, 2004). In this study the detection limit of PCR was the equivalent of approximately 0.1 *D. fragilis* trophozoites per sample (Peek *et al.*, 2004). No real time PCR assays have been developed to date for *D. fragilis*.

As permanent stains are time consuming and require a highly trained microscopist to read the stains, another diagnostic method would be helpful. Care must be taken when reading the slides as *D. fragilis* may be difficult to distinguish from non-pathogenic protozoa such as *E. nana*. Molecular techniques could provide an additional tool to be used in diagnosing *D. fragilis* infections.

As with other enteric protozoan infections the collection of multiple stool specimens is essential to aid in diagnosis. Intermittent shedding of *D. fragilis* occurs regularly with the daily shedding of trophozoites being highly variable (Van Gool *et al.*, 2003; Peek *et al.*, 2004). Hiatt *et al.* (1995) compared the sensitivity of examining one stool specimen to that of three specimens. Using conventional permanent staining it was found that the additional stool examinations increased the percentage of positive results by 31.1% for *D. fragilis*. This data suggests that even in symptomatic patients the examination of a single stool specimen could miss a large number of *D. fragilis* infections (Hiatt *et al.*, 1995).

Overall the diagnostic tools available for *D. fragilis* are limited. The same methods for diagnosis are still being used as they were at the turn of last century. Where many advances have been made in the diagnosis of other pathogenic parasites once again *D. fragilis* has been neglected. Newer diagnostic methods would be a welcome addition for both laboratories and physicians. Current diagnostic techniques are laborious, time consuming, require highly trained staff, and are prone to human error. Correct and prompt diagnosis is essential given the potential for chronic long-term infections. Recognition is also important for clinical management, as specific treatment is often required.

1.9 THERAPY

Many studies have shown that the elimination of *D. fragilis* with antimicrobial agents usually relieves clinical symptoms (Johnson and Windsor, 1999). As such the treatment of symptomatic patients with *D. fragilis* infections is warranted.

Hakansson was one of the first parasitologists to advocate the use of antimicrobials for the treatment of *D. fragilis*. He successfully treated himself and 6 patients with the arsenic compound carbarsone (Hakansson, 1936, Hakansson, 1937). Knoll and Howell (1945) administered carbarsone to 3 children and 3 adults with acute and *chronic D. fragilis* infection. In all patients the clinical symptoms improved quickly after treatment. Carbarsone is no longer available for human use.

A newer arsenic compound, diphetarsone, was shown to be 100% effective in treating *D. fragilis* infections. However side effects were seen, in particular transient liver function abnormalities (Keystone *et al.*, 1983). Due to the limited availability of this drug and of the reluctance to use arsenic based compounds the drug has not been used widely and is not recommended for *D. fragilis* treatment.

Tetracycline was recommended for the treatment of *D. fragilis* infections by Dardick *et al.* (1983) due to its safety and efficacy. This recommendation was based on a single case report of a 35-year-old male who was successfully treated with tetracycline (Dardick *et al.*, 1983). Tetracycline use is not recommended in children or pregnant women due to its deleterious effect on dental development. No large-scale studies have examined the efficacy of tetracycline in regards to *D. fragilis*, and until such evaluations are undertaken one could not recommend tetracycline with much confidence.

Iodoquinol (diidohydroxyquin) was widely used to treat *D. fragilis* infections particularly in North America (Shein and Gelb, 1983; Butler, 1996). Millet *et al.* (1983) treated 12 patients suffering with *D. fragilis* infections with iodoquinol. 10 of the 12 treated eliminated the parasite, although three subjects required a second course of therapy.

Spencer *et al.* (1979) showed that therapy with iodoquinol or metronidazole was effective in 18 paediatric patients. In another study on paediatric patients Cuffari *et al.* (1998) showed that metronidazole was effective in five patients and iodoquinol in four others. In one patient neither iodoquinol nor metronidazole was effective in resolving symptoms. Successful eradication of both symptoms and parasite were obtained with paromomycin.

Metronidazole was used in three patients with dientamoebiasis in New Zealand. The treatment eradicated the parasite in all patients, however one needed a further course of metronidazole in combination with oxytetracycline to finally eradicate the organism (Oxner *et al.*, 1987). In a study from Sweden 32 patients infected with *D. fragilis* were treated with metronidazole. The drug was given at various doses for various lengths of time. Only four patients responded to the metronidazole treatment (Norberg *et al.*, 2003). No details were given to the exact dosages or duration of treatment so it is difficult to comment on the clinical effect of metronidazole under these circumstances.

Preiss *et al.* (1990) studied 123 paediatric patients with *D. fragilis* infections. The efficacy of 5 antimicrobial agents is shown in Table III. They found metronidazole to be effective with 70% of patients eliminating the parasite and symptoms after one treatment. A second treatment was required for 21 patients with another drug. While10 patients had to be treated a third time to eliminate *D. fragilis* and accompanying abdominal complaints. They recommended a 10-day treatment with metronidazole for *D. fragilis* infections.

A recent study in Turkey evaluated the use of secnidazole, a newer nitromidazole derivative, in 35 patients with *D. fragilis* infection. *D. fragilis* was eradicated in all but one patient with a single dose of secnidazole, and a second dose was necessary in one patient. This data suggested that secnidazole is effective in achieving parasitological and clinical cure.

Table III. Efficacy of antimicrobials in patients with *D. fragilis* infections (adapted from Preiss *et al.*, 1991)

Antimicrobial	Patient Numbers	Cured	
		Number	%
Metronidazole	91	64	70
Oxytetracycline	9	8	90
Doxycycline	4	3	75
Erythromycin	6	3	50
Hydroxychinoline	5	1	20

Susceptibility testing of *D. fragilis* ATCC 30948 was performed with iodoquinol, paromomycin, tetracycline, and metronidazole in a dixenic culture. The minimal inhibitory concentrations were as follows; iodoquinol 128μg/ml, paromomycin 16 μg/ml, tetracycline 32μg/ml, and metronidazole 32μg/ml. It is difficult to correlate these MIC's to clinical responses. This study was undertaken in a dixenic culture system containing *Klebsiella pneumoniae* and *Bacteroides vulgatus*. There is potential for antimicrobial effects on the bacterial flora, which supports the growth of *D. fragilis*. Therefore the significance of these MIC's would be questionable at best.

To date most studies involving antimicrobial treatment have been case studies or small-scale studies. More large scale randomised, double-blinded controlled studies are needed to determine the true efficacy of several of the antimicrobial agents mentioned above in successfully treating *D. fragilis* infections.

1.10 CONCLUSION

Numerous clinical and epidemiological studies have substantiated *D. fragilis* as a significant enteropathogen. It is therefore inexcusable that so little research has been undertaken on this organism.

Clifford Dobell, one of the parasitologists to first describe *D. fragilis*, wrote the following about this organism in 1940.

> To the protozoologist - if not the physician - *D. fragilis* is now, perhaps, the most interesting of all the intestinal amoebae of man: for we know less about it than about any of the others, and its life history and activities are still mysterious. Ever since I first saw this curious organism in 1917, I have been intrigued by its peculiarities and have taken every opportunity of studying it further: yet after more than 20 years of work and cogitation, I am still baffled...'

Over 65 years have passed since the distinguished parasitologist wrote these remarks and remarkably little has changed.

Of all the pathogenic protozoan parasites that infect humans the least amount of knowledge that we have acquired over the years concerns *D. fragilis*. Its life cycle and mode of transmission are both poorly defined. Pathogenesis of the organism and its exact mode of action are unknown. No animal models or axenic culture systems have been developed for the study of this organism. The diagnostic tests available are limited when compared to other protozoa.

More research is needed on the epidemiology, clinical syndromes and pathology of dientamoebiasis. The life cycle of *D. fragilis* still needs to be determined. Molecular epidemiology studies also should be undertaken as there is already evidence that two genetic stains of *D. fragilis* exist. Animal models would provide a greater understanding

in various aspects of not only the life cycle of *D. fragilis* but also in the pathogenesis involved with this organism. Such research will provide a better understanding of the epidemiology, pathogenicity, diagnosis, and treatment of *D. fragilis* infections.

1.11 PROJECT AIM

The overall aim's of this project were to determine the prevalence, genetic diversity and clinical relevance of *D. fragilis* infections in an Australian population. These answers were met by conducting research on the following objectives.

1.12 OBJECTIVES

The specific objectives of the research undertaken and described are to;

- Sequence the SSU rRNA gene of Australian *D. fragilis* isolates.
- Develop a conventional and real-time PCR method that is highly sensitive and specific for the detection of *D. fragilis* in unpreserved faecal specimens.
- Evaluate three diagnostic methods, including microscopy, conventional PCR and real-time PCR, for the detection of *D. fragilis* in stool specimens.
- Determine the genetic diversity of *D. fragilis* isolates by genotyping with RFLP-PCR
- Investigate the clinical relevance of *D. fragilis* infections.

CHAPTER 2

DETECTION OF *DIENTAMOEBA FRAGILIS* IN FRESH STOOL SPECIMENS USING PCR

2.1 INTRODUCTION

Dientamoeba fragilis is a pathogenic protozoan that has a worldwide cosmopolitan distribution (Windsor and Johnson, 1999). The prevalence of this organism varies widely, occurring in up to 8.8% of faecal specimens from patients with diarrhoea (Girginkardesler *et al.*, 2003).

On the basis of light and electron microscopy, quantitative fluorescent antibody and gel diffusion methods *D. fragilis* was shown to be closely related to the trichomonads (Talis, 1967; Dwyer, 1972a,b; Camp *et al.*, 1974). Sequence analysis of small subunit ribosomal DNA confirmed that *D. fragilis* clustered with the trichomonads, but its exact phylogenic position is still to be determined (Silberman *et al.,* 1996; Delgado-Viscogliosi *et al.*, 2000).

Dientamoeba fragilis seems to only exist as a trophozoite and no cyst stages have been observed. The mode of transmission of this organism is unknown although some researchers postulate that transmission may occur through the ova of *Enterobius vermicularis* or by the faecal-oral route (Yang and Scholten, 1977).

Dientamoeba fragilis infection may be symptomatic with both acute and chronic infections being reported in children and adults. The most common clinical symptoms include diarrhoea and abdominal pain (Girginkardesler *et al.*, 2003). Numerous studies have shown that treatment which eliminates the organism results in clinical improvement; thus treatment is recommended for symptomatic patients. Antimicrobial agents that have been successfully and commonly used include metronidazole, tetracycline and iodoquinol (Priess *et al.*, 1991; Butler, 1996).

Definitive diagnosis of *D. fragilis* requires permanently stained smears as demonstration of the characteristic nuclear structure cannot be achieved in unstained faecal specimens (Dobell, 1940). Fresh faecal specimens are needed as the trophozoites degenerate rapidly within hours of being passed. Such techniques are time consuming as staining can take

over 1 hour and require experienced laboratory personnel to interpret the stained smears with at least 200 to 300 oil immersion fields examined microscopically (Garcia, 2001). Care must be taken when reading the slides, as *D. fragilis* may be difficult to distinguish from non-pathogenic protozoa such as *Endolimax nana*. As with many intestinal protozoa, daily shedding of trophozoites is highly variable with intermittent shedding occurring regularly which necessitates multiple sampling for maximum yield (Van Gool *et al.*, 2003; Peek *et al.*, 2004).

Several studies have indicated that culture is more sensitive for diagnosis than permanent stains (Sawangjaroen *et al.*, 1993; Windsor *et al.*, 2003). However the cultivation of luminal parasitic protists is technically difficult, time consuming and often unrewarding (Clark and Diamond, 2002). As such, these techniques are usually restricted to specialist parasitology laboratories, and are not offered by routine diagnostic laboratories. One draw-back of culture systems for *D. fragilis* is that specimens need to be inoculated promptly after stool collection as reduced temperatures adversely affect *D. fragilis*. Specimens cannot be refrigerated because it reduces recovery rates (Sawangjaroen *et al.*, 1993).

Molecular biology techniques offer the potential of a highly sensitive and specific alternative to traditional diagnostic approaches such as microscopy. Regions of ribosomal DNA (rDNA) are ideal targets for PCR development as they exhibit interspecific variability yet are amongst the most ubiquitous and conserved DNA sequences in nature. For this reason they have been extensively used for phylogenic analysis and as targets for diagnostic probes. PCR techniques have been used for the detection of a variety of parasites from clinical samples, including: *Cryptosporidium parvum* (Limor *et al.*, 2002) and *Entamoeba histolytica* (Troll *et al.*, 1997).

Only one study has reported the development of a PCR to detect *D. fragilis*; however the sensitivity of this PCR was not determined and no large scale testing was undertaken to determine the specificity of the assay (Peek *et al.*, 2004). In this study the detection limit of PCR was the equivalent of approximately 0.1 *D. fragilis* trophozoites per sample by

cloning the amplicon and a known number of copies were then amplified (Peek *et al.*, 2004).

As current diagnostic methods such as permanent stains and xenic culture systems are cumbersome and time-consuming, a PCR based method for the detection of *D. fragilis* would provide a useful diagnostic tool for the detection of *D. fragilis* in patient samples. The aim of this study was to develop a PCR method that is highly sensitive and specific for the detection of *D. fragilis* in unpreserved faecal specimens.

2.2 MATERIALS AND METHOD

2.2.1 Stool specimens

Stool specimens used in this study were those submitted to St Vincent's Hospital Department of Microbiology, Sydney, for investigation of diarrhoea. Portions of all stool samples were fixed in sodium acetate acetic acid formalin (SAF) and permanently stained using a modified iron-haematoxylin stain (Fronine, Australia) according to manufacturer's recommendations.

Definitive diagnosis of *D. fragilis* was based on the morphology of parasites observed in the permanently stained smears. The following criteria were used to determine positive samples by microscopy. *Dientamoeba fragilis* is a pleomorphic amoeba with most trophozoites ranging from 5-15μm in size. The trophozoites are typically binucleate with 30-40% uninucleate. In stained smears the nuclear membrane is delicate and does not possess any peripheral chromatin. The karyosome contains chromatin granules often appearing as packets. The cytoplasm of *D. fragilis* appears granular and may be vacuolated and contain food inclusions.

Twenty two specimens that were fixed in SAF for various amounts of time (5 days to several years) where *D. fragilis* was detected by modified iron-haematoxylin stain were obtained from Concord Hospital, Sydney, John Hunter Hospital in Newcastle, NSW and St Vincent's Hospital in Sydney NSW, to determine the effect of SAF on the PCR reaction.

2.2.2 DNA extraction

All faeces positive for *D. fragilis* trophozoites by microscopy underwent direct DNA extraction on both the fresh specimen (<24 h old) and a portion of the same stool that had been fixed in SAF for 24 h. DNA was extracted from both fresh faeces and SAF fixed specimens using the QIAamp™ DNA stool minikit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

2.2.3 Small subunit rRNA gene amplification and sequencing

Molecular characterisation was achieved by sequencing the small-subunit rRNA (SSU rRNA) gene (SSUrDNA) of seven isolates chosen randomly. Oligonucleotide primers that were previously described for the amplification of trichomonad SSU rDNA were used:

TRD5 (5'GATACTTGGTTGATCCTGCCAAGG3') and

TRD3 (5'GATCCAACGGCAGGTTCACCTACC3') (Johnson and Clarke, 2000).

PCR amplifications (25µl) were performed using pureTaq Ready-To-Go™ (Amersham Pharmacia Biotech) PCR beads (each containing ~1.5 units Taq DNA polymerase, 10 mM Tris–HCl at pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each dNTP and stabilisers, including BSA), 1.0µl of genomic DNA extract and 0.5 µM of each PCR primer using the following thermocycling profile: 3 min denaturation hold at 94°C; 30 cycles of 1 min at 94°C, 1.5 min at 57°C, 2 min at 72°C. The PCR product was analysed by electrophoresis on 1.0% ReadyAgarose™ Gels (Bio-Rad, Marnes la Coquette, France).

The PCR products were then purified using the QIAquick™ PCR Purification Kit (Qiagen) as per manufacturer's instructions. The PCR product was sequenced in both directions on an ABI Prism 3700 automated sequencer at the SUPAMAC facility (Royal Prince Alfred Hospital, Sydney). The SSU rDNA sequences obtained from the seven samples were aligned together, along with an existing sequence from *D. fragilis* deposited in GenBank (accession number U37481) using the PILEUP program (Genetics Computer Group, Version 8). The aligned sequences were then compared to those available in the GenBank databases using the BLASTN program run on the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/).

2.2.4 *D. fragilis* PCR

Based on the sequence data derived from the SSU rDNA of *D. fragilis*, a set of oligonucleotide primers were designed manually to amplify only *D. fragilis* SSU rDNA
DF400 (5'TATCGGAGGTGGTAATGACC3') and
DF1250 (5'CATCTTCCTCCTGCTTAGACG3').
PCR amplification was performed as described for the SSU rRNA gene amplification using TRD5 and TRD3 primers.

2.3 RESULTS

DNA isolated from seven stool samples containing *D. fragilis* detected by microscopy were amplified by PCR using the primers TRD5 and TRD3. All seven isolates produced a product of approximately 1.7kb, which contains the SSU rRNA gene. The PCR product was sequenced in both directions from all seven isolates (Fig. 2.1). These sequences showed no variation and were compared to those available in the GenBank databases with the BLASTN program. The nucleotide sequences of the seven identical *D. fragilis* isolates were deposited in the GenBank database under one accession number (AY730405). All seven sequences which were identical showed a 96% similarity with that of the *D. fragilis* strain Bi/PA (ATCC 30948), deposited under GenBank accession no. U37461 (Fig.2.2). Other matches included *Histomonas meleagridis* (GenBank accession no. AF293056) and *Tritrichomonas species* (GenBank accession nos. AY0559799, AY055800, AY055801, AY055802, AY055803). The closest matches to those protozoa that are capable of infecting humans were *Trichomonas vaginalis* (GenBank accession no. U17510), *Trichomonas tenax* (GenBank accession no. U37711) and *Pentatrichomonas hominis* (GenBank accession no. AF124609). This SSU rDNA data and subsequent BLASTN searches highlights the close relationship that *D. fragilis* has with other trichomonads.

The identical SSU rDNA sequences were used to develop sensitive and species specific primers for PCR. The rDNA sequences were aligned against those from other trichomonad parasites and the primers DF400 and DF1250 were designed to amplify the region from positions approximately 400 to 1250 of the SSU rDNA of *D. fragilis*. Both sets of primers contain several nucleotides that only match the *D. fragilis* sequence and not other trichomonad species or other intestinal protozoa that infect humans.

To determine the sensitivity of the PCR, stool samples positive by microscopy for *D. fragilis* underwent DNA extraction and subsequent PCR using F400 and R1250. Out of the samples submitted to the laboratory, *D. fragilis* was detected by permanent stain in 37. With six of the 37 specimens there was a delay in undertaking the DNA extraction, (more than 7 days; aged specimens), whereas 31 specimens had DNA isolated within 24

hours (fresh specimens). Twenty-nine of the 31 fresh specimens produced a PCR product using the *D. fragilis* specific primers DF400 and DF1250 (Fig. 2.3). Nine out of 31 stool specimens positive for *D. fragilis* also contained other protozoan parasites. Six had *Blastocystis hominis*, two with *B. hominis* and *Endolimax nana* and one with *Giardia intestinalis* as determined by microscopy. Six aged specimens (>seven days) failed to amplify and did not give a PCR product.

To determine the specificity of the PCR, 29 specimens containing various other protozoan parasites (Table IV) underwent direct DNA extraction from fresh stool samples. PCR was then performed using DF400 and DF1250, and the products obtained were analysed by agarose gel electrophoresis. No PCR products were obtained from any of these specimens. A further 29 specimens containing no protozoa underwent direct DNA extraction on fresh stool samples. No PCR products were detected in this group either. To rule out any inhibitory effect due to the faecal material, inhibition controls were carried out and specimens were spiked with an equal volume of DNA from a known positive sample. All samples produced a PCR product showing these specimens were not inhibitory to the PCR reaction.

Twenty known positive *D. fragilis* samples along with 20 known negative samples underwent the PCR reaction under double-blinded conditions; all positive samples were detected by PCR while all the negative samples did not amplify.

Time course experiments were used to evaluate the PCR further. DNA was extracted every 24 h for one week from seven specimens stored at 4-8°C where *D. fragilis* was detected by permanent stain. Of the seven stored specimens that underwent daily DNA extraction: two specimens produced a PCR product from samples extracted only at 24hr and 48hrs. Three specimens produced PCR products from samples extracted at 24, 48 and 72 h. One specimen produced a PCR product at 24, 48, 72, 96 and 120h. Only one specimen produced PCR product from samples extracted at 24, 48, 72, 96, 120 and 144h.

The suitability of SAF fixative on the PCR reaction was also evaluated. No specimens fixed in SAF gave a PCR product including the 22 stored specimens and the 24 fresh specimens fixed in SAF overnight.

```
ATACTTGGTT GATCCTGCCA AGGAAGCACA CTATGGTCAT AGATTAAGCC
ATGCAAGTGT AAGTTCAGGT AACTAAACTG CGAATAGCTC ATTAACACAC
TCATAATCTA CTTGGAACCA ATTTTTTAAA AATTTTAAAT GGATAGCAGG
AGTAATTCTC GTGCTAATAC ATGAAATTTT AATAATCTTA AATTAAATCA
GATTATTTTT AATACCTTTT AATAGGTAAT CCAATCGAAT GAGTGACCTA
TCAGGCCAGT ACTTAGGGTC TTTACCTAAG TAAGCTATCA CGGGTAACGG
GCGGTTACCG TCGTACTGCC GGAGAAGGCG CCTGAGAGAT AGCGACTATA
TCCACGGGTA GCAGCAGGCG CGAAACTTAC CCACTCGAGA CTATCGGAGG
TGGTAATGAC CAGTTATAAT TAAGGAATTT TCCTTATTAT ATAGGAATAT
ACTTTTCCAG TATATTGTAA CCTAGCAGAG GGCCAGTCTG GTGCCAGCAG
CTGCGGTAAT TCCAGCTCTG CAAGTTTGCT CCCATATTGT TGTAGTTAAA
ACGCTCGTAG TCTGAATTAT TTTAATTTAA ATTTTTTAAA TTAAAATTTA
GTTTTTATTT TATAAAAACG TTCACTGTGG AACAAATCAG AACGCTTAAA
GTAATTTTCT TTATTGAATG ATTTAGCGCA GTATGAAATT TTTACCTTTT
AAATTTTAAT TAATTTAACA AGTAATATCA AAGAGAATAA TCGGGGATAG
ATCTATTTCA TGGCGAACAG CGAAATGTTT TGACCCATGA GAGAGAAACG
AAGGCGAAAG CATCTATCAA GTGTATTTCT ATCGATCAAG GGCGAGAGTA
GGAGTATCCA ACCGGATCAG AGACCCGGGT AGTTCCTACC TTAAACTATG
CCGACAAGGT TTTGTTTTTT TTAATAAAAG CAGTACCATA GGAGAAATCA
TAGTTCATGG GCTCTGGGGG AACTACGACC GCAAGGCTGA AACTTGAAGG
AATTGACGGA AGGGCACACC AGGGGTGGAG CTTGTGGCTT AATTTGAATC
AACACGGGAA AACTTACCAG GACCAGATAT TTTTAATGAC TGATCAGGCT
ATAGGTCTTT CAGGATATGA TTTTTGGTGG TGCATGGCCG TTGGTGGTGC
GTGGGTTGAC CTGTCTAGCG TTGATTCAGA TAACGAGCGA GATTATCACC
AATTAAATAT ATAAATATTT TTATTAAAAT AATTTATTTT CTAATTGGGA
CTCCCTGCGT CTAAGCAGGA GGAAGATGGT AGCAATAACA GGTCCGTGAT
GTCCTTTAGA TGCTCTGGGC TGCACGCGCG CTACAATGTT ATAATCAAAG
AGGTTTGCTA AATCGATAGA TTATCTTTTT TTTTAAAGAT TTCATAGCTA
CTCTGTTAAT ATATAACGTA GTTGGGATTG ATAATTGTAA TCATTATCAT
GAACCAGGAA TCCCTTGTAA ATGCGTGTCA ACAACGCGCG TTGAATACGT
CCCTGCCCTT TGTACACACC GCCCGTCGCT CCTACCGATT GAATGACTCG
GTGAAATCAT TGGATCATTT TTTTTTAAAT GAAAAGGTGA TTAAATCACG
TTATTTAGAG GAAGGAGAAG TCGTAACAAG GTAACGGTAG GTGAACCTGC
CGTTGGATCA A
```

Figure 2.1: Nucleotide sequence of the entire SSurRNA gene of Australia *D. fragilis* isolates.

```
             1                                                  50
D.fragilis   ATACTTGGTT GATCCTGCCA AGGAAGCACA CTATGGTCAT AGATTAAGCC
    u37461   ATACTTGGTT GATCCTGCCA AGGAAGCACA CTATGGTCAT AGATTAAGCC

             51                                                100
D.fragilis   ATGCAAGTGT AAGTTCAGGT AACTAAACTG CGAATAGCTC ATTAACACAC
    u37461   ATGCAAGTGT AAGTTCAGGT AACTAAACTG CGAATAGCTC ATTAACACAC

             101                                               150
D.fragilis   TCATAATCTA CTTGGAACCA ATTTTTTAAA AATTTTAAAT GGATAGCAGG
    u37461   TCATAATCTA CTTGGAACCA ATTTTTTAAA AATTTTAAAT GGATAGCAGG

             151                                               200
D.fragilis   AGTAATTCTC GTGCTAATAC ATGAAATTTT AATAATCTTA AATTAAATCA
    u37461   AGTAATTCTC GTGCTAATAC ATGAAATTTT AATAATCTTA AATTATTTTA

             201                                               250
D.fragilis   GATTATTTTT AATACCTTTT AATAGGTAAT CCAATCGAAT GAGTGACCTA
    u37461   GATTATTTTT AATACCTTTT AATAGGTAAT CCAATCGAAT GAGTGACCTA

             251                                               300
D.fragilis   TCAGGCCAGT ACTTAGGGTC TTTACCTAAG TAAGCTATCA CGGGTAACGG
    u37461   TCAGGCCAGT ACTTAGGGTC TTTACCTAAG TAAGCTATCA CGGGTAACGG

             301                                               350
D.fragilis   GCGGTTACCG TCGTACTGCC GGAGAAGGCG CCTGAGAGAT AGCGACTATA
    u37461   GCGGTTACCG TCGTACTGCC GGAGAAGGCG CCTGAGAGAT AGCGACTATA

             351                                               400
D.fragilis   TCCACGGGTA GCAGCAGGCG CGAAACTTAC CCACTCGAGA CTATCGGAGG
    u37461   TCCACGGGTA GCAGCAGGCG CGAAACTTAC CCACTCGAGA CTATCGGAGG

             401                                               450
D.fragilis   TGGTAATGAC CAGTTATAAT TAAGGAATTT TCCTTATTAT ATAGGAATAT
    u37461   TGGTAATGAC CAGTTATAAA TAAGGAATTT TCCTTTTTAT ATAGGAATAT

             451                                               500
D.fragilis   ACTTTTCCAG TATATTGTAA CCTAGCAGAG GGCCAGTCTG GTGCCAGCAG
    u37461   ACTTTTCCAG TATATTGTAA CCTAGCAGAG GGCCAGTCTG GTGCCAGCAG

             501                                               550
D.fragilis   CTGCGGTAAT TCCAGCTCTG CAAGTTTGCT CCCATATTGT TGTAGTTAAA
    u37461   CTGCGGTAAT TCCAGCTCTG CAAGTTTGCT CCCATATTGT TGTAGTTAAA

             551                                               600
D.fragilis   ACGCTCGTAG TCTGAATTAT TTTAATTTAA ATTTTTTAAA TTAAAATTTA
    u37461   ACGCTCGTAG YCTGAATTAT TTTAATTTAA ATTTTTTAAA TTAAAATTAA

             601                                               650
D.fragilis   GTTTTTATTT TATAAAAACG TTCACTGTGG AACAAATCAG AACGCTTAAA
    u37461   GTTTTTATTT TATAAAAACG TTCACTGT.G AACAARTCAG AACGCTTAGA

             651                                               700
D.fragilis   GTAA.TTTTC TTTATTGAAT GATTTAGCGC AGTATGAAAT TTTTACCTTT
    u37461   GTAATTTTTA ATTATTGAAT GATTTAGCGC AGTATGAAAT TTTTACTTTT
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                701                                                750
D.fragilis  TAAA...... .........T TTTAATTAAT TTAACAAGTA ATATCAAAGA
    u37461  TAAAAAAAAA AAATTTATTT TTTTTTTTTT TTAACAAGTA ATATCAAAGA

            751                                                800
D.fragilis  GAATAATCGG GGATAGATCT ATTTCATGGC GAACAGCGAA ATGTTTTGAC
    u37461  GAATAATCGG GGATAGRTCT ATTTCATGGC GAACAGCGAA ATGTTTTGAC

            801                                                850
D.fragilis  CCATGAGAGA GAAACGAAGG CGAAAGCATC TATCAAGTGT ATTTCTATCG
    u37461  CCATGAGAGA GAAACTAAGG CGAAAGCATC TATCAAGTGT ATTTCYATCG

            851                                                900
D.fragilis  ATCAAGGGCG AGAGTAGGAG TATCCAACCG GATCAGAGAC CCGGGTAGTT
    u37461  ATCAAGGGCG AGAGTAGGAG TATCCAACCG GATCAGAGAC CCGGGTAGTT

            901                                                950
D.fragilis  CCTACCTTAA ACTATGCCGA CAAGGTTTTG TTTTTTTTAA TAAAAGCAGT
    u37461  CCTACCTTAA ACGATGCCGA CAAGRTTTTG TTTTTYTTTA TAAAAGCAGT

            951                                               1000
D.fragilis  ACCATAGGAG AAATCATAGT TCATGGGCTC TGGGGGAACT ACGACCGCAA
    u37461  ATCATAGGAG AAATCATAGT TCATGGGCTC TGGGGGAACT ACGACCGCAA

            1001                                              1050
D.fragilis  GGCTGAAACT TGAAGGAATT GACGGAAGGG CACACCAGGG GTGGAGCTTG
    u37461  GGCTGAAACT TGAAGGAATT GACGGAAGGG CACACCAGGG GTGGAGCTTG

            1051                                              1100
D.fragilis  TGGCTTAATT TGAATCAACA CGGGAAAACT TACCAGGACC AGATATTTTT
    u37461  TGGCTTAATT TGAATCAACA CGGGAAAACT TACCAGGACC AGATATTTTT

            1101                                              1150
D.fragilis  AATGACTGAT CAGGCTATAG GTCTTTCAGG ATATGATTTT TGGTGGTGCA
    u37461  AATGACYRAT CAGGCTATAG GTCTTTCAGG ATATGATTTT TGGTGGTGCA

            1151                                              1200
D.fragilis  TGGCCGTTGG TGGTGCGTGG GTTGACCTGT CTAGCGTTGA TTCAGATAAC
    u37461  TGGCCGTTGG TGGTGCGTGG GTTGACCTGT CTAGCGTTGA TTCAGATAAC

            1201                                              1250
D.fragilis  GAGCGAGATT ATCACCAATT AAATATATAA ATATTTTTAT TAAAATAATT
    u37461  GAGCGAGATT ATCACCAATT AAATATATAA TAATTTTTTT TAAAATTAAT

            1251                                              1300
D.fragilis  TATTTTCTAA TTGGGACTCC CTGCGTCTAA GCAGGAGGAA GATGGTAGCA
    u37461  TATTTTCTAA TTGGGACTCC CTGCGTCTAA GCAGGAGGAA GATGGTAGCA

            1301                                              1350
D.fragilis  ATAACAGGTC CGTGATGTCC TTTAGATGCT CTGGGCTGCA CGCGCGCTAC
    u37461  ATAACAGGTC CGTGAYGTCC TTTAGATGCT CTGGGCTGCA CGCGCGCTAC

            1351                                              1400
D.fragilis  AATGTTATAA TCAAAGAGGT TTGCTAAATC GATAGATTAT CTTTTTTTTT
    u37461  AATGTTATAA TCAAAGAGTT TTGCTAAATC GATAGATTAT CTYTTTTTTT
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             1401                                               1450
D.fragilis  AAAGATTTCA TAGCTACTCT GTTAATATAT AACGTAGTTG GGATTGATAA
    u37461  TAAGATTTAA TAGCTACTCT GTTAATATAT AACGTAGTTG GGATTGATAA

             1451                                               1500
D.fragilis  TTGTAATCAT TATCATGAAC CAGGAATCCC TTGTAAATGC GTGTCAACAA
    u37461  TTGTAATCAT TATCATGAAC CAGGAATCCC TTGTAAATGC GTGTCAACAA

             1501                                               1550
D.fragilis  CGCGCGTTGA ATACGTCCCT GCCCTTTGTA CACACCGCCC GTCGCTCCTA
    u37461  CGCGCGTTGA ATACGTCCCT GCCCTTTGTA CACACCGCCC GTCGCTCCTA

             1551                                               1600
D.fragilis  CCGATTGAAT GACTCGGTGA AATCATTGGA TCATTTTTTT TTAAATGAAA
    u37461  CCGATTGAAT GACTCGGTGA AATCATTGGA TCATTTTTTT TTAAATGAAA

             1601                                               1650
D.fragilis  AGGTGATTAA ATCACGTTAT TTAGAGGAAG GAGAAGTCGT AACAAGGTAA
    u37461  AGGTGATTAA ATCACGTTAT TTAGAGGAAG GAGAAGTCGT AACAAGGTAA

             1651                     1677
D.fragilis  CGGTAGGTGA ACCTGCCGTT GGATCAA
    u37461  CGGTAGGTGA ACCTGCCGTT GGATCAA
```

Figure 2.2: Nucleotide sequence alignment of the SSUrRNA gene of *D. fragilis* (AY730405) vs *D. fragilis* (U37461). The specific insertion/deletion referred to in the text is underlined.

M 1 2 3 ---31

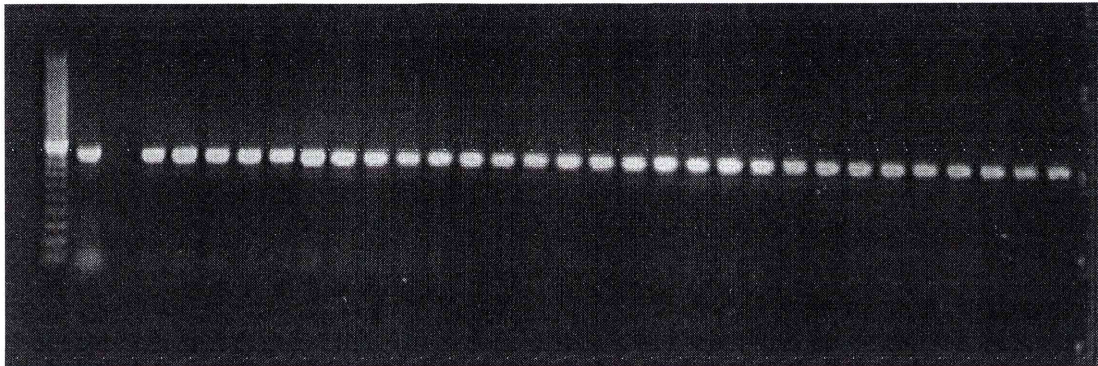

Figure 2.3: Detection of *D. fragilis* in faeces by PCR with primers DF400 and DF1250. M – Molecular Marker (100bp ladder, Bio-Rad), Lane 1- positive control, Lane 2 – negative control, Lanes 3-31, *D. fragilis* microscopy positive samples.

No.	Protozoan parasites present
1	*Blastocystis hominis*
2	*Entamoeba histolytica*
3	*Entamoeba dispar*
4	*Entamoeba coli*
5	*Entamoeba hartmanni*
6	*Giardia intestinalis*
7	*Endolimax nana*
8	*Iodamoeba butschlii*
9	*Cryptosporidium* species
10	*Cyclospora* species
11	*Chilomastix mesnili*
12	*E. histolytica/dispar* complex, *E. coli, E. nana, B. hominis* and *Enteromonas hominis*
13	*E. histolytica/dispar* complex, *E. coli, E. nana* and *I. Butschlii*
14	*E. hartmanni, E. nana, E. homins* and *B. hominis*
15	*E. nana, I. Butschlii* and *B. hominis*
16	*Cryptosporidium* species and *B. hominis*
17	*E. nana, I. Butschlii, C. mesnili* and *B. hominis*
18	*G. intestinalis, E. nana* and *B. hominis*
19	*E. coli, E. nana* and *B. hominis*
20	*G. intestinalis, E. coli, E. hartmanni* and *E. hominis*
21	*E. coli, E. nana, I. Butschlii* and *B. hominis*
22	*E. histolytica/dispar* complex, *E. hartmanni, E. hominis* and *B. hominis*
23	*E. histolytica/dispar* complex, *E. nana, E. hominis, B. hominis*
24	*E. coli, E. hartmanni, E. nana, I. Butschlii* and *B. hominis*
25	*E. hartmanni* and *E. nana*
26	*G. intestinalis*, *E. histolytica/dispar* complex, *E. hartmanni, E. coli, E. hominis, I . butschlii, B .hominis*
27	*E. histolytica/dispar* complex, *E. nana, B. hominis* and *Cryptosporidium* species
28	*C. mesnili, E. hominis* and *E. nana*
29	*G. intestinalis, E. nana* and *B. hominis*

Table IV. List of specimens containing various other protozoan parasites that were used in this study.

2.4 DISCUSSION

In this study a PCR was developed for *D. fragilis* from rDNA sequence data. The sequence data generated from the entire SSU rDNA region from the seven Australian isolates that were sequenced showed no variation and supports the notion that *D. fragilis* is a clonal species. All of the sequences from the 7 Australian isolates however differed from the sequence of the *D. fragilis* strain Bi/PA (ATCC 30948) GenBank accession No.U37461. At position 705 the sequence data from the ATCC strain 30948 has a 15bp insert that is not present in any of the Australian isolates. The Australian isolates were found to be similar to those found in a recent study in the Netherlands and do not contain the polymorphic *Dde*I restriction site (CTTAG) at position 644 found in the *D. fragilis* strain Bi/PA (Peek *et al.*, 2004). The sequence data generated in this study supports the notion of at least two distinct genetic variants in *D. fragilis* (those with and without the insert). Johnson and Clarke (2000) estimated a sequence divergence of 2% between the two SSUrRNA genotypes of *D. fragilis*; this was later supported by Peek *et al.*, (2004) by sequencing a 558bp region of the SSUrDNA. Our sequence data shows a greater sequence divergence of 4% between the Australian genotypes and the *D. fragilis* ATCC strain Bi/PA. Further studies are needed to determine the incidence of the variants in Australia and to determine if such variation has any influence on the pathogenicity of the parasite.

The sequence data also supports the findings that *D. fragilis* has a low G+C content relative to other trichomonads and contains on average an extra 100 nucleotides in the SSU rDNA region (Silberman *et al.*, 1996). This is due to three stretches of adenine and uracil in expansion segments known to be hypervariable regions. The SSU rDNA sequence data also highlights the close relationship between *D. fragilis* and *H. meleagridis*, an amoboeflagellate that is a common pathogen of domestic fowl (Silberman *et al.*, 1996).

The PCR for *D. fragilis* was evaluated by using stool specimens where *D. fragilis* was detected by permanent stain. Of the 31 fresh faecal samples positive with *D. fragilis* on

microscopy, 29 gave PCR products, thus giving a sensitivity of 93.5% from fresh specimens (extraction less than 24 h). Aged specimens did not produce a PCR product. The remaining 2 specimens that did not amplify were processed and underwent extraction within 24 hours of receipt in the laboratory. These specimens were spiked with an equal volume of DNA from a known positive sample. All produced a PCR product. Thus the specimens were not inhibitory to the PCR reaction. These two specimens were processed promptly (within 24 h); however the time of collection was unknown as no time was recorded on the specimen jar and attempts to contact the patients failed. Therefore the failure of amplification may be explained by the age of the specimens.

The specificities of the primers for *D. fragilis* were tested using 29 specimens containing human protozoan parasites (Table IV) and 29 human stool samples containing no protozoan parasites. No non-specific priming was seen with any of the specimens. Inhibition controls were carried out to exclude the possibility of inhibitory substances, and all were negative. Thus the PCR was shown to have 100% specificity.

A number of specimens were analysed by PCR in a double blind experimental design. All 20 positive specimens gave positive PCR results while all the negative samples gave negative PCR results.

The effect of storage and the use of fixatives on the PCR was also investigated. All of the specimens fixed in SAF failed to produce a PCR product. The stored specimens that had been fixed in SAF for various time periods, ranging from 5 days to 3 years, did not amplify. The 29 fresh specimens that produced a PCR product failed to do so having been fixed in SAF overnight. Therefore the PCR amplification of *D. fragilis* is inhibited by SAF fixation. Inhibition of PCR due to formalin has been described by other researchers. For examples a recent study concluded that formalin fixation has a direct effect on the suitability of material as a template (Ramos *et al.*, 1999), with formalin causing the DNA to fragment during the fixation process or the DNA to become cross-linked to its associated proteins. Therefore SAF fixed specimens are not suitable for *D. fragilis* PCR.

The time course experiments clearly demonstrated that the sensitivity of the PCR was affected when aged specimens were used as the DNA source. Most of the isolates failed to produce a PCR product after 72 hours, indicating that aged specimens that have been stored at 4-8°C are unsuitable for this PCR. DNA extraction on fresh samples <24 hours old is recommended as the trophozoites degenerate rapidly. Peek *et al.*, (2004) found that *D. fragilis* DNA could be detected up to one week, after which the signal could not be detected or became very weak. The specimens for Peek's PCR assay were stored at room temperature. *D. fragilis* has been shown to rapidly degenerate once refrigerated and this could account for the fact that the *D. fragilis* DNA degenerated more rapidly in our study than in the study from the Netherlands (Peek *et al.*, 2004).

These findings are supported by other research which demonstrated that *D. fragilis* does degenerate rapidly once outside the human body (Dobell, 1940), with the failure to amplify the PCR product due to nuclear material degrading in the ageing specimens. Unlike many other intestinal protozoa, *D. fragilis* may not have a cyst stage. Estimates of the survival time of trophozoites in faecal specimens vary from six to 48 hours (Hakansson, 1936). Wenrich conducted a number of experiments and was unable to keep *D. fragilis* trophozoites alive in boiled pond water or boiled hay infusion and in tap water they swell and burst within minutes (Wenrich, 1944). Yang and Scholten failed to keep *D. fragilis* trophozoites alive in simulated gastric juice (Yang and Scholten, 1977).

When using culture systems it was shown that positive cultures were only obtained from stools 8-11 hours old that had not been refrigerated (Sawangjaroen *et al.*, 1993). Brug (1936) found that cultures of *D. fragilis* that were exposed to room temperature were adversely affected. Dobell (1940) also described the fragile nature of this organism.

As inhibitors of PCR are commonly found in faecal specimens, a method for DNA purification that removes faecal inhibitors is needed. These inhibitors include heme compounds, acidic complex polysaccharides, protein, proteinases, DNAses, fats and interference from the DNA of other organisms or mucosal cells (Lantz *et al.*, 1997). The QIAamp™ DNA stool minikit has been evaluated and used successfully in a number of

studies (McOrist *et al.*, 2002). These studies include a number where protozoa have undergone direct DNA extraction from faeces including the extraction of *Cryptosporidium parvum* DNA from faeces (Limor *et al.*, 2002), *Entamoeba histolytica/dispar* complex (Blessmann *et al.*, 2002). It has also been shown to be successful in extracting DNA from *D. fragilis* in the study described here. No inhibitory effect of faecal material was detected in any of the negative samples in this study.

Current techniques that are used to diagnose *D. fragilis* infection are laborious, time consuming, require highly trained staff, and are prone to human error. Correct and prompt diagnosis is essential given the potential for chronic long-term infections. Recognition is also important for clinical management, as specific treatment is often required. This PCR technique will allow the rapid identification of *D. fragilis* in clinical specimens with results available in several hours. It will also provide a quick, simple and effective method to investigate the molecular epidemiology of *D. fragilis*. By using a direct extraction method, culture systems that are often laborious, time consuming and technically difficult need not be used when studying *D. fragilis*.

This work is the first attempt to extract DNA from *D. fragilis* in clinical stool samples and development of a PCR assay specific for *D. fragilis* in Australia. On fresh stools that had undergone direct DNA extraction promptly (within 24 hours) the sensitivity of the PCR was 93.5% and the specificity was 100%. Optimal extraction occurs on fresh faeces less than 24 hours old. In summary this PCR method is quick, simple, shows excellent sensitivity and specificity and offers another diagnostic tool other than permanent stains for the diagnosis of dientamoebiasis.

CHAPTER 3

THE MOLECULAR EPIDEMIOLOGY, GENETIC DIVERSITY AND CLINIAL RELEVANCE OF *DIENTAMOEBA FRAGILIS* INFECTIONS

3.1 INTRODUCTION

Dientamoeba fragilis is a trichomonad parasite found in the gastrointestinal tract of humans and implicated as a cause of gastrointestinal disease. *Dientamoeba fragilis* has been found in most parts of the world in both rural and cosmopolitan areas (Johnson *et al.*, 2004). The prevalence of this organism in Australia varies greatly from 0.4% to 16.8% (Anonymous, 1992; Walker *et al.*, 1985).

No cyst stage has been observed and only the trophozoites are detected in stool samples. Definitive diagnosis is based on prompt fixation and permanent staining as the trophozoites degenerate rapidly within hours of been passed and demonstration of their characteristic nuclear structure cannot be achieved in unstained preparations (Yang and Scholten, 1977). Daily shedding of *D. fragilis* trophozoites has been shown to be highly variable with intermittent shedding occurring regularly necessitating the need for multiple sampling for maximum chances of detection (van Gool *et al.*, 2003).

Molecular techniques for the diagnosis of *D. fragilis* show much promise with PCR demonstrating excellent sensitivity and specificity (Stark et *al.*, 2005a). Such techniques have been used successfully for the diagnosis of other pathogenic protozoa (Troll *et al.*, 1997; Limor *et al.*, 2002).

Molecular genotyping and sequence analysis has demonstrated that *D. fragilis* exists as at least two genetically distinct forms (Johnson and Clarke, 2000; Peek *et al.*, 2004; Stark *et al.*, 2005a). Stark *et al.* (2005a) sequenced the SSU rRNA gene of seven Australian *D. fragilis* isolates. The sequence data generated from the seven isolates showed no variation and supports the notion that *D. fragilis* is a clonal species. The sequences from the Australian isolates however differed from the sequence of the *D. fragilis* strain Bi/PA (ATCC 30948) GenBank accession No.U37461, and were found to be similar to those found in a recent study in the Netherlands (Peek *et al.*, 2004). The true incidence of the wild type and variant forms in Australia needs to be established and to determine if such variation has any influence on the pathogenicity of the parasite.

A prospective study was undertaken to determine the prevalence and clinical relevance of *D. fragilis* infections in an Australian population and to determine the genetic diversity of these isolates obtained at the SSU rDNA locus.

3.2 MATERIALS AND METHODS

3.2.1 Stool Specimens

All faecal specimens submitted to the Department of Microbiology at St. Vincent's Hospital, Sydney, for investigation of diarrhoea from March 2002 until July 2004, were included in the study. Specimens from outpatients were collected by the patient and submitted to the laboratory as a fresh specimen along with a portion mixed with Sodium Acetate Acetic Acid Formalin (SAF) preservative. Specimens from inpatients or received without a portion fixed in SAF were immediately preserved in SAF upon arrival at the laboratory.

3.2.2 Microbiological investigation

Faecal specimens were cultured for the following bacterial pathogens: *Salmonella* spp, *Shigella* spp, *Campylobacter* spp, Aeromonas spp, *Yersinia* spp, *Clostridium difficile* and culture for *Vibrio* spp was performed where indicated using standard laboratory procedures and techniques.

An immunochromatographic screening test, the Adeno/Rota STAT-PAK™ (Chembio Diagnostic Systems Inc., Sydney) for the detection of adenovirus and rotavirus antigen in faeces was used according to the manufacturer's recommendations.

Approximately 1 gram of faeces was placed into SAF and fixed overnight. The fixed specimens were then stained using a modified iron haematoxylin stain (Fronine, Australia) according to the manufacturer's recommendations. Formalin-ethyl acetate concentration was used for the detection of any helminth ova. In addition any specimens

from HIV-infected patients were examined for microsporidial spores using the Uvitex 2B stain (Van Gool *et al.*, 1993).

3.2.3 PCR for *D. fragilis*

All specimens where *D. fragilis* was detected by permanent stain underwent DNA extraction and PCR for *D. fragilis* specific DNA using primers DF400 and DF1250 as previously described (Stark *et al.*, 2005a).

3.2.4 Restriction fragment length polymorphism

RFLP analysis was undertaken on all positive PCR products. Eight µl of the PCR product was digested with 10 U of *Dde*I (Roche, Australia) in a final volume of 15 µl for 1 hour at 37°C. Samples were analysed by electrophoresis on 3% ReadyAgarose™ Gels (Bio-Rad, Sydney).

3.2.5 Follow up data

Clinical data was collected from all patients diagnosed with *D. fragilis*. Wherever possible, sticky-tape tests were conducted for the detection of *Enterobius vermicularis*.

3.2.6 Control group

A control group comprising of 900 faecal samples from patients without diarrhoea or symptoms of gastroenteritis (submitted for occult blood testing and faecal reducing substances) were used. These specimens were processed as above and stained using a modified iron haematoxylin stain. Ninety of these specimens underwent PCR using *D. fragilis* specific primers as described by Stark *et al.* (2005a).

3.2.7 Questionnaire

Questionnaires were distributed to 26 laboratories in the Sydney metropolitan area. Information requested for the calendar years 1996-2002 included: total number of faecal samples processed for ova cyst and parasites, total number of specimens positive for *D.*

fragilis, use of permanent stain, fixation method used in this period, and the situation in which a fixation method would be used.

3.3 RESULTS

A total of 6,750 faecal specimens were submitted between March 2002 and July 2004. Sixty patients were diagnosed with *D. fragilis* infection from the permanent stains giving a prevalence of infection of 0. 9%. The results found in this study are summarised in Table V.

Of the 60 patients infected with *D. fragilis* six (10%) had a history of recent overseas travel – three to South East Asia, one to Timor, one to Fiji and one to Papua New Guinea. The remaining 54/60 patients (90%) had no recent history of travel outside Australia.

A total of 24/60 (40%) patients had other parasites detected (Table VI). The only other pathogenic protozoan was *Giardia intestinalis* which was found concurrently with *D. fragilis* in three samples. The remaining 36 patients (60%) had only *D. fragilis* detected. All faecal samples were semi-formed or liquid.

The most frequent clinical symptoms associated with *D. fragilis* infection were diarrhoea, abdominal pain and loose bowel motions. Vomiting was only reported in one patient. Chronic persistent infections were common, with 19/60 (32%) patients having diarrhoea over 2 weeks duration and one patient claimed to have intermittent diarrhoea for several years. Five patients had recurrent *D. fragilis* infections. One patient was diagnosed with irritable bowel syndrome. All patients were symptomatic. Only one patient was immunosuppressed (HIV-infected) with all the others being immunocompetent. No *Microsporidia* were detected in the HIV-infected patient.

Thirty patients were female and 30 were male with the age range 3 to 79 years (Fig.3.1). The average age was 39.8 years, with a median of 44.5 years. No seasonal variation was found with *D. fragilis* infection.

No helminth ova were detected in the 60 patients using a formalin ethyl acetate concentration technique and no *Enterobius vermicularis* adults or ova were found. 33/60 (55%) patients submitted a sticky tape test for *E. vermicularis* ova, all of which were negative.

No bacterial pathogens were isolated from the patients with *D. fragilis* infection. The immunochromatographic tests for both adenovirus and rotavirus were also negative for all of the patients.

PCR was performed on 54 of the 60 samples; for six specimens there was a delay (> 7 days) in undertaking the DNA extraction so these specimens were excluded from PCR testing. A specific *D. fragilis* PCR product of approximately 870 bp was detected in 50 out 54 samples using the *D. fragilis* specific primers designed by Stark *et al.* (2005a). RFLP was performed on the 50 positive PCR samples. All gave identical RFLP patterns (Fig. 3.2).

Nine-hundred faecal samples from patients without gastrointestinal symptoms was used as a control group. No *D. fragilis* was detected by permanent staining. However non-pathogenic protozoa were detected in the control group. *Blastocystis hominis* was found in 47 patients and *Endolimax nana* in 19, while *B. hominis* and *E. nana* were found concurrently in 12 patients. One patient was found to have *Entamoeba hartmanni*. PCR using *D. fragilis* specific primers was undertaken on 90 samples randomly chosen from the control group. All ninety specimens were negative for *D. fragilis* DNA by PCR.

Out of the 26 laboratories that were sent the questionnaire only 11 responded. The remaining 15 laboratories were contacted and 4 agreed to participate in a phone interview using the same questions as the written questionnaire. Of the 15 laboratories it was determined that only 3 laboratories in the Sydney metropolitan area routinely performed permanent stains on faeces for ova, cysts and parasite examinations.

No.	Age (yr)	Sex[b]	Faeces[c]	Ethyl-acetate [d]	Sticky tape test (no. tapes)	Culture [e]	Rotavirus / Adenovirus [f]	Clinical symptoms	Other parasites present
1	15	F	Uf	Neg	N/A	NSP	Nd	PUO, Diarrhoea	No
2	6	M	Uf	Neg	Neg (4)	NSP	Nd	Abdominal pains	No
3	52	F	Uf	Neg	Neg(2)	NSP	Nd	Gastroenteritis ?Giardia	No
4	7	M	Uf	Neg	Neg(5)	NSP	Nd	Diarrhoea ?Giardia	*E. nana*
5	7	M	Uf	Neg	Neg(3)	NSP	Nd	Diarrhoea ?Giardia	*E. nana*
6	47	M	Uf	Neg	N/A	NSP	Nd	Chronic GIT symptoms	*I. butschlii,* *E.nana,* *E. hominis* *B. hominis*
7	27	M	Uf	Neg	Neg(3)	NSP	Nd	Gastroenteritis	*I. butschlii,* *E.nana,* *E. hominis,* *B. hominis,* *E. coli,* *G.intestinalis*
8	58	F	Uf	Neg	Neg(2)	NSP	Nd	Gastroenteritis	No
9	7	F	Uf	Neg	N/A	NSP	Nd	Diarrhoea	*B. hominis*
10	58	F	Uf	Neg	Neg(2)	NSP	Nd	Gastroenteritis	No
11	54	F	Uf	Neg	Neg(2)	NSP	Nd	Diarrhoea 3-4/52, Abdominal pains	No
12	53	M	Uf	Neg	N/A	NSP	Nd	Gastroenteritis	No
13	8	F	Fl	Neg	Neg(4)	NSP	Nd	Gastroenteritis ?Giardia	No
14	9	F	Uf	Neg	Neg(3)	NSP	Nd	Gastroenteritis , Previous *D. fragilis*	*B. hominis*

[b] F – Female, M – Male.
[c] Uf – Unformed, Fl – Fluid.
[d] Neg – Negative.
[e] NSP – No significant bacterial pathogens isolated.
[f] Nd – Not detected by immunochromatographic assay.

15	33	F	Uf	Neg	N/A	NSP	Nd	Recurrent gastroenteritis ?parasites	No
16	29	F	Fl	Neg	N/A	NSP	Nd	Diarrhoea, recent travel to South East Asia	No
17	34	F	Uf	Neg	Neg(2)	NSP	Nd	Diarrhoea for 5 weeks	No
18	45	M	Uf	Neg	Neg(3)	NSP	Nd	Chronic gastroenteritis	*E. nana,* *B. hominis*
19	45	M	Uf	Neg	N/A	NSP	Nd	Diarrhoea	*E. nana,* *B. hominis*
20	9	F	Uf	Neg	Neg(5)	NSP	Nd	Persistent diarrhoea	No
21	74	M	Uf	Neg	Neg(2)	NSP	Nd	Persistent loose bowel movements, overseas travel	No
22	45	M	Uf	Neg	Neg(3)	NSP	Nd	Previous *D. fragilis*	*B. hominis*
23	49	F	Uf	Neg	N/A	NSP	Nd	Diarrhoea	No
24	63	F	Uf	Neg	N/A	NSP	Nd	Diarrhoea	*B. hominis*
25	74	M	Uf	Neg	N/A	NSP	Nd	Previous *D. fragilis*, symptomatic	No
26	46	M	Fl	Neg	N/A	NSP	Nd	?Irritable bowel syndrome	No
27	45	F	Uf	Neg	N/A	NSP	Nd	Abnormal stools, camping in Fiji	No
28	36	F	Fl	Neg	Neg(2)	NSP	Nd	Diarrhoea, abdominal pains	No
29	47	F	Fl	Neg	Neg(2)	NSP	Nd	Diarrhoea	No
30	10	M	Uf	Neg	N/A	NSP	Nd	Chronic Gastroenteritis	*B. hominis*

31	62	F	Uf	Neg	Neg(3)	NSP	Nd	Intermittent diarrhoea for years, ?Giardia	*B. hominis*
32	75	M	Uf	Neg	Neg(4)	NSP	Nd	Ongoing loose bowel movements – previous *D. fragilis*	No
33	38	M	Uf	Neg	N/A	NSP	Nd	Watery diarrhoea for 3 weeks - travel to Vietnam	No
34	64	M	Uf	Neg	Neg(3)	NSP	Nd	Diarrhoea, ?parasites	No
35	38	F	Fl	Neg	N/A	NSP	Nd	Diarrhoea	No
36	11	F	Uf	Neg	Neg(2)	NSP	Nd	Diarrhoea and vomiting	No
37	45	M	Uf	Neg	N/A	NSP	Nd	Diarrhoea, Recent trip to Papua New Guinea	No
38	45	M	Uf	Neg	N/A	NSP	Nd	Loose stools	No
39	7	F	Fl	Neg	Neg(2)	NSP	Nd	Diarrhoea	*G. intestinalis*
40	49	M	Uf	Neg	Neg(3)	NSP	Nd	Diarrhoea, fever	No
41	75	M	Uf	Neg	N/A	NSP	Nd	Recurrent *D. fragilis* infection	No
42	68	F	Uf	Neg	Neg(2)	NSP	Nd	Diarrhoea, abdominal pain ?Giardia	*B. hominis*
43	4	M	Uf	Neg	Neg(4)	NSP	Nd	Abdominal pain, diarrhoea intermittently for 3 months	*G. intestinalis*, *B.hominis*
44	25	M	Fl	Neg	Neg(2)	NSP	Nd	Diarrhoea	*B. hominis*
45	69	F	Uf	Neg	N/A	NSP	Nd	Diarrhoea, abdominal pain	No
46	79	F	Uf	Neg	Neg(2)	NSP	Nd	Diarrhoea >1 week	No

47	41	F	Fl	Neg	N/A	NSP	Nd	Diarrhoea ?Giardia	*B. hominis*
48	56	F	Uf	Neg	N/A	NSP	Nd	Diarrhoea ?Giardia	No
49	61	M	Fl	Neg	N/A	NSP	Nd	Diarrhoea, abdominal pain ? Giardia	*B. hominis*
50	43	F	Uf	Neg	N/A	NSP	Nd	Diarrhoea, recurrent *Dientamoeba*, *Blastocystis*	*B. hominis*
51	3	M	Uf	Neg	Neg(5)	NSP	Nd	Diarrhoea	*G. intestinalis*, *B. hominis*
52	56	F	Uf	Neg	Neg(3)	NSP	Nd	Intermittent diarrhoea, abdominal pain	No
53	43	M	Fl	Neg	N/A	NSP	Nd	Diarrhoea	No
54	20	M	Fl	Neg	Neg(2)	NSP	Nd	Diarrhoea, Abdominal pain	*B. hominis*
55	22	M	Uf	Neg	N/A	NSP	Nd	Chronic diarrhoea, >2 weeks duration	B. hominis
56	47	M	Uf	Neg	Neg(2)	NSP	Nd	Diarrhoea, cramps	*B. hominis, E. nana, E. coli*
57	32	M	Fl	Neg	Neg(2)	NSP	Nd	Loose motions and wind for > 6 months	No
58	42	F	Uf	Neg	Neg(3)	NSP	Nd	Diarrhoea, >1 week	*B. hominis*
59	31	M	Uf	Neg	N/A	NSP	Nd	Diarrhoea after visit to Timor, mild chronic Gastritis >4 weeks. Ulcerative proctitis.	No
60	44	F	Uf	Neg	N/A	NSP	Nd	Abdominal cramps, wind, loose motions	No

Table V. Summary of results from patients with *D. fragilis* infection.

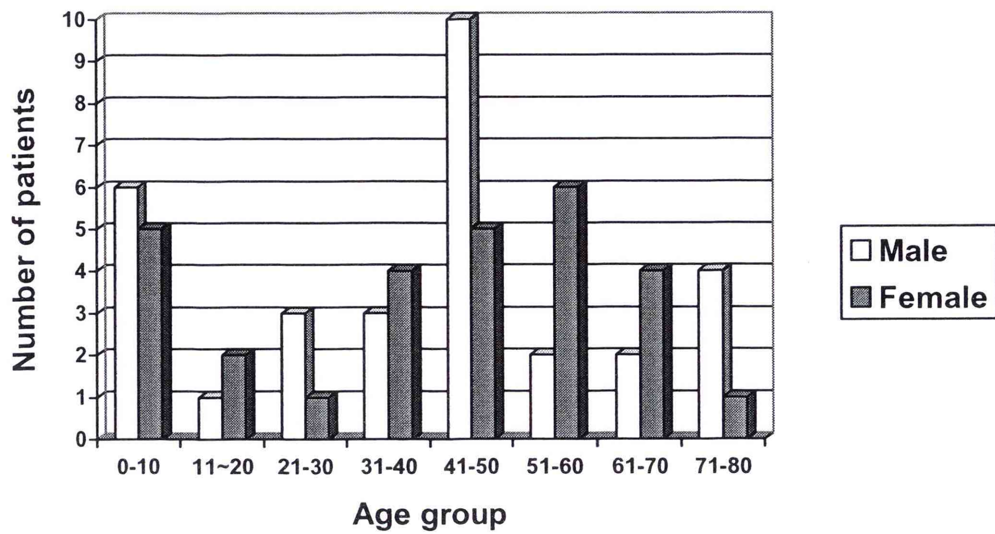

Figure 3.1: Prevalence of *D. fragilis* in different age groups.

14 *Blastocystis hominis*
2 *Endolimax nana*
2 *B. hominis, E. nana*
2 *G. intestinalis, B. hominis*
1 *B. hominis, E. nana, Entamoeba coli*
1 *G. intestinalis*
1 *G. intestinalis, I. butschlii, E.nana, E. hominis, B. hominis, E. coli*
1 *G. intestinalis, I. butschlii, E. nana, B. hominis*

Table VI. Parasites found in 24 patients with *D. fragilis* infection.

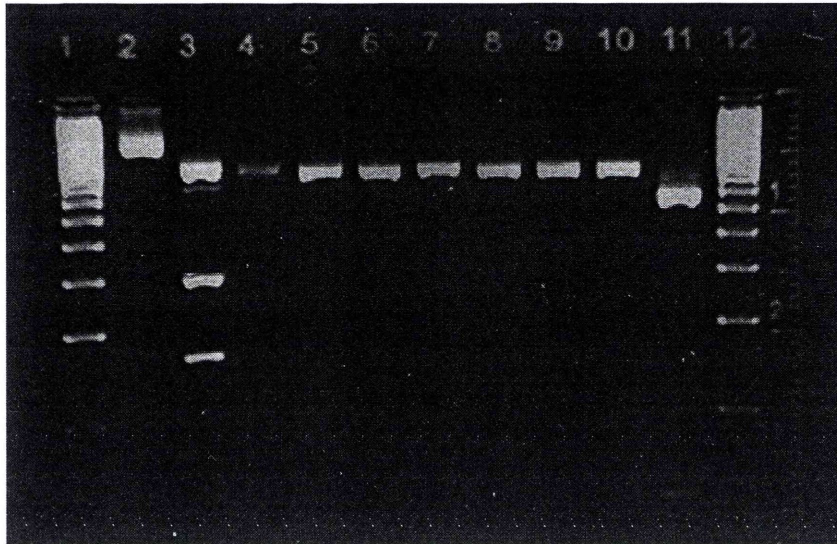

Figure 3.2. Restriction endonuclease digestion of DF400/DF1250 products (lane 5-10) digested with *Dde* I, Lane 2 undigested TRD5/TRD3 products (entire 1.7kb *D. fragilis* SSUrRNA gene), Lane 3 TRD3/TRD5 products digested with *Dde* I, Lane 4 DF400/DF1250 product undigested, Lane 11 DF400/DF1250 product digested with *Hae* III. The size marker (Lane 1 and 12) is a 100pb ladder.

351 400
A. TCCACGGGTA GCAGCAGGCG CGAAACTTAC CCACTCGAGA CTATCGGAGG
B. TCCACGGGTA GCAGCAGGCG CGAAACTTAC CCACTCGAGA CTATCGGAGG

401 450
A. TGGTAATGAC CAGTTATAAT TAAGGAATTT TCCTTATTAT ATAGGAATAT
B. TGGTAATGAC CAGTTATAAT TAAGGAATTT TCCTTATTAT ATAGGAATAT

451 500
A. ACTTTTCCAG TATATTGTAA CCTAGCAGAG GGCCAGTCTG GTGCCAGCAG
B. ACTTTTCCAG TATATTGTAA CCTAGCAGAG GGCCAGTCTG GTGCCAGCAG

501 550
A. CTGCGGTAAT TCCAGCTCTG CAAGTTTGCT CCCATATTGT TGTAGTTAAA
B. CTGCGGTAAT TCCAGCTCTG CAAGTTTGCT CCCATATTGT TGTAGTTAAA

551 600
A. ACGCTCGTAG TCTGAATTAT TTTAATTTAA ATTTTTTAAA TTAAAATT**T**A
B. ACGCTCGTAG YCTGAATTAT TTTAATTTAA ATTTTTTAAA TTAAAATTAA

601 650
A. GTTTTTATTT TATAAAAACG TTCACTGT**G**G AACAAATCAG AACGCTTA**A**A
B. GTTTTTATTT TATAAAAACG TTCACTGT.G AACAAATCAG AACGCTTAGA

651 700
A. GTAA.TTTT**C** **T**TTATTGAAT GATTTAGCGC AGTATGAAAT TTTTAC**C**TTT
B. GTAATTTTTA ATTATTGAAT GATTTAGCGC AGTATGAAAT TTTTACTTTT

701 750
A. TAAA...... ....T TTT**AA**TT**AA**T TTAACAAGTA ATATCAAAGA
B. TAAAAAAAAA AAATTTATTT TTTTTTTTTT TTAACAAGTA ATATCAAAGA

751 800
A. GAATAATCGG GGATAGATCT ATTTCATGGC GAACAGCGAA ATGTTTTGAC
B. GAATAATCGG GGATAGRTCT ATTTCATGGC GAACAGCGAA ATGTTTTGAC

801 850
A. CCATGAGAGA GAAAC**G**AAGG CGAAAGCATC TATCAAGTGT ATTTCTATCG
B. CCATGAGAGA GAAACTAAGG CGAAAGCATC TATCAAGTGT ATTTCYATCG

851 900
A. ATCAAGGGCG AGAGTAGGAG TATCCAACCG GATCAGAGAC CCGGGTAGTT
B. ATCAAGGGCG AGAGTAGGAG TATCCAACCG GATCAGAGAC CCGGGTAGTT

901 950
A. CCTACCTTAA AC**T**ATGCCGA CAAGGTTTTG TTTTTTTT**A**A TAAAAGCAGT
B. CCTACCTTAA ACGATGCCGA CAAGRTTTTG TTTTTYTTTA TAAAAGCAGT

```
    951                                              1000
A.  ACCATAGGAG AAATCATAGT TCATGGGCTC TGGGGGAACT ACGACCGCAA
B.  ATCATAGGAG AAATCATAGT TCATGGGCTC TGGGGGAACT ACGACCGCAA

    1001                                             1050
A.  GGCTGAAACT TGAAGGAATT GACGGAAGGG CACACCAGGG GTGGAGCTTG
B.  GGCTGAAACT TGAAGGAATT GACGGAAGGG CACACCAGGG GTGGAGCTTG

    1051                                             1100
A.  TGGCTTAATT TGAATCAACA CGGGAAAACT TACCAGGACC AGATATTTTT
B.  TGGCTTAATT TGAATCAACA CGGGAAAACT TACCAGGACC AGATATTTTT

    1101                                             1150
A.  AATGACTGAT CAGGCTATAG GTCTTTCAGG ATATGATTTT TGGTGGTGCA
B.  AATGACYRAT CAGGCTATAG GTCTTTCAGG ATATGATTTT TGGTGGTGCA

    1151                                             1200
A.  TGGCCGTTGG TGGTGCGTGG GTTGACCTGT CTAGCGTTGA TTCAGATAAC
B.  TGGCCGTTGG TGGTGCGTGG GTTGACCTGT CTAGCGTTGA TTCAGATAAC

    1201                                             1250
A.  GAGCGAGATT ATCACCAATT AAATATATAA ATATTTTTAT TAAAATAATT
B.  GAGCGAGATT ATCACCAATT AAATATATAA TAATTTTTTT TAAAATTATT

    1251                                           1300
A.  TATTTTCTAA TTGGGACTCC CTGCGTCTAA GCAGGAGGAA GATGGTAGCA
B.  TATTTTCTAA TTGGGACTCC CTGCGTCTAA GCAGGAGGAA GATGGTAGCA
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Figure 3.3: Sequence alignment of part of the SSUrRNA gene sequence (between positions 351 and 1300 that is amplified using the *D. fragilis* specific primers). The A genotype sequence represents several Australian *D. fragilis* isolates, while the B genotype sequence represents the *D. fragilis* strain Bi/PA (ATCC 30948). The nucleotide differences between the two genotypes are in bold, while the *Dde*I restriction site (CTTAG) is underlined. The position of primers used for the PCR are underlined.

3.4 DISCUSSION

Dientamoeba fragilis has a worldwide cosmopolitan distribution. In Australia and New Zealand the reported prevalence rate ranges from 0.4% in Western Australia (Anonymous, 1992) and 1.5% in an urban community in Brisbane (Sawangjaroen *et al.*, 1993) to 2.2% in Christchurch, New Zealand (Oxner *et al.*, 1987) and 16.8% in suburban Sydney (Walker *et al.*, 1985). A longitudinal study of parasite infections in Aboriginal children from the Queensland outback found a prevalence of 5.0% for *D. fragilis* (Welch and Stuart, 1976). In this present study a prevalence of 0.9% was found, this is in stark contrast to the prevalence of 16.8% that was found by Walker *et al.* (1985) during an outbreak of dientamoebiasis in unsewered areas of the Sydney suburb of French's Forrest.

In this study *D. fragilis* infection was closely associated with diarrhoea, abdominal pain and loose bowel motions. All patients with *D. fragilis* infection were symptomatic and bacterial and viral causes of these symptoms are unlikely as routine microbiological cultures, adenovirus and rotavirus testing were negative. Three patients were also infected with *G. intestinalis* which could have caused the gastrointestinal symptoms described in those patients.

One important finding of this study was that chronic persistent *D. fragilis* infections were common. Thirty-two percent of patients had diarrhoea for greater than 2 weeks duration, and one patient claimed to have had intermittent diarrhoea for several years. Five patients had recurrent *D. fragilis* infection during the course of the study. It is unknown whether these recurrences were due to treatment failure or reinfection from a common source. One patient was diagnosed with irritable bowel syndrome and was subsequently found to have *D. fragilis* infection. A recent Australian study by Borody *et al.* (2002) showed a link between *D. fragilis* and irritable bowel syndrome (IBS). Twenty-one patients diagnosed with IBS and concurrent *D. fragilis* infection were treated with iodoquinol and doxycycline. Complete elimination of *D. fragilis* with marked clinical improvement occurred in the majority of patients.

Only one patient was immunosuppressed (HIV-infected) with all the others being immunocompetent. This is in contrast to a study from Argentina which suggested that the incidence of *D. fragilis* infections may be higher in immunocompromised patients (Mendez *et al.*, 1994). In all other studies conducted, including this present one, immunosuppression does not seem to be a contributing factor for infection with *D. fragilis*.

Ten percent of patients diagnosed with *D. fragilis* infection had a history of recent overseas travel including Southeast Asia, Papua New Guinea, Timor and Fiji. *Dientamoeba fragilis* has been implicated as a cause of diarrhoea in returning Swedish travellers with Norberg *et al.* (2003) finding 63% of patients in a retrospective study had been infected outside the country. Most patients were infected in Africa, South America and the Middle East.

In our study *D. fragilis* occurred in older patients with the average age of 39.8 years, and a median of 44.5 years. This is in contrast to other studies that have found higher prevalence rates in children (Preiss *et al.*, 1991; Norberg *et al.*, 2003). However the true incidence of infection in younger children may not be truly reflected. Although the microbiology laboratory services a number of general practices with paediatric clients, St.Vincent's Hospital does not have a paediatric department so cases from this age group are under-represented in this study.

No parasites were detected by formalin ethyl acetate concentrations performed on faecal specimens from the *D. fragilis* infected patients. Fifty-five percent of the patients submitted a sticky-tape test for the detection of *Enterobius vermicularis* ova and no *E. vermicularis* ova were detected. Many researchers have postulated that pin worm is a vector for *D. fragilis* transmission. Burrows *et al.* (1954) were the first to propose that *E. vermicularis* might be a vector for *D. fragilis*. Several other researchers also found a higher than expected concurrence of *D. fragilis* and *E. vermicularis* infections (Burrows and Swerdlow, 1956; Yang and Scholten, 1977; Preiss *et al.*, 1991).

In contrast a recent study of 25 paediatric cases of *D. fragilis* found no infections were associated with *E. vermicularis* (Cuffari *et al*., 1998). These results along with the findings from this present study would argue against the hypothesis that *E. vermicularis* plays a significant role in the transmission of *D. fragilis*. Most studies that have examined *D. fragilis* infection have inadequately examined for *E. vermicularis*. It has yet to be proven what role helminth ova play in the transmission of *D. fragilis*. Further study is required to ascertain the true mode of transmission of this organism.

Other enteric protozoa were present in 40% of patients with *D. fragilis* infection. The most common organism was *B. hominis*. Other protozoa present included *E. nana*, *E. hominis*, *E. coli*, *Iodamoeba butschlii* and *G. intestinalis*. All of these parasites are known to be transmitted via the faecal-oral route. Other researchers have found similar rates of coinfection of *D. fragilis* with other parasites that are transmitted via the faecal-oral route. Windsor *et al.* (1998) found 54% of patients with *D. fragilis* had other parasites or enteropathogens present. These findings would provide evidence to support a hypothesis for a faecal-oral route of transmission for *D. fragilis*.

No *D. fragilis* trophozoites were detected in the control group of 900 patients without gastrointestinal symptoms. This is in contrast to other studies where *D. fragilis* was detected in patients with no clinical symptoms (Colea *et al*., 1980) and in a case control study on gastroenteritis from the Netherlands where *D. fragilis* was recovered more frequently from controls than case patients (De Wit *et al*., 2001). These findings may be attributed to the fact that asymptomatic carriage of intestinal protozoa can often occur.

The permanent stained smears positive for *D. fragilis* were confirmed by PCR. A sensitivity of 93% was obtained using a previously published method (Stark *et al*., 2005a). All 90 negative samples from the control group failed to produce a PCR product.

Sequence data generated in several studies supports the notion of at least two distinct genetic variants in *D. fragilis*. Johnson and Clarke (2000) estimated a sequence divergence of 2% between the two SSUrRNA genotypes of *D. fragilis*; this was later

supported by Peek *et al.*, (2004) by sequencing a 558bp region of the SSU rDNA. Sequence data generated by Stark *et al.*, (2005a) from the entire SSU rDNA region of Australian isolates of *D. fragilis* showed a greater sequence divergence of 4% between the Australian genotypes and the *D. fragilis* ATCC strain Bi/PA (ATCC 30948). All Australian strains sequenced were identical and supports the notion that *D. fragilis* is a clonal species. The Australian isolates were found to be similar to those found in a recent study in the Netherlands and do not contain the polymorphic *Dde* I restriction site (CTTAG) at position 644 found in the *D. fragilis* strain Bi/PA (Peek *et* al., 2004). RFLP analysis was undertaken on all 50 Australian samples to determine the genotypes present in the Australian population and the extent of genetic diversity. The PCR used in this study amplifies the SSU rDNA region from approximately position 400 to position 1270. This PCR product contains a *Dde* I restriction sites (CTTAG) that are present in the *D. fragilis* ATCC 30948 strain yet are absent in the Australian genotypes (Fig. 3.3). All 50 *D. fragilis* samples showed no variation and corresponded to genotype A. These findings suggest that *D. fragilis* in Sydney, Australia, display only a single genotype in faecal samples from various groups including inpatients, outpatients and travellers. Further studies are needed to identify the presence of other genotypes throughout Australia.

Dientamoeba fragilis has no recognised cyst stage and as such diagnosis is dependent on detecting the trophozoites. As these trophozoites degenerate rapidly, prompt fixation of the specimen is necessary (Yang and Scholten, 1977). Successful diagnosis of *D. fragilis* is closely associated with the use of permanent stains of faecal smears. Failure to use permanent staining and fixation techniques will inevitably preclude identification of *D. fragilis.* The aim of the questionnaire sent to the Sydney laboratories was to determine how many laboratories routinely undertake permanent staining and therefore how many laboratories are able to report the presence of *D. fragilis*. Of the 26 Sydney laboratories 58% participated in the survey and only three routinely performed permanent staining for ova, cyst and parasites on faecal specimens. Those three laboratories were the only institutions that detected *D. fragilis* in routine samples. Therefore the true extent of *D. fragilis* infection must be greatly underestimated as most laboratories do not use techniques to adequately identify this organism.

This is the first prospective study of *D. fragilis* in Australia to examine clinical data in addition to the genetic diversity of the isolates. Diagnosis was based on permanent staining of fixed faecal smears and confirmed by PCR which demonstrated good sensitivity. All persons infected with *D. fragilis* were symptomatic and *D. fragilis* infections were most commonly associated with diarrhoea and abdominal pain. Concurrent infections with other protozoa were common, occurring in 40% of samples. The occurrence of *D. fragilis* with other protozoa that are transmitted via the faecal oral route would strengthen the case for *D. fragilis* also being transmitted via this route. No correlation was found with *E. vermicularis* or any other helminths, questioning the role, if any, pin worm has in the transmission of *D. fragilis*. The genetic diversity of 50 samples was examined by PCR followed by RFLP. This data indicated that a single genotype of *D. fragilis* was represented, one that is genetically different to the North American *D. fragilis* strain Bi/PA (ATCC 30948). The evidence that *D. fragilis* is a pathogenic protozoa is overwhelming and as such all laboratories should attempt to identify this protozoa by the use of permanent staining techniques or molecular methods.

CHAPTER 4

EVALUATION OF THREE DIAGNOSTIC METHODS, INCLUDING, REAL-TIME PCR, FOR THE DETECTION OF *DIENTAMOEBA FRAGILIS* IN STOOL SPECIMENS

4.1 INTRODUCTION

Dientamoeba fragilis is a pathogenic protozoan parasite that causes gastrointestinal disease in humans (Johnson *et al.*, 2004; Stark *et al.*, 2005b). Two distinct genetic forms of *D. fragilis* have been described by analysis of the small subunit ribosomal RNA gene (Johnson and Clarke, 2000; Peek *et al.*, 2004; Windsor *et al.*, 2004; Stark *et al.*, 2005b) with only one genotype predominant in Australia (Stark *et al.*, 2005b).

Dientamoeba fragilis infection can be responsible for diarrhoea in children and adults with chronic infection common. The most frequent clinical symptoms associated with *D. fragilis* infection are diarrhoea, abdominal pain and overall looseness of stools (Johnson *et al.*, 2004; Stark *et al.*, 2005b). Several studies have shown that *D. fragilis* is more prevalent than *Giardia intestinalis* as a cause of gastrointestinal infection (Girginkardesler *et al.*, 2003; Crotti *et al.*, 2005). In a recent Australian study Stark *et al.*, (2005b) found that chronic persistent infections were also common, with 32% patients in this study having diarrhoea for greater than 2 week's duration. *Dientamoeba fragilis* was also implicated as one possible cause of irritable bowel syndrome (Borody *et al.*, 2002). Numerous studies have shown that chemotherapeutic treatment which eliminates the organism results in clinical improvement; thus treatment is recommended for symptomatic patients (Preiss *et al.*, 1991; Borody *et al.*, 2002; Girginkardesler *et al.*, 2003; Johnson *et al.*, 2004). Due to the propensity of this organism to cause gastrointestinal infection particularly chronic infection, it is essential that correct diagnosis occurs promptly.

The diagnosis of *D. fragilis* relies on direct visualisation of the trophozoites in stained fixed faecal smears by light microscopy as demonstration of the characteristic nuclear structure cannot be achieved in unstained faecal specimens (Butler, 1996). Fresh faecal specimens or specimens fixed in a preservative are needed as the trophozoites degenerate rapidly within hours of being passed. Permanent stains are time consuming and require experienced laboratory personnel to interpret the stained smears. Care must be taken

when reading the slides, as *D. fragilis* may be difficult to distinguish from non-pathogenic protozoa (Johnson *et al.*, 2004).

It was not until 1996 that molecular techniques were used as an alternative to traditional phenotypic markers to determine the taxonomic position of *Dientamoeba*. Silberman *et al.*, (1996) constructed molecular phylogenies based upon the complete small subunit (SSU) rRNA gene sequences (rDNA) of *D. fragilis*, several trichomonad groups and a variety of other eukaryotes. Similarity calculations demonstrated a clear association between *D. fragilis* and other parabasalid flagellates. All phylogenic constructions showed *D. fragilis* to be closely related to trichomonads. Further molecular studies of the SSU rDNA failed to resolve the exact position of *D. fragilis* compared to other parabasalids however the sequence data showed significant similarity to other parabasalids sequences (Delgao-Viscogliosi *et al.*, 2000).

Molecular techniques such as PCR also provide alternative methods for specific detection of pathogens in stools and show much promise for the diagnosis of dientamoebiasis with conventional PCR demonstrating good sensitivity and specificity (Peek *et al.*, 2004; Stark *et al.*, 2005a). The sensitivity of PCR has been shown to be greater than that of microscopy, making it of great use for detecting low numbers of parasites in stool specimens (Bialek *et al.*, 2002). A recent advancement in PCR-based methodology is the development of real-time PCR, which allows continual monitoring of amplicon formation throughout the reaction. In addition, closed tube real-time methods do not require further processing of the amplicon, which is time consuming, prone to cross-contamination and the generation of false positive results.

The aim of this study was to develop a real-time PCR method that is rapid, highly sensitive and specific for the detection of *D. fragilis* in faecal specimens. Results from the real-time assay were compared to those derived using an established conventional PCR assay and microscopic examination by a traditional modified iron-haematoxylin staining procedure in order to determine the usefulness and practicality of PCR-based detection methods for clinical diagnosis.

4.2 MATERIALS AND METHODS

4.2.1 Culture of Enteric Protozoa and extraction of genomic DNA

The passage of the following species and strains was performed in TYI-S-33 broth: *Entamoeba histolytica* HM-1:IMSS (ATCC strain 30459) and *Trichomonas vaginalis* (ATCC strain F1623). Genomic DNA was extracted from cultured parasites using the QIAamp™ DNA minikit (Qiagen, Hilden, Germany).

4.2.2 Stool specimens

Stool specimens used in this study were those submitted to St Vincent's Hospital Department of Microbiology, Sydney, for investigation of diarrhoea. Portions of all stool samples were fixed in sodium acetate acetic acid formalin (SAF) and permanently stained using a modified iron-haematoxylin stain (Fronine, Australia) according to manufacturer's recommendations.

4.2.3 DNA extraction from stool specimens

DNA was extracted from fresh faecal specimens (<24 h old) using the QIAamp™ DNA stool minikit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To ensure that we were using "best practice" form stool specimens using this kit, we also evaluated a modification of the manufacturers instructions (Gookin *et al.*, 2002) using 4 samples, positive for *D. fragilis* by microscopy. Briefly, the modified method included an extended proteinase K digestion (20μl of proteinase K at 56 C for 1h), and a second wash in guanidinium chloride (buffer AW1), plus an additional centrifugation step following the final wash. As a control, these same samples were also extracted following the manufacturer's instructions and the two sets of DNA were then tested with conventional and real-time PCR.

4.2.4 Cloning of small subunit ribosomal DNA from *D. fragilis*

The SSUrDNA from *D. fragilis* was amplified using primers TRD3/TRD5 (Stark *et al.*, 2005a) and the 1.8kb product was cloned into the PCR cloning TA vector, as described by the manufacturer (Invitrogen). After transformation into *Escherichia coli* (strain DH5α) individual transformants were screened for the presence of cloned DNA by PCR. Plasmid DNA from one of these clones (pDf18S rDNA) was purified from bacterial cultures grown in L-broth using standard procedures. The purified recombinant DNA was quantified and used for the sensitivity testing of the conventional and real-time PCR.

4.2.5 Conventional PCR

Conventional PCR and DNA sequencing, using primers DF 400/DF1250 and DF3/DF4, was performed according to Stark *et al.*, (2005a). Inhibition controls, comprising of patient faecal samples spiked with cloned *D. fragilis* SSUrDNA, were also run to rule out PCR inhibition.

4.2.6 Real-time PCR

The SSUrDNA sequences present in GenBank from enteric protozoa normally associated with clinical signs of disease in humans were aligned using the computer program Pileup. From this multiple sequence alignment, *D. fragilis* specific primers and dual labelled fluorescent probe were designed so as to amplify by PCR a 78bp region of the SSU rRNA gene of *D. fragilis*.

The following primers and probe were developed using a computer program from PROLIGO and used in the real-time PCR DF3 (5'-GTTGAATACGTCCCTGCCCTTT-3') and DF4 (5'-TGATCCAATGATTTCACCGAGTCA-3'). The dual labelled fluorescent probe was labelled at the 5' end with a reporter dye 5-carboxyfluorescein (FAM) and at the 3' end with a quencher dye, 6-carboxytetramethylrhodamine (5'-FAM-CACACCGCCCGTCGCTCCTACCG-TAMRA-3').

Real-time PCR was performed using the LightCycler (Roche) in a 20-μl reaction volume in a glass capillary tube containing 2μl of FastStart reaction mix hybridisation probes (a component of the FastStart DNA master hybridisation probes kit; Roche Diagnostics), 3mM $MgCl_2$, 0.25μM of forward and reverse primer, 0.2μM of dual labelled fluorescent probe and 2μl of DNA extract.

Reaction conditions were as follows; 10 min at 95°C, followed by 35 cycles of 10 s at 57°C and 3 s at 72°C. Temperature change rates were at 20°C/s. Readout was performed in channel F1.

To determine the sensitivity and detection limit of the PCR assay the purified recombinant DNA was quantified and known concentrations were serially diluted down to approximately 1 plasmid copy. These known concentrations were then run with both the conventional PCR and the real-time PCR.

4.3 RESULTS

The SSUrDNA sequence data generated from a previous study (Stark *et al.*, 2005a) was used to develop sensitive and species-specific primers for real-time PCR. The rDNA sequences were aligned with those derived from other enteric protozoa and the primers DF3 and DF4 were designed to amplify the region from approximately 1,490 – 1,567 of the SSU rRNA gene of *D. fragilis*. In initial experiments using cloned DNA as a target template, real-time PCR was used to construct a standard curve that related PCR detection levels to known amounts of cloned DNA (Fig. 4.1).

Optimal extraction of DNA from human faeces was achieved by using the commercial kit with no modifications to the extraction procedure. All 4 specimens that underwent extraction using both the modified and non-modified method produced amplicons in both conventional and real-time PCR. Given that the modified extraction procedure greatly increased the time taken to process the specimens (> 1h), it was deemed that the extra steps taken were not needed. This is in contrast to a study that used the modified technique for extraction of a closely related Trichomonad, *Tritrichomonas foetus*, from feline faeces (Gookin *et al.*, 2002).

A total of 170 faecal samples were screened by microscopy and conventional and real-time PCR. All 170 faecal samples spiked with positive control *D. fragilis* DNA amplified the correct size band indicating that PCR inhibition was not an issue in this study.

Real-time PCR analysis of the samples detected a total of 51 positives (Fig. 4.2) while conventional PCR detected 48 positive samples. Microscopy detected a total of 50 *D. fragilis* positive samples. Other protozoa were detected by permanent staining including; *Blastocystis hominis*, *Chilomastix mesnili*, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Endolimax nana*, *Entamoeba histolytica/dispar* complex, *Entamoeba coli*, *Entamoeba hartmanni*, *Enteromonas hominis*, *Giardia intestinalis*, *Iodamoeba butschlii*, and *Pentatrichomonas hominis*. One sample positive by microscopy and negative by both PCR methods was subsequently deemed a false positive when the permanently stained

smear was re-examined by an independent experienced microscopist who concluded that the non-pathogenic *Endolimax nana* was misidentified as *D. fragilis*. Two samples of the 120 deemed negative for *D. fragilis* by permanent staining produced amplicons by real time PCR. One of these samples gave a product with conventional PCR. Upon sequencing these amplicons, PCR products were confirmed to be derived from *D. fragilis* DNA by DNA sequence comparisons. One of the 120 samples negative by microscopy was positive by conventional PCR while 2 of the 120 microscopy samples were positive by real-time PCR. One of these samples contained *B. hominis*, and *E. nana* while the other contained *E. histolytica/dispar* complex and on subsequent review of each permanent slide no *D. fragilis* was detected by microscopy. The second sample had a crossing point of 25.12 compared to the positive control of 19.48 and the first sample with 19.62 which would indicate a low number of *D. fragilis* parasites present in the sample which fell below the detection limits of both conventional PCR and microscopy. In summary, based on the analysis of the faecal samples, microscopy showed 92.5% sensitivity and 99.2% specificity, conventional PCR showed 92.3% sensitivity and 100% specificity compared with 100% sensitivity and specificity for real-time PCR (Table VII).

To determine the sensitivity of both conventional and real-time PCR, the entire SSUrRNA gene was cloned and a known number of copies were then amplified using the same conditions as for the patients' samples. This showed that the detection limit was 100 plasmid copies or an equivalent of approximately 1.0 *D. fragilis* trophozoite for conventional PCR. The detection limit for the real-time PCR was determined at 1 plasmid copy (a crossing point of 27.87) of the SSUrRNA gene which is equivalent to approximately 0.01 *D. fragilis* trophozoite (Fig. 4.1). This shows that the real-time PCR was 100 times more sensitive than the conventional PCR.

To determine any cross reactivity of the real-time assay, DNA extractions were also performed on live cultures of *E. histolytica* HM-1:IMSS (ATCC strain 30459) and *T. vaginalis* (ATCC strain F1623) propagated in TYI-S-33 broth. Real-time PCR was alsoperformed on genomic DNA from *Trichomonas foetus* (ATCC strain 3000). Only *T. vaginalis* and *T. foetus* DNA produced a PCR product by this technique.

Method	No. of samples examined	No. of positives detected	Sensitivity (%) [a]	Specificity (%) [b]
Real-time PCR	120	51	100	100
Conventional PCR	120	48	92.3	100
Microscopy [c]	120	50	92.5	99.2

[a] Calculated as follows: [number of true positives/(number of true positives + number of false negatives)] x 100.
[b] Calculated as follows: [number of true negatives/(number of true negatives + number of false positives)] x 100.
[c] Microscopy was performed using a modified-iron haematoxylin stain (Fronine, Australia).

Table VII. Comparison of PCR, conventional PCR and microscopy for detection of *Dientamoeba fragilis*.

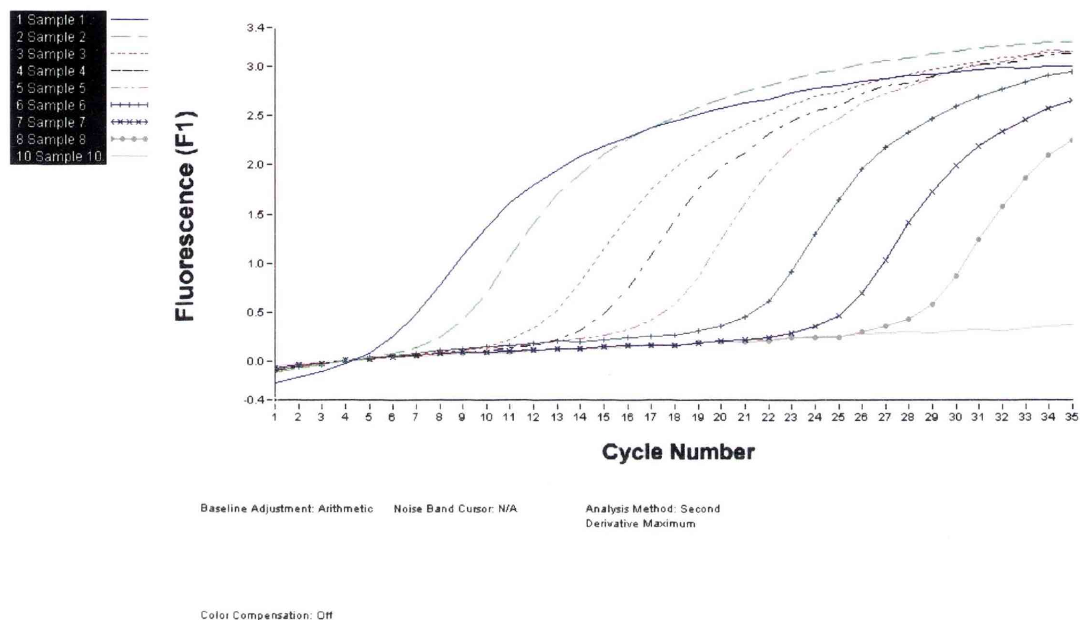

Figure 4.1. Evaluation of sensitivity of real-time PCR using cloned DNA. **The results show that the following amounts of target are detectable: Sample 1 – 10,000000 rDNA copies, Sample 2 – 1,000,000 rDNA copies, Sample 3 – 100,000 rDNA samples, Sample 4 – 10,000 rDNA copies, Sample 5 – 1,000 r DNA copies, Sample 6 – 100 r DNA copies, Sample 7 – 10 rDNA copies, Sample 8 – 1 rDNA copy, Sample 10 – negative control**

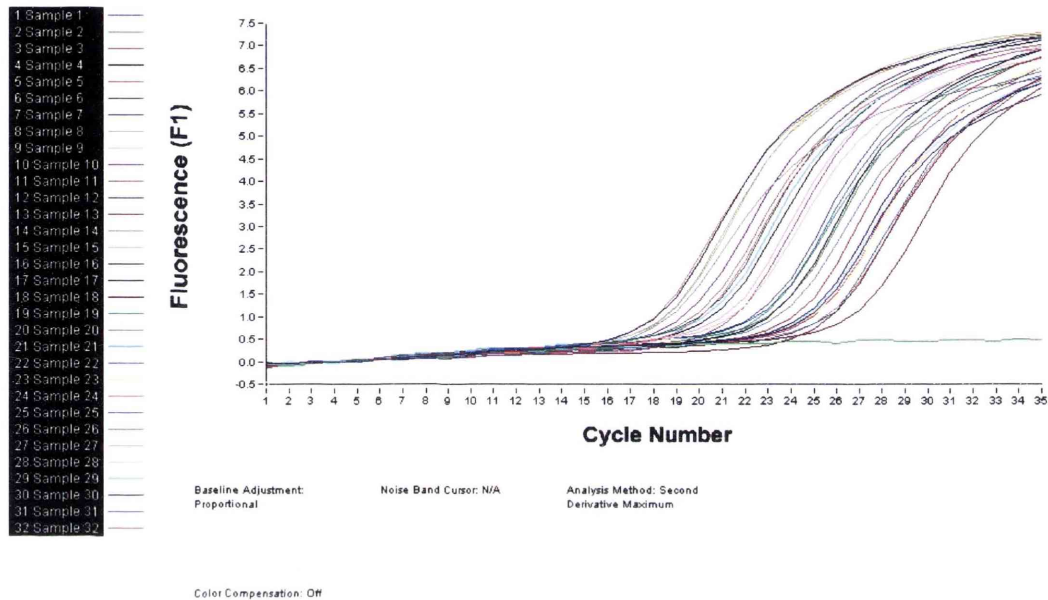

Figure 4.2. Detection of *D. fragilis* in faeces by real-time PCR. Sample 1- positive control, Sample 2 – negative control, Samples 3-32, *D. fragilis* microscopy positive samples.

4.4 DISCUSSION

In this study we developed a new 5' nuclease (TaqMan) based real-time PCR assay, targeting the small-subunit ribosomal RNA gene, for the detection of *D. fragilis* in human stool specimens. We then evaluated and compared the ability of real-time PCR to detect *D. fragilis* in faecal specimens with conventional PCR and microscopy. We took advantage of a cloned rDNA to initially determine the sensitivity of the assay. Other researchers have estimated that *D. fragilis* contains 100 copies of the rDNA repeat, so this was used in our calculations (Peek *et al.*, 2004).

In the comparison, microscopy missed 2 positive samples and also gave a false positive. The preparation of each slide and the staining procedure using a modified iron-haemotoxylin stain can take upwards of 1 hour. In addition to this is the reading of the slide which requires approximately 10 minutes per slide. Considerable expertise is also required in the reading and interpretation of the slides by the microscopist. Microscopy showed a sensitivity of 92.5% and this high sensitivity can be attributed to the highly experienced microscopists that were reading the slides. The conventional PCR only detected 48 positive samples in comparison to the 51 samples detected by the real-time PCR. The higher crossing-point values of those specimens that were positive by real-time PCR but negative by conventional PCR and microscopy indicate that the low number of parasites in those samples fell below the detection limit of both the traditional PCR and microscopy. Thus real-time PCR was shown to possess a higher level of sensitivity (approximately 100 times) for the detection of *D. fragilis* in faeces. Real-time PCR methods have been utilised in several areas of clinical parasitology, including the detection of faecal parasites. These real-time PCR assays have been shown to be more sensitive and specific than conventional methods. A real-time PCR method targeting the β-tubulin gene of *C. parvum* was shown to have a detection limit of 1 oocyst per reaction (Tanriverdi *et al.*, 2002). Varma *et al.*, (2003) developed a 5'nuclease assay which targeted the 18S rRNA of *Cyclospora cayetanensis*, the detection limit of this assay was 1 oocyst per reaction. Real-time assays targeting *E. histolytica* and *E. dispar* were sensitive enough to detect 0.1 parasite per gram of faeces (Blessman *et al.*, 2002). This

study represents the first report of the application of a real-time PCR assay for the detection of *D. fragilis* in human faecal specimens.

Of the cultured organisms that were studied by PCR, *T. vaginalis* and *T. foetus* DNA produced an amplicon with the *D. fragilis* primers, and subsequent alignments of *T. vaginalis*, *T. foetus* and *D. fragilis* DNA SSUrDNA showed the priming site to be highly conserved and almost identical among the three species. This shows the close relationship *D. fragilis* has with other trichomonads. However as *T. vaginalis* and *T. foetus* are not found in human stool samples, and other enteric trichomonads found in the stool specimens used in this study (*C. mesnili*, *P. homins* and *E.hominis*) did not produce an amplicon with the primers, this cross-reactivity does not affect the usefulness of this real-time assay in a clinical setting. Inhibition controls were carried out to exclude the possibility of inhibitory substances, and all were negative. Thus the PCR was shown to have 100% specificity for enteric specimens. On a similar note we have yet to evaluate the use of this PCR for *T. vaginalis* in clinical specimens.

Real-time PCR offers several advantages over conventional PCR for a diagnostic laboratory. As amplification and detection is in an integrated system the technology obviates the need for gel electrophoresis. This coupled with sealed reaction capillaries greatly reduces the risk of contamination from previously amplified products. The TaqMan probes allow constant reaction monitoring, and rapid cycling times allows for high specimen throughput and greatly reduces the time it takes for diagnosis of pathogens. However the major advantage real-time PCR has over conventional PCR is increased sensitivity (Templeton *et al.*, 2003; Pang *et al.*, 2004; Roy *et al.*, 2005).

This method coupled with the use of a simple commercially available DNA extraction kit enhances the practicability of the assay for diagnostic laboratories. Numerous studies have shown that the commercial Qiagen kit allows reproducible preparations of PCR-amplifiable DNA from stool samples as greater than 99% of all extracted samples do not contain inhibitory activity (Verweij *et al.*, 2000). This was confirmed in this study as no inhibition was detected in any samples. The entire assay, including the DNA extraction,

can be performed in less than 2 hours, providing an attractive alternative for the diagnosis of *D. fragilis*. As the assay is rapid it can be adapted to high throughput detection for screening of a large numbers of samples. This approach has much wider applicability in that it can also be applied to the detection of many other pathogens.

In summary this work is the first report of a real-time PCR assay specific for *D. fragilis*. On fresh stools that had undergone direct DNA extraction promptly (within 24hours) the sensitivity of the PCR was 100% and the specificity was 100%. The PCR method is quick (less than 2 h) and simple, and so offers another diagnostic tool other than light microscopy and conventional PCR for the diagnosis of dientamoebiasis.

CHAPTER 5

GENERAL DISCUSSION

In this study rDNA sequence data was generated from the entire SSU rDNA region from seven Australian *D. fragilis* isolates. All isolates that were sequenced showed no variation at this gene loci. The sequences from the Australian isolates however differed from the sequence of the *D. fragilis* strain Bi/PA (ATCC 30948) GenBank accession No.U37461. At position 705 the sequence data from the ATCC strain 30948 has a 15bp insert that is not present in any of the Australian isolates. In contrast, the Australian isolates were found to be similar to those found in a recent study in the Netherlands which also did not contain the polymorphic *Dde*I restriction site (CTTAG) at position 644 found in the *D. fragilis* strain Bi/PA (Peek *et al.*, 2004). The sequence data generated in all these studies supports the notion of at least two distinct genetic variants in *D. fragilis* (those with and without the insert). Johnson and Clarke (2000) estimated a sequence divergence of 2% between the two SSUrRNA genotypes of *D. fragilis*; this was later supported by Peek *et al.* (2004) by sequencing a 558bp region of the SSUrDNA. Our sequence data shows a greater sequence divergence of 4% between the Australian genotypes and the *D. fragilis* ATCC strain Bi/PA.

The sequence data also supports the findings that *D. fragilis* has a low G+C content relative to other trichomonads and contains on average an extra 100 nucleotides in the SSU rDNA region (Silberman *et al.*, 1996). This is due to three stretches of adenine and uracil in expansion segments known to be hypervariable regions. The SSU rDNA sequence data also highlights the close relationship between *D. fragilis* and *H. meleagridis*, an amoeboflagellate that is a common pathogen of domestic fowl (Silberman *et al.*, 1996). Whether or not *D. fragilis* is also found in fowl remains to be investigated.

The sequences data obtained from the Australian isolates were aligned with other enteric protozoan SSUrDNA sequences, available in GenBank. Primers were then designed targeting the SSUrDNA region for both conventional and real-time PCR assays.

Two DNA extraction methods for faecal specimens were also evaluated; the first using a commercial kit and the second using the commercial kit with modifications. Optimal

extraction of DNA from human faeces was achieved by using the commercial kit with no modifications to the extraction procedure. Given that the modified extraction procedure greatly increased the time taken to process the specimens (> 1h), it was deemed that the extra steps taken were not needed. This is in contrast to a study that used the modified technique for extraction of a closely related Trichomonad, *Tritrichomonas foetus*, from feline faeces (Gookin *et al.*, 2002).

The conventional PCR for *D. fragilis* was evaluated by using stool specimens where *D. fragilis* was detected by permanent stain. Of the 31 fresh faecal samples positive with *D. fragilis* on microscopy, 29 gave PCR products, thus giving a sensitivity of 93.5% from fresh specimens (extraction within 24 h). Aged specimens did not produce a PCR product. The remaining two specimens that did not amplify were processed and underwent extraction within 24 hours of receipt in the laboratory. These specimens were spiked with an equal volume of DNA from a known positive sample. All produced a PCR product. Thus the specimens were not inhibitory to the PCR reaction. These two specimens were processed promptly (within 24 h); however the time of collection was unknown as no time was recorded on the specimen jar and attempts to contact the patients failed. Therefore the failure of amplification may be explained by the age of the specimens.

The specificities of the primers for *D. fragilis* were tested using 29 specimens containing human protozoan parasites and 29 human stool samples containing no protozoan parasites. No non-specific amplification was seen with any of the specimens. Inhibition controls were carried out to exclude the possibility of inhibitory substances, and all were negative. Thus the PCR was shown to have 100% specificity.

A number of specimens were analysed by PCR in a double blind experimental design. All 20 positive specimens gave positive PCR results while all the negative samples gave negative PCR results.

The effect of storage and the use of fixatives on the PCR was also investigated. All of the specimens fixed in SAF failed to produce a PCR product. The stored specimens that had been fixed in SAF for various time periods, ranging from 5 days to 3 years, did not amplify. The 29 fresh specimens that produced a PCR product failed to do so having been fixed in SAF overnight. Therefore the PCR amplification of *D. fragilis* is inhibited by SAF fixation. Inhibition of PCR due to formalin has been described by other researchers. For example a recent study concluded that formalin fixation has a direct effect on the suitability of material as a template (Ramos *et al.*, 1999), with formalin causing the DNA to fragment during the fixation process or the DNA to become cross-linked to its associated proteins. Therefore SAF fixed specimens are not suitable for *D. fragilis* PCR.

The time course experiments clearly demonstrated that the sensitivity of the PCR was affected when aged specimens were used as the DNA source. Most of the isolates failed to produce a PCR product after 72 hours, indicating that aged specimens that have been stored at 4-8°C are unsuitable for this PCR. DNA extraction on fresh samples less than 24 hours old is recommended as the trophozoites degenerate rapidly. Peek *et al.* (2004) found that *D. fragilis* DNA could be detected up to one week, after which the signal could not be detected or became very weak. The specimens for Peek's PCR assay were stored at room temperature. *D. fragilis* has been shown to rapidly degenerate once refrigerated and this could account for the fact that the *D. fragilis* DNA degenerated more rapidly in our study than in the study from the Netherlands (Peek *et al.*, 2004).

These findings are supported by other research which demonstrated that *D. fragilis* does degenerate rapidly once outside the human body (Dobell, 1940), with the failure to amplify the DNA template due to nuclear material degrading in the ageing specimens. Unlike many other intestinal protozoa, *D. fragilis* may not have a cyst stage. Estimates of the survival time of trophozoites in faecal specimens vary from six to 48 hours (Hakansson, 1936). Wenrich (1944) conducted a number of experiments and was unable to keep *D. fragilis* trophozoites alive in boiled pond water or boiled hay infusion and in tap water they swell and burst within minutes. Yang and Scholten (1977) failed to keep *D. fragilis* trophozoites alive in simulated gastric juice.When using culture systems it was

shown that positive cultures were only obtained from stools 8-11 hours old that had not been refrigerated (Sawangjaroen *et al.*, 1993). Brug (1936) found that cultures of *D. fragilis* that were exposed to room temperature were adversely affected. Dobell (1940) also described the fragile nature of this organism.

As inhibitors of PCR are commonly found in faecal specimens, a method for DNA purification that removes faecal inhibitors is needed. These inhibitors include haeme compounds, acidic complex polysaccharides, protein, proteinases, DNAses, fats and interference from the DNA of other organisms or mucosal cells (Demeke and Adams 1992: Akane *et al.*, 1994; Stacy-Phipps *et al.*, 1995: Lantz *et al.*, 1997; Monterio *et al.*, 1997). The QIAamp™ DNA stool minikit has been evaluated and used successfully in a number of studies (Holland *et al.*, 2000; McOrist *et al.*, 2002). These studies include a number where protozoa have undergone direct DNA extraction from faeces including the extraction of *Cryptosporidium parvum* DNA from faeces (Limor *et al.*, 2002), and *Entamoeba histolytica/dispar* complex (Blessmann *et al.*, 2002). It has also been shown to be successful in extracting DNA from *D. fragilis* in the study described here. No inhibitory effect of faecal material was detected in any of the negative samples in this study. Numerous studies have shown that the commercial QIAGEN kit allows reproducible preparations of PCR-amplifiable DNA from stool samples as more than 99% of all extracted samples do not contain inhibitory activity (Gookin *et al.*, 2002). This was confirmed in this study as no inhibition was detected in any samples. This method coupled with the use of a simple commercially available DNA extraction kit enhances the practicability of the assay for diagnostic laboratories.

I developed a new 5' nuclease (TaqMan) based real-time PCR assay, amplifying a 78bp region of the small-subunit ribosomal RNA gene, for the detection of *D. fragilis* in human stool specimens. Of the cultured organisms that were studied by real-time PCR, *T. vaginalis* and *T. foetus* DNA produced an amplicon with the *D. fragilis* primers, and subsequent alignments of *T. vaginalis*, *T. foetus* and *D. fragilis* DNA SSUrDNA showed the priming site to be highly conserved and almost identical among the three species. This shows the close relationship *D. fragilis* has with other trichomonads. However,. as

T. vaginalis and *T. foetus* are not found in human stool samples, and other enteric trichomonads found in the stool specimens used in this study (*C. mesnili*, *Pentatrichomonas. homins*, and *E.hominis*) did not produce an amplicon with the primers this cross-reactivity does not affect the usefulness of this real-time assay in a clinical setting. Inhibition controls were carried out to exclude the possibility of inhibitory substances, and all were negative. Thus the PCR was shown to have 100% specificity for enteric specimens. On a similar note I have yet to evaluate the use of this PCR for *T. vaginalis* in clinical specimens.

To determine the sensitivity of both conventional and real-time PCR, the entire SSUrRNA gene was cloned and a known number of copies were then amplified using the same conditions as for the patients' samples. This showed that the detection limit was 100 plasmid copies or an equivalent of approximately one *D. fragilis* trophozoite for conventional PCR. The detection limit for the real-time PCR was determined at one plasmid copy (a crossing point of 27.87) of the SSUrRNA gene which is equivalent to approximately 0.01 *D. fragilis* trophozoite. This shows that the real-time PCR was 100 times more sensitive than the conventional PCR.

In the comparison of the three diagnostic methods, microscopy missed two positive samples and also gave a false positive. The preparation of each slide and the staining procedure using a modified iron-haemotoxylin stain can take upwards of one hour. In addition to this is the reading of the slide which requires approximately 10 minutes per slide. Considerable expertise is also required in the reading and interpretation of the slides by the microscopist. Microscopy showed a sensitivity of 92.5% and this high sensitivity can be attributed to the highly experienced microscopists that were reading the slides. The conventional PCR only detected 48 positive samples in comparison to the 51 samples detected by the real-time PCR. The higher crossing-point values of those specimens that were positive by real-time PCR, but negative by conventional PCR and microscopy, indicate that the low number of parasites in those samples fell below the detection limit of both the traditional PCR and microscopy. Thus real-time PCR was shown to possess a higher level of sensitivity (approximately 100 times) for the detection

of *D. fragilis* in faeces. Real-time PCR methods have been utilised in several areas of clinical parasitology, including the detection of faecal parasites. These real-time PCR assays have been shown to be more sensitive and specific than conventional methods. A real-time PCR method targeting the β-tubulin gene of *C. parvum* was shown to have a detection limit of one oocyst per reaction (Tanriverdi, *et al.*, 2002). Varma *et al.* (2003) developed a 5'nuclease assay which targeted the 18S rRNA of *Cyclospora cayetanensis*, the detection limit of this assay was one oocyst per reaction. Real-time assays targeting *E. histolytica* and *E. dispar* were sensitive enough to detect 0.1 parasite per gram of faeces (Blessman *et al.*, 2002). This study represents the first report of the application of a real-time PCR assay for the detection of *D. fragilis* in human faecal specimens.

Current techniques that are used to diagnose *D. fragilis* infection are laborious, time consuming, require highly trained staff, and are prone to human error. Correct and prompt diagnosis is essential given the potential for chronic long-term infections. Recognition is also important for clinical management, as specific treatment is often required. These PCR techniques will allow the rapid identification of *D. fragilis* in clinical specimens with results available in several hours. It will also provide a quick, simple and effective method to investigate the molecular epidemiology of *D. fragilis*. By using a direct extraction method, culture systems that are often laborious, time consuming and technically difficult need not be used when studying *D. fragilis*.

Real-time PCR offers several advantages over conventional PCR for a diagnostic laboratory. As amplification and detection is in an integrated system, the technology obviates the need for gel electrophoresis. This coupled with sealed reaction capillaries greatly reduces the risk of contamination from previously amplified products. The TaqMan probes allow constant reaction monitoring, and rapid cycling times allows for high specimen throughput and greatly reduces the time it takes for diagnosis of pathogens. However, the major advantage real-time PCR has over conventional PCR is increased sensitivity.

This work is the first attempt to extract DNA from *D. fragilis* in clinical stool samples and development of a conventional and real-time PCR assay specific for *D. fragilis* in Australia. On fresh stools that had undergone direct DNA extraction promptly (within 24 hours) the sensitivity of the conventional PCR was 93.5% and the specificity was 100%. While the real-time PCR showed a sensitivity and specificity of 100%. Optimal extraction occurs on fresh faeces less than 24 hours old using a commercially available kit. The PCR methods are quick and simple, and so offers another diagnostic method in addition to light microscopy and conventional PCR for the diagnosis of dientamoebiasis

Dientamoeba fragilis has a worldwide cosmopolitan distribution. In Australia and New Zealand the reported prevalence rate ranges from 0.4% in Western Australia (Anonymous, 1992) to 16.8% in suburban Sydney (Walker *et al.*, 1985). In this present study a prevalence of 0.9% was found, this is in stark contrast to the prevalence of 16.8% that was found by Walker *et al.* (1985) in the Sydney suburb of French's Forrest. It was our aim to determine the prevalence, epidemiology and genotypes of *D. fragilis* within Sydney, Australia

In this study *D. fragilis* infection was closely associated with diarrhoea, abdominal pain and loose bowel motions. All patients with *D. fragilis* infection were symptomatic and bacterial and viral causes of these symptoms are unlikely as routine microbiological cultures, adenovirus and rotavirus testing were negative. Three patients were also infected with *G. intestinalis* which could have caused the gastrointestinal symptoms described in those patients.

One important finding of this study was that chronic persistent *D. fragilis* infections were common. Thirty-two percent of patients had diarrhoea for greater than 2 weeks duration, and one patient claimed to have had intermittent diarrhoea for several years. Five patients had recurrent *D. fragilis* infection during the course of the study. It is unknown whether these recurrences were due to treatment failure or reinfection from a common source. One patient was diagnosed with irritable bowel syndrome and was subsequently found to have *D. fragilis* infection. A recent Australian study by Borody *et al.*, (2002) showed a

link between *D. fragilis* and irritable bowel syndrome (IBS). Twenty-one patients diagnosed with IBS and concurrent *D. fragilis* infection were treated with iodoquinol and doxycycline. Complete elimination of *D. fragilis* with marked clinical improvement occurred in the majority of patients.

Only one patient was immunosuppressed (HIV-infected) with all the others being immunocompetent. This is in contrast to a study from Argentina which suggested that the incidence of *D. fragilis* infections may be higher in immunocompromised patients (Mendez *et al.*, 1994). In all other studies conducted, including this present one immunosuppression does not seem to be a contributing factor for infection with *D. fragilis*.

Ten percent of patients diagnosed with *D. fragilis* infection had a history of recent overseas travel including Southeast Asia, Papua New Guinea, Timor and Fiji. *Dientamoeba fragilis* has been implicated as a cause of diarrhoea in returning Swedish travellers with Norberg *et al.* (2003) finding 63% of patients in a retrospective study had been infected outside the country. Most patients were infected in Africa, South America and the Middle East.

In our study *D. fragilis* occurred in older patients with the average age of 39.8 years, and a median of 44.5 years. This is in contrast to other studies that have found higher prevalence rates in children (Preiss, *et al.*, 1991; Norberg, *et al.*, 2003). However the true incidence of infection in younger children may not be truly reflected. Although the microbiology laboratory services a number of general practices with paediatric clients, St.Vincent's Hospital does not have a paediatric department so cases from this age group are under-represented in this study.

No parasites were detected by formalin ethyl acetate concentrations performed on faecal specimens from the *D. fragilis* infected patients. Fifty-five percent of the patients submitted a sticky-tape test for the detection of *E. vermicularis* ova and no *E. vermicularis* ova were detected. Many researchers have postulated that pin worm is a

vector for *D. fragilis* transmission. Burrows *et al.* (1954) were the first to propose that *E. vermicularis* might be a vector for *D. fragilis*. Several other researchers also found a higher than expected concurrence of *D. fragilis* and *E. vermicularis* infections (Burrows and Swerdlow, 1956; Yang and Scholten, 1977; Preiss *et al.*, 1991).

In contrast a recent study of 25 paediatric cases of *D. fragilis* found no infections were associated with *E. vermicularis* (Cuffari *et al.*, 1998). These results along with the findings from this present study would argue against the hypothesis that *E. vermicularis* plays a significant role in the transmission of *D. fragilis*. Most studies that have examined *D. fragilis* infection have inadequately examined for *E. vermicularis*, as appropriate testing methods for the detection of pinworm were not used. It has yet to be proven what role helminth ova play in the transmission of *D. fragilis*. Further study is required to ascertain the true mode of transmission of this organism.

Other enteric protozoa were present in 40% of patients with *D. fragilis* infection. The most common organism was *B. hominis*. Other protozoa present included *E. nana*, *E. hominis*, *E. coli*, *Iodamoeba butschlii* and *G. intestinalis*. All of these parasites are known to be transmitted via the faecal-oral route. Other researchers have found similar rates of coinfection of *D. fragilis* with other parasites that are transmitted via the faecal-oral route. Windsor *et al.* (1998) found 54% of patients with *D. fragilis* had other parasites or enteropathogens present. These findings would provide evidence to support a hypothesis for a faecal-oral route of transmission for *D. fragilis*.

No *D. fragilis* trophozoites were detected in the control group of 900 patients without gastrointestinal symptoms. This is in contrast to other studies where *D. fragilis* was detected in patients with no clinical symptoms (Colea *et al.*, 1980) and in a case control study on gastroenteritis from the Netherlands where *D. fragilis* was recovered more frequently from controls than case patients (De Wit *et al.*, 2001). These findings may be attributed to the fact that asymptomatic carriage of intestinal protozoa may occur.

The permanent stained smears positive for *D. fragilis* were confirmed by PCR. A sensitivity of 93% was obtained using a previously published method (Stark *et al.*, 2005a). All 90 negative samples from the control group failed to produce a PCR product.

Sequence data generated in several studies supports the notion of at least two distinct genetic variants in *D. fragilis*. Johnson and Clarke (2000) estimated a sequence divergence of 2% between the two SSUrRNA genotypes of *D. fragilis*; this was later supported by Peek *et al.* (2004) by sequencing a 558bp region of the SSU rDNA. Sequence data generated by Stark *et al.* (2005a) from the entire SSU rDNA region of Australian isolates of *D. fragilis* showed a greater sequence divergence of 4% between the Australian genotypes and the *D. fragilis* ATCC strain Bi/PA (ATCC 30948). All Australian strains sequenced were identical and supports the notion that *D. fragilis* is a clonal species. The Australian isolates were found to be similar to those found in a recent study in the Netherlands and do not contain the polymorphic *Dde* I restriction site (CTTAG) at position 644 found in the *D. fragilis* strain Bi/PA (Peek *et al.*, 2004). RFLP analysis was undertaken on all 50 Australian samples to determine the genotypes present in the Australian population and the extent of genetic diversity. The PCR used in this study amplifies the SSU rDNA region from approximately position 400 to position 1270. This PCR product contains a *Dde* I restriction sites (CTTAG) that are present in the *D. fragilis* ATCC 30948 strain yet are absent in the Australian genotypes. All 50 *D. fragilis* samples showed no variation and corresponded to genotype A. These findings suggest that *D. fragilis* in Sydney, Australia display only a single genotype in faecal samples from various groups including inpatients, outpatients and travellers. Further studies are needed to identify the presence of other genotypes throughout Australia.

Dientamoeba fragilis has no recognised cyst stage and as such diagnosis is dependent on detecting the trophozoites. As these trophozoites degenerate rapidly, prompt fixation of the specimen is necessary (Yang and Scholten, 1977). Successful diagnosis of *D. fragilis* is closely associated with the use of permanent stains of faecal smears. Failure to use permanent staining and fixation techniques will inevitably preclude identification of *D. fragilis*. The aim of the questionnaire sent to the Sydney laboratories was to determine

how many laboratories routinely undertake permanent staining and therefore how many laboratories are able to report the presence of *D. fragilis*. Of the 26 Sydney laboratories 58% participated in the survey and only three routinely performed permanent staining for ova, cyst and parasites in faecal specimens. Those three laboratories were the only institutions that detected *D. fragilis* in routine samples. Therefore the true extent of *D. fragilis* infection must be greatly underestimated as most laboratories do not use techniques to adequately identify this organism.

This is the first prospective study of *D. fragilis* in Australia to examine clinical data in addition to the genetic diversity of the isolates. Diagnosis was based on permanent staining of fixed faecal smears and confirmed by PCR which demonstrated good sensitivity. All persons infected with *D. fragilis* were symptomatic and *D. fragilis* infections were most commonly associated with diarrhoea and abdominal pain. Concurrent infections with other protozoa were common, occurring in 40% of samples. The occurrence of *D. fragilis* with other protozoa that are transmitted via the faecal oral route would strengthen the case for *D. fragilis* also being transmitted via this route. No correlation was found with *E. vermicularis* or any other helminths, questioning the role, if any, pin worm has in the transmission of *D. fragilis*. The genetic diversity of 50 samples was examined by PCR followed by RFLP. This data indicated that a single genotype of *D. fragilis* was represented, one that is genetically different to the North American *D. fragilis* strain Bi/PA (ATCC 30948). The evidence that *D. fragilis* is a pathogenic protozoa is overwhelming and as such all laboratories should attempt to identify this protozoa by the use of permanent staining techniques or molecular methods.

CHAPTER 6

APPENDIX

Dientamoeba Fragilis Laboratory Data Collection Sheet

Name of institution	
Contact person	

Contact Details

Address:
Phone:
Fax:
Email:

1 JANUARY 1996 TO 31 DECEMBER 1996 RESULTS

Q1 Total number of stool samples processed for ova cyst and parasites: ☐

Q2 Total number of stools positive for *D. fragilis*: ☐

Q3 Are permanent stained smears routinely performed?

☐1 **Yes, please go to Q4** ☐2 **No, please go to Q5**

Q4 Which of the following permanent stains are used?

(please tick any appropriate boxes)

☐1 Iron haemotoxylin

☐2 Modified Iron haemotoxylin (incorporating carbol fuchsin step eg. Medivet stain)

☐3 Trichrome stain

☐4 Other, please specify: ______________________

Q5 Fixation method used in this period

(please tick one box only)

☐1 PVA

☐2 SAF

☐3 Schaudinn's fixative

☐4 5% or 10% formalin

☐5 Merthiolate-iodine-formaldehyde (MIF)

☐6 No fixative used

☐7 Other fixative used, please specify: ______________________

Q6 In which of the following situation would a fixation method be used?

(please tick one box only)

☐1 All stool samples

☐2 Semiform stool samples

☐3 Liquid stool samples only

☐4 Other, please specify: ______________________

1 JANUARY 1997 TO 31 DECEMBER 1997 RESULTS

Q7 Total number of stool samples processed for ova cyst and parasites: ☐

Q8 Total number of stools positive for *D. fragilis*: ☐

Q9 Are permanent stained smears routinely performed?

☐1 **Yes, please go to Q10** ☐2 **No, please go to Q11**

Q10 Which of the following permanent stains are used?
(please tick any appropriate boxes)

☐1 Iron haemotoxylin

☐2 Modified Iron haemotoxylin (incorporating carbol fuchsin step eg. Medivet stain)

☐3 Trichrome stain

☐4 Other, please specify: ______________________

Q11 Fixation method used in this period
(please tick one box only)

☐1 PVA

☐2 SAF

☐3 Schaudinn's fixative

☐4 5% or 10% formalin

☐5 Merthiolate-iodine-formaldehyde (MIF)

☐6 No fixative used

☐7 Other fixative used, please specify: ______________________

Q12 In which of the following situation would a fixation method be used?

(please tick one box only)

☐1 All stool samples

☐2 Semiform stool samples

☐3 Liquid stool samples only

☐4 Other, please specify: ____________________

1 JANUARY 1998 TO 31 DECEMBER 1998 RESULTS

Q13 Total number of stool samples processed for ova cyst and parasites: ☐

Q14 Total number of stools positive for *D. fragilis*: ☐

Q15 Are permanent stained smears routinely performed?

☐1 **Yes, please go to Q16** ☐2 **No, please go to Q17**

Q16 Which of the following permanent stains are used?
(please tick any appropriate boxes)

☐1 Iron haemotoxylin

☐2 Modified Iron haemotoxylin (incorporating carbol fuchsin step eg. Medivet stain)

☐3 Trichrome stain

☐4 Other, please specify: ______________________

Q17 Fixation method used in this period
(please tick one box only)

☐1 PVA

☐2 SAF

☐3 Schaudinn's fixative

☐4 5% or 10% formalin

☐5 Merthiolate-iodine-formaldehyde (MIF)

☐6 No fixative used

☐7 Other fixative used, please specify: ______________________

Q18 In which of the following situation would a fixation method be used?
(please tick one box only)

☐1 All stool samples

☐2 Semiform stool samples

☐3 Liquid stool samples only

☐4 Other, please specify: ______________________

1 JANUARY 1999 TO 31 DECEMBER 1999 RESULTS

Q19 Total number of stool samples processed for ova cyst and parasites: ☐

Q20 Total number of stools positive for *D. fragilis*: ☐

Q21 Are permanent stained smears routinely performed?

☐1 **Yes, please go to Q22** ☐2 **No, please go to Q23**

Q22 Which of the following permanent stains are used?

(please tick any appropriate boxes)

☐1 Iron haemotoxylin

☐2 Modified Iron haemotoxylin (incorporating carbol fuchsin step eg. Medivet stain)

☐3 Trichrome stain

☐4 Other, please specify: ____________________

Q23 Fixation method used in this period

(please tick one box only)

☐1 PVA

☐2 SAF

☐3 Schaudinn's fixative

☐4 5% or 10% formalin

☐5 Merthiolate-iodine-formaldehyde (MIF)

☐6 No fixative used

☐7 Other fixative used, please specify: ____________________

Q24 In which of the following situation would a fixation method be used?

(please tick one box only)

- ☐1 All stool samples
- ☐2 Semiform stool samples
- ☐3 Liquid stool samples only
- ☐4 Other, please specify: ______________________

1 JANUARY 2000 TO 31 DECEMBER 2000 RESULTS

Q25 Total number of stool samples processed for ova cyst and parasites: ☐

Q26 Total number of stools positive for *D. fragilis*: ☐

Q27 Are permanent stained smears routinely performed?

☐1 **Yes, please go to Q28** ☐2 **No, please go to Q29**

Q28 Which of the following permanent stains are used?
(please tick any appropriate boxes)

☐1 Iron haemotoxylin

☐2 Modified Iron haemotoxylin (incorporating carbol fuchsin step eg. Medivet stain)

☐3 Trichrome stain

☐4 Other, please specify: ______________________

Q29 Fixation method used in this period
(please tick one box only)

☐1 PVA

☐2 SAF

☐3 Schaudinn's fixative

☐4 5% or 10% formalin

☐5 Merthiolate-iodine-formaldehyde (MIF)

☐6 No fixative used

☐7 Other fixative used, please specify: ______________________

Q30 In which of the following situation would a fixation method be used?

(please tick one box only)

- ☐1 All stool samples
- ☐2 Semiform stool samples
- ☐3 Liquid stool samples only
- ☐4 Other, please specify: ______________________

1 JANUARY 2001 TO 31 DECEMBER 2001 RESULTS

Q31 Total number of stool samples processed for ova cyst and parasites: ☐

Q32 Total number of stools positive for *D. fragilis*: ☐

Q33 Are permanent stained smears routinely performed?

☐1 **Yes, please go to Q34** ☐2 **No, please go to Q35**

Q34 Which of the following permanent stains are used?

(please tick any appropriate boxes)

☐1 Iron haemotoxylin

☐2 Modified Iron haemotoxylin (incorporating carbol fuchsin step eg. Medivet stain)

☐3 Trichrome stain

☐4 Other, please specify: ____________________

Q35 Fixation method used in this period

(please tick one box only)

☐1 PVA

☐2 SAF

☐3 Schaudinn's fixative

☐4 5% or 10% formalin

☐5 Merthiolate-iodine-formaldehyde (MIF)

☐6 No fixative used

☐7 Other fixative used, please specify: ______________________

Q36 In which of the following situation would a fixation method be used?
(please tick one box only)

☐1 All stool samples

☐2 Semiform stool samples

☐3 Liquid stool samples only

☐4 Other, please specify: ______________________

1 JANUARY 2002 TO 31 DECEMBER 2002 RESULTS

Q31 Total number of stool samples processed for ova cyst and parasites: ☐

Q32 Total number of stools positive for *D. fragilis*: ☐

Q33 Are permanent stained smears routinely performed?

☐1 **Yes, please go to Q34** ☐2 **No, please go to Q35**

Q34 Which of the following permanent stains are used?
(please tick any appropriate boxes)

☐1 Iron haemotoxylin

☐2 Modified Iron haemotoxylin (incorporating carbol fuchsin step eg. Medivet stain)

☐3 Trichrome stain

☐4 Other, please specify: ______________________

Q35 Fixation method used in this period
(please tick one box only)

☐1 PVA

☐2 SAF

☐3 Schaudinn's fixative

☐4 5% or 10% formalin

☐5 Merthiolate-iodine-formaldehyde (MIF)

☐6 No fixative used

☐7 Other fixative used, please specify: ______________________

Q36 In which of the following situation would a fixation method be used?
(please tick one box only)

☐1 All stool samples

☐2 Semiform stool samples

☐3 Liquid stool samples only

☐4 Other, please specify: ______________________

THANK YOU FOR COMPLETING THIS FORM

CHAPTER 6

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