# New discovery and ultrastructural description of *Dientamoeba fragilis* cysts and the establishment of an animal model for their study

**By**

**Varuni Subashini Munasinghe**

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**School of Life sciences,**

**Ithree Institute,**

**Faculty of Science,**

**University of Technology**

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## Certificate of original authorship

This study was conducted in the School of Medical and Molecular Biosciences and i3 institute, Faculty of Science, University of Technology, Sydney and in the Microbiology Department, St. Vincent's Hospital Sydney, under the supervision of Professor John T. Ellis and Dr. Damien Stark.

I certify that the work in this thesis are all done in part for the fulfilment of this thesis and has not been submitted as part of requirements for a degree except within this thesis.

Finally, I certify that the thesis has been written by me with editorial support from my supervisors, Professor Michael Wallach, Professor John Ellis and Dr. Damien Stark as acknowledged in individual chapters. I have acknowledged all the help, support and resources that I received in fulfilment of this work. Finally, I certify all literature and information sources used have been indicated in this thesis.

Varuni Subashini Munasinghe

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## Table of contents

## List of figures

## Referred publications arising from this thesis

- **Munasinghe, V.S**., Stark, D., Ellis, J.T., 2012. New advances in the in-vitro culture of Dientamoeba fragilis. Parasitology 139, 864-869.

- **Munasinghe, V.S**., Vella, N.G.F., Ellis, J.T., Windsor, P.A., Stark, D., 2013. Cyst formation and faecal–oral transmission of Dientamoeba fragilis – the missing link in the life cycle of an emerging pathogen. Int J Parasitol. 43, 879-883

## Conference Proceedings

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## Abbreviations

**Terms:**

| | |
|---|---|
| Ax | Axostyle |
| ATCC | American Type Culture Collection |
| BB | Basal Body |
| Co | Costa |
| CWP | Cyst wall protein |
| DNA | Deoxyribonucleic Acid |
| DNase | Deoxyribonuclease |
| EBSS | Earle's Balanced Salt Solution |
| Gc | Golgi Complex |
| HCl | Hydrochloric Acid |
| IBS | Irritable bowel syndrome |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| Pf | Parabasal Filament |
| RNA | Ribonucleic Acid |
| RNase | Ribonuclease |
| rRNA | Ribosomal RNA |
| TEM | Transmission Electron Microscopy |

**Units:**

| | |
|---|---|
| °C | Degree Celsius |
| G | Relative Centrifugal Force |
| KDa | Kilo Daltons |

| | |
|---|---|
| Kg | kilogram |
| M | Molar |
| $\mu$ M | Micromolar |
| $\mu$ m | Micrometre |
| $\mu$ L | Microlitre |
| mg | Milligram |
| mL | Millilitre |
| mM | Millimolar |
| min | Minute |
| ng | Nanogram |
| nm | Nanometer |

## Abstract

*Dientamoeba fragilis* is a pathogenic protozoan parasite which causes diarrhoea and gastrointestinal disease in humans with a propensity for chronic infections. Although *Dientamoeba* was discovered over a century ago, its life cycle and mode of transmission are poorly defined. No cyst stage has been described in the scientific literature and no animal models were available for the further study of this parasite in the past. The clinical and pathologic features of Dientmaoebiasis, along with the pathogenic mechanisms of the disease and the nature of the host defence weren't fully elucidated.

In this study the *in vitro* culture for *D. fragilis* was established and further improved, which increased the trophozoite numbers to large and sufficient numbers for further study. A new overlay was designed for the *in vitro* culturing of *D. fragilis* trophozoites which is Earle's balanced salt solution (EBSS) enriched with ferric ammonium citrate and cholesterol. The large trophozoite numbers obtained from the *in vitro* culture using this overlay enabled their use to inoculate experimental animals in order to develop an animal model. A rodent model was developed using BALB/C mice and rats to study the mode of transmission of this parasite, which remained a mystery in the past. This was an important step in this research as attempts to establish an animal model for this parasite have been unsuccessful in the past. Moreover, the animal model s enabled us to fulfil three criteria of Koch's postulates for *D. fragilis*. The most important finding of this study was the discovery of a cyst stage of *D. fragilis,* adding to the evidence on the mode of transmission of *D. fragilis* via cysts. Ultrastructural observations of the cysts were carried out in detail using transmission electron microscopy. These studies of cysts showed a clear cyst wall surrounding an encysted parasite. The cyst wall was double layered with an outer fibrillar layer and an inner layer enclosing the parasite. Hydrogenosomes, endoplasmic reticulum and nuclei were present in the cysts. Pelta-axostyle structures, costa and axonemes were identifiable and internal flagella were present. These cysts shared similar morphological characteristics to those of *Giardia*, *Histomonas,* which belong to the same family as *D. fragilis* showing its phylogenetic relationship with these parasites. This study provides additional novel details and knowledge of the ultrastructure of the cyst stage of *D. fragilis,* that plays an important role in the mode of transmission of this pathogen.

The data support the pathogenic potential of this organism, demonstrates chronic infection and parasite carriage along with prolonged shedding of the organism. The recurrent nature of Dientamoebiasis in human hosts could be attributable to the cysts stage which is more resistant to the environmental conditions than the trophozoite stage. Further research is needed to study the biology and the virulence of the cyst stage of *D. fragilis*. The discovery of the cyst stage and the establishment of an animal model have major implications for the potential control of Dientamoebiasis in humans and in gaining a better understanding of the disease itself.

# Chapter 1:

# Literature Review on *Dientamoeba fragilis* and cysts of parasitic protozoa

## 1.1 Introduction

*Dientamoeba fragilis* is a pathogenic protozoan which inhabits the gut and causes gastrointestinal disease in humans (Stark et al., 2010b; Stark et al., 2009). It was first described by Jepps and Dobell in 1918 as a commensal, which inhabits the gut of humans (Jepps and Dobell, 1918a). Recent studies have shown its pathogenic potential in infected humans presenting with a wide range of gastrointestinal symptoms including diarrhoea, loose stool, and abdominal pain (Crotti et al., 2005; Norberg et al., 2003; Stark et al., 2005b; Vandenberg et al., 2006). Chronic symptoms are common with persistent diarrhoea as described by Stark et al 2005 (Stark et al., 2005b). *Dientamoeba fragilis* has a worldwide distribution with a prevalence varying between 0.3- 52% (Barratt et al., 2011b). The reported prevalence in Australia and New Zealand varies from 0.4% in Western Australia, and 1.5% in an urban community in Brisbane to 2.2% in Christchurch, New Zealand and 16.8% in suburban Sydney (Stark et al., 2005b). In contrast to many pathogenic protozoa, which are more prevalent in developing countries where hygiene and health standards are poor, this parasite is more prevalent in the countries with higher health standards such as Italy, Belgium, United States and Sweden (Crotti and D'Annibale, 2007b; Spencer et al., 1979; Stensvold et al., 2007). It has been suggested that between 6.3% to 29.8% of people who are suffering from intestinal parasitosis will harbour *Dientamoeba,* which further suggests that *D. fragilis* is a commonly occurring parasite in relation to other gastrointestinal parasites (Rayan et al., 2007; Schuster and Jackson, 2009; Stensvold et al., 2007; Vandenberg et al., 2006). Moreover, due to recent advances in diagnostic methods to detect *D. fragilis,* it is now often described as being more prevalent than *Giardia* (Barratt et al., 2011b; Chan et al., 1996; Girginkardesler et al., 2003; Millet et al., 1983; Stark et al., 2010b; Stensvold et al., 2007).

## 1.2 Taxonomy

This parasite has a detailed history of reclassification (Hopkins, 2006). Initially *D. fragilis* was included in the subphylum Sarcodina (Johnson et al., 2004). Later on, Camp et al (1974) observed the ultrastructure of *D. fragilis* through transmission electron microscopy for the first time and indicated its close similarity to the Trichomonads (Stark et al., 2006a). Later studies show that *D. fragilis* is closely related to *Histomonas meleagridis,* which is a Trichomonad flagellate (Delgado-Viscogliosi et al., 2000). Further studies based on the sequence analysis of SSU rDNA showed that it is closely related to Trichomonads (Gerbod et al., 2002; Ohkuma et al., 2005).

Current Classification of *Dientamoeba fragilis* (Stark et al., 2006b)

Kingdom: Protista
Subkingdom:protozoa
Phylum:sarcomastigophora
Subphylum: Mastigophora
Class: Zoomastigophora
Order: Trichomonadida
Family:monocercomonadidae
Genus: *Dientamoeba*
Species: *fragilis*

## 1.3 Morphology

*Dientamoeba fragilis* is a single celled, pleomorphic trophozoite ranging from 5µm to 15µm in diameter (Stark et al., 2006a). *Dientamoeba* may contain one to four nuclei as found in light microscopic studies (Banik et al., 2012). The trophozoites are binucleate in stained smears and contained a large, fragmented, central karyosome (Sawangjaroen et al., 1993). It was long believed that *D. fragilis* does not have a cyst stage (Barratt et al., 2011a). In this study the cyst of *D. fragilis* was detected for the first time along with the ultrastructural description of the cyst wall. The cyst of *D. fragilis* has a thick cyst wall with one to two nuclei and measured to be 5µm in diameter as observed in iron hematoxylin stained smears (Figure 1) (Munasinghe et al., 2013). The cyst wall is filamentous in nature, which is similar to that of *Giardia* (Munasinghe et al., 2013). A recent study detected the basal body components of *D. fragilis* trophozoites although no flagellar axonemes were visible (Banik et al., 2012). Adding to this information, this

study was able to detect flagellar axonemes in the cysts of *D. fragilis* (Munasinghe et al., 2013), confirming that *D. fragilis* is a flagellate.

### 1.4 Clinical aspects

Initially it was thought that *D. fragilis* is non-pathogenic (Stark et al., 2006b). In many clinical settings there is still reluctance to accept the pathogenic potential of this organism and many doubt its clinical importance as a disease causing pathogen in humans (Stark et al., 2006b). Therefore, this pathogen has been neglected for decades (Johnson et al., 2004).

An Australian study carried out in 2002 observed an association between *D. fragilis* and irritable bowel syndrome (Borody et al., 2002). Twenty one patients diagnosed with IBS and concurrent *D. fragilis* infection were treated with iodoquinol and doxycycline (Borody et al., 2002). They all showed complete elimination of the parasites and improvement in their clinical symptoms (Stark et al., 2006b). However, there is no other convincing evidence to support this association between IBS and *D. fragilis* infection, which need further investigation.

Previous studies have documented the pathogenic role of *D. fragilis*. It causes colonic irritation provoking an inflammatory response that leads to fibrosis (Vassalou et al., 2016). Another study has shown that *D. fragilis* excretes potential cytotoxic molecules that causes symptoms in infected individuals (Barratt et al., 2012).

A prospective study conducted in Australia examined 6750 faecal samples over a 30 month period for the presence of *D. fragilis* trophozoites. This study includeddata collection and patient follow up. Trophozoites of *D. fragilis* were detected in 60 patients by permanent staining using iron haematoxylin smears and this result was also confirmed by PCR (Stark et al., 2006b). Gastrointestinal symptoms, including abdominal pain and diarrhoea, were present in all patients and 32% of them had chronic symptoms (Stark et al., 2005b). Studies using fixation and permanent staining methods to detect *D. fragilis* have shown *D. fragilis* to be more prevalent than *Giardia lamblia* as a cause of gastrointestinal infection (Crotti et al., 2005; Girginkardesler et al., 2003).

Many studies have shown that treatment which eliminated *D. fragilis* results in clinical improvement (Windsor and Johnson, 1999). There is substantial evidence that suggests *D. fragilis* causes disease in humans with a range of symptoms including diarrhoea, abdominal pain, vomiting and chronic infection (Stark et al., 2006b). These studies suggest the pathogenic nature of the organism no longer can be neglected.

## 1.5 Dientamoebiasis

The infection caused by *Dientamoeba fragilis* can be acute or chronic and can be asymptomatic, which was reported in both adults and children (Fletcher et al., 2012; Stark et al., 2005a). The ability of this parasite to cause chronic symptoms ranging from weeks to months has been documented in the literature (Stark et al., 2014). The most prevalent clinical symptoms are persistant diarrhea, abdominal pain, weight loss, flatulence and loss of appetite (Fletcher et al., 2012; Stark et al., 2005a). These symptoms are similar to those in irritable bowel disease (IBS), therefore *D. fragilis* infection should be taken into account in the diagnosis of IBS (Stark et al., 2007).

## 1.6 Diagnostic methods

### 1.6.1 Fixing, staining and microscopy analysis

The traditional method of diagnosis is mainly through microscopic detection of trophozoites in fresh and fixed stool samples (Fletcher et al., 2012). For accurate diagnosis prompt fixation of the clinical specimen is essential as *D. fragilis* trophozoites degrade rapidly in the environment (Stark et al., 2010a). Different fixatives and stains have been used over the years to detect *D. fragilis* in clinical specimens. The stains that are currently used in the laboratories are polyvinyl alcohol and sodium acetate acetic acid formalin along with iron hematoxylin and trichrome stains (Johnson et al., 2004).

The gold standard diagnostic method was microscopy of fixed smears of infected stool samples with iron haematoxylin staining (Fletcher et al., 2012). This method was time consuming and relatively insensitive compared to molecular techniques.

### 1.6.2 Molecular diagnosis

There are only a limited number of molecular assays developed for the detection of *D. fragilis* compared to other protozoans. For example, there are a variety of molecular assays such as monoclonal antibodies and enzyme immune assays available for the detection of antigens of protozoa such as *Cryptosporidium parvum*, *Giardia lamblia* and *E. histolytica* in stools, unlike for *D. fragilis* (Stark et al., 2006b). Molecular techniques, such as conventional and real time PCR targeting the 18S rRNA of *D. fragilis,* were developed (Peek et al., 2004; Stark et al., 2006a; Verweij et al., 2007).

The current gold standard is to detect the small subunit rRNA gene of *D. fragilis* using fresh infected stool samples by conventional and real time PCR, which is more rapid, sensitive and specific than microscopy (Stark et al., 2011). It has been suggested that real time PCR is the most sensitive method of all to detect *D. fragilis* (Stark et al., 2010a). Indeed, a PCR assay was developed, which did not cross react with other protozoan parasites and showed a specificity of 100% and a sensitivity of 93.5% (Stark et al., 2005a). A 5' nuclease (Taq man) based real time PCR assay targeting the small subunit rRNA gene was also developed.(Stark et al., 2006a). Moreover, nested PCR and Multiplex Tandem Real Time PCR could also be considered as a diagnostic tool for the rapid detection of *D. fragilis* in stool samples (Stark et al., 2011).

## 1.7 *In vitro* culture of *D. fragilis*

Recent advances in the *in vitro* culture of *D. fragilis* have enabled researchers to unravel more information about the mode of transmission of this parasite. There are three different types of culture media developed in general for the cultivation of microorganisms, namely: xenic, mono xenic and axenic culture media. For *D. fragilis* there has been a wide range of xenic and poly xenic culture media developed, but no mono xenic or axenic culture media has been developed yet (Munasinghe et al., 2012).

The first xenic culture medium was a biphasic medium consisting of an inspissated horse serum slope overlaid with a liquid phase consisting of egg whites diluted in

Ringer's solution (Dobell, 1940b). Other xenic culture media that were developed over the years for the culture of *D. fragilis* are listed below.

### 1.7.1 Modified Boeck and Drbohlav's medium

This is a biphasic medium that consists of Locke's solution (made with sodium chloride; calcium chloride; potassium chloride; magnesium chloride; sodium phosphate, dibasic;sodium bicarbonate; and potassium phosphate) containing heat inactivated horse serum overlaid on an inspissated egg slope made with fresh hen's eggs (Clark and Diamond, 2002). This medium showed an average growth of $2.5 \times 10^5$/ml of *D. fragilis* trophozoites (Barratt et al., 2010).

### 1.7.2 Robinson's medium

This is a complex medium that has been widely used for the isolation of enteric amoebae. The liquid overlay consists of erythromycin, phthalate-bacto peptone, BRS medium and is overlaid on top of an agar slant (Clark and Diamond, 2002). The average growth of *Dientamoebafragilis* in Robinson's medium was higher than that of modified BD (Barratt et al., 2010).

### 1.7.3 TYSGM broth

This is a monophasic medium, which contains potassium phosphate, sodium chloride; casein digest peptone; yeast extract and gastric mucin (Clark and Diamond, 2002) and showed poor growth of *D. fragilis* (Barratt et al., 2010).

### 1.7.4 Loeffler's medium

This is a biphasic medium that is currently used for the *in vitro* cultivation of *D. fragilis* (Barratt et al., 2010; Munasinghe et al., 2012). This medium consists of an inspissated horse serum slope overlayed with 1X PBS along with rice starch. It has been proven to give higher trophozoite numbers (twice that obtained in other media) as shown previously (Barratt et al., 2010). A recent advancement was made to this biphasic medium by replacing the PBS with Earl's balanced salt solution, which consists of $NaHCO_3$, cholesterol and ferric ammonium citrate (Munasinghe et al., 2012) and there

is a significant increase in the trophozoite numbers compared to the overlay used previously which is phosphate buffered saline(Munasinghe et al., 2012).

### 1.8 Treatment

Despite the discovery of *D. fragilis* more than 100 years ago little research has been done to study the susceptibility of this parasite to different antimicrobial agents (Stark et al., 2014). There is no gold standard treatment regime for *D. fragilis* and the treatment options to date are based on limited studies carried out to date (Nagata et al., 2012). A recent study has shown the resistance of *D. fragilis* to benzimidazole derivatives, which are anti helminthic drugs (Nagata et al., 2012).

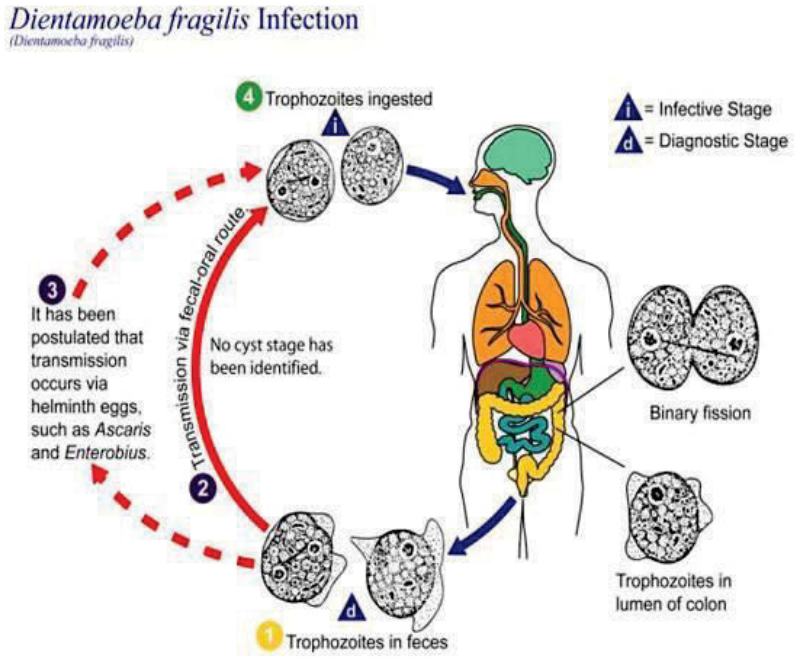
Antimicrobial agents such as metronidazole, tetracycline and iodoquinol are commonly used as the first line therapy for treating *D. fragilis* infections in symptomatic patients (Stark et al., 2005a). However, most of those studies were based on small size case reports (Nagata et al., 2012). A recent study has shown that metronidazole, ornidazole, tinidazole and ronidazole are the most active antimicrobials against *D. fragilis* (Nagata et al., 2012). Iodoquinol and metronidazole are commonly used to treat *D. fragilis* infections in children (Banik et al., 2011; Vandenberg et al., 2007).

Treatment of dientamoebiasis is recommended in symptomatic patients and asymptomatic family members to prevent reinfection (Fletcher et al., 2012). Various antimicrobial and antiprotozoal drugs have been effective in clearing the symptoms (Fletcher et al., 2012). Paramomycin, iodoquinol, secnidazole, tetracycline, ornidazole have been used successfully to treat *D. fragilis* infections (Fletcher et al., 2012). However, there is evidence of treatment failures of metronidazole among *D.fragilis* isolates, suggesting the importance of introducing combination therapy for *D. fragilis*. Combination therapy has been effective in resolution of the symptoms (Stark et al., 2009a).

### 1.9 Life cycle of *D. fragilis*

#### 1.9.1 Mode of transmission

*D. fragilis* life cycle (CDC, 2015)




Despite the discovery of *D. fragilis* almost a century ago, the life cycle of this parasite has not been completely defined yet. The only known stage previously identified was the trophozoite stage and no cyst stage had been identified (Barratt et al., 2011a). As the trophozoites of *D. fragilis* are fragile in nature it has led many researchers to suggest that this mode of transmission is unlikely (Yang and Scholten, 1977). Several theories have been proposed to explain how *Dientamoeba* trophozoites could survive outside their host for a sufficient period of time to allow transmission. One theory suggested that the parasite is transmitted via the ova of a helminth. Another theory suggested the existence of a cyst stage (Barratt et al., 2011a). Jepps and Dobell in 1918 commented on the fragile nature of the trophozoite of *D. fragilis* although they were unable to find a cyst stage in the infected stools of humans (Jepps and Dobell, 1918a). These authors suggested that *D. fragilis* may form cysts in an unidentified species of animal (Jepps and Dobell, 1918a). Several animals have been reported to carry *D. fragilis* parasites

even though no cyst stage had been reported (Crotti et al., 2007; Dobell, 1940a; Knowles and Gupta, 1936; Lankester et al., 2010; Myers and Kuntz, 1968; Stark et al., 2008).

Several studies have documented the role of animals in the transmission of *D. fragilis* (Barratt et al., 2011a). *D. fragilis* has been detected in several differentspecies including macaques, wild monkeys and baboons (Stark et al., 2008). Other studies reported the presence of *D. fragilis* in sheep and pigs (Stark et al., 2008) highlighting the involvement of animal hosts in the life cycle and the zoonotic potential of *D. fragilis*.

Several authors have described what appeared to be cysts, pseudocysts and cystlike stages in *Dientamoeba* infections (Knoll and Howell, 1945; Kofoid, 1923; Piekarski, 1948; Silard et al., 1979; Wenrich, 1936). It was suggested that these cystlike forms could be degenerating trophozoites and their true identity could not be determined (Johnson et al., 2004). Therefore, many years after the original identification of *D. fragilis*, two theories were proposed to explain the mode of transmission in *D. fragilis*: transmission via the ova of helminths and transmission via cysts, which is explained below.

**1.9.1a Transmission via helminths**

Dobell (1940) was the first to postulate that *D. fragilis* could be transmitted via the ova of a nematode such as *Trichuris trichura* or *Ascaris lumbricoides* (Dobell, 1940a). This was based on the fact that *D. fragilis* closely resembles *Histomonas meleagridis,* a protozoan parasite which infects poultry and is known to be transmitted via the ova of the caecal nematode *Heterakis gallinae* (Dobell, 1940a). Burrows and Swerdlow in 1956 carried out a study of twenty two appendices containing *D. fragilis* and found that twelve of these were co-infected with *Enterobius vermicularis*. *Dientamoeba fragilis* bodies were found in the *E. vermicularis* eggs from the co-infected appendices but not in the appendices infected only with *E. vermicularis,* suggesting the ova of *E. vermicularis* to be a possible vector of *D. fragilis* (Burrows and Swerdlow, 1956). Further research on this issue supported the hypothesis that *D. fragilis* was transmitted via the ova of *E. vermicularis* (Ockert, 1972; Ockert and Schmidt, 1976b).

One researcher claimed to infect himself and two others with *Dientamoeba* using pin worm eggs from a patient infected with both *Dientamoeba* and *E. vermicularis* (Ockert,

1972; Ockert, 1975). Another study showed that the nuclei and cytoplasmic amoeboid bodies, which were found in *Enterobius* ova and trophozoites from *D. fragilis* culture, had identical isoelectric points (Johnson et al., 2004; Ockert and Schmidt, 1976a). A later survey done with 43000 individuals further confirmed that there is a strong association between *D. fragilis* infections and *E. vermicularis* infections (Yang and Scholten, 1977). Furthermore, Girginkardesler et al. 2008 confirmed a clear relationship between *D. fragilis* and *E. vermicularis* infections, supporting the hypothesis that the pin worm acts as a vector in the transmission of *D. fragilis* (Girginkardesler et al., 2008). Sukanahaketu (1977) reported *Dientamoeba*like structures inside the ova of *Ascaris lumbricoides* of patients co infected with *D. fragilis,* while those structures were absent in the ova of patients that were infected only with *S. lumbricoides* (Sukanahaketu, 1977).

In contrast, some studies found no association between *Dientamoeba* and *E. vermicularis,* making the pin worm hypothesis controversial (Cuffari et al., 1998; Kean and Malloch, 1966; Oxner et al., 1987; Stark et al., 2010b; Stark et al., 2009; Stark et al., 2006b; Walker et al., 1985). A recent study demonstrated that *E. vermicularis* eggs carried *D. fragilis* DNA, as they were able to isolate and amplify *D. fragilis* DNA inside surface-sterilised *E. vermicularis* eggs (Röser et al., 2012). Despite this finding there are questions that still remained unanswered; Does *E. vermicularis* serve as a vector to transmit *D. fragilis*? Does it undergo development and multiplication inside the pinworm egg? Do other nematodes harbour *D. fragilis*? Further research is required in order to answer those questions.

### 1.9.1b Transmission via cysts

The other proposed mechanism of transmission of *D. fragilis* is via a resistant pseudocyst or a cyst stage (Barratt et al., 2011a). The first encysted stage of *D. fragilis* was described in 1923 based on observations through light microscopy (Kofoid, 1923). Three years later Kudo discovered forms of the parasite that are 4µm in diameter and he described them as "small spherical amoebas without food particles" and he thought these were precystic forms of the parasite (Kudo, 1926). Wenrich (1936) described a pseudocyst stage of *D. fragilis,* which is 5µm in size and finely granular with a distinct endosome (Wenrich, 1936). This description is not sufficient to prove that this stage exists since there are no distinct features described as seen in the pseudocysts of

*Tritrichomonas foetus,* which consist of multiple nuclei (Pereira-Neves et al., 2011). Pierkaski in 1948 reported the occurrence of precystic and cystic forms via iron haematoxylin smears (Piekarski, 1948), but later it was shown that these were more likely to be degenerating trophozoites (Johnson et al., 2004). It has been theorized that *Dientamoeba fragilis* may form cysts in an unidentified animal species (Jepps and Dobell, 1918b), suggesting the potential of a zoonotic transmission. Several animal species have been reported that carry *Dientamoeba* including non-human primates, sheep and pigs (Stark et al., 2008). A recent study confirmed that pigs are a natural host of *D. fragilis* harbouring the genotypes found in infected humans (Caccio et al., 2012).

## 1.10 Literature review on Pseudocysts, precysts and cysts in other protozoa

### 1.10.1 Pseudocysts in Trichomonads

*Trichomonas foetus* and *Trichomonas vaginalis* are flagellated parasitic protozoans that inhabit the urogenital tract of cattle and humans respectively and belong to the same family as *D. fragilis* (Pereira-Neves et al., 2003). The life cycle of these two species consist of a motile trophozoite stage and a pseudocyst stage (Brugerolle, 1973; Pereira-Neves et al., 2003). However, true cysts were found in other intestinal trichomonads such as *Trichomitus batachorum*, *Trichomitus sanguisugae* (Brugerolle, 1973). Monocercamonads, which are close relatives belonging to the phylum parabasalia, also display cyst formation (Brugerolle, 1973). The pseudocyst form is a morphological transformation of the trophozoite stage into a compact non-motile form that lacks a true cyst wall (Pereira-Neves et al., 2003). For years pseudocysts were thought to be irreversible degenerative forms of the parasite (Honigberg and Brugerolle, 1990; Mattern et al., 1973). However, recent studies have shown that the pseudocyst stage is reversible and its formation represents a defence mechanism against adverse environmental conditions (Mattern and Daniel, 1980; Pereira-Neves and Benchimol, 2009). The trophozoite forms, which are polar and flagellate, become rounded and internalise their flagella in pseudocyst formation as a response to environmental stress. It has been shown that *T. foetus* treated with Hydroxyurea (which inhibits DNA synthesis) or subjected to cold/warm cycles, exhibits pseudocyst formation *in vitro* (Pereira-Neves et al., 2003). Light and electron microscopy studies have shown that the

three anterior flagella and the recurrent flagellum of *T. foetus* can be internalised and the pseudocysts can revert back to trophozoite forms upon rewarming (Granger et al., 2000). Pseudocyst formation can be triggered by desiccation, increased oxygen tension, or lower temperature and might increase the survival time in the feces (Granger et al., 2000).

The trophozoite stage of *T. foetus* and *T. vaginalis* has a characteristic teardrop shape with four and five flagella respectively (Pereira-Neves et al., 2003). During pseudocyst formation the shape of the parasite becomes spherical and all the flagella are internalised. The axostyle and costa, which were axial in the trophozoite stage, becomecurved during the pseudocyst stage. The flagella were located in endocytic vacuoles or canals (Granger et al., 2000) without direct contact with the cytoplasm. The spindle is visible as a straight ribbon between the two cell poles. The pseudocysts are mostly 7-10µm in diameter, and contain one or two nuclei. Organisms with three to four nuclei are present but rare. Some *in vitro* studies have shown that pseudocysts generated from long term *T. foetus* cultures were capable of generating multinucleated cells that release single organisms through a budding process (Pereira-Neves and Benchimol, 2009). It has been shown in *in vitro* culture the bi nucleated pseudocysts forms and polymastigont pseudocyst forms are composed of a large cytosolic mass containing up to eight nuclei (Pereira-Neves and Benchimol, 2009). Polymastigont organisms have been reported in several trichomonads including *T. foetus* (Mariante et al., 2003) and *T. vaginalis* (Abonyi, 1995). There are controversial arguments regarding these polymastigont forms. Some authors suggest that these are degenerative forms (Honigberg and Brugerolle, 1990; Samuels, 1959). However, evidence suggests that these polymastigont forms represent a normal part of the trichomonad life cycle (Pereira-Neves and Benchimol, 2009). Several authors observed more details on the nuclear division by light and electron microscopy in which single organisms were budding out of the polymastigont forms until all of them were completely separated (Abonyi, 1995).

All dividing Trichomonad pseudocyst forms display pole to nucleus spindle structures on the nuclear envelope which are involved in mitosis (Pereira-Neves et al., 2003). The true cysts display a resistant cell wall surrounding the parasite as a means of enduring

harsh environmental conditions for prolonged periods of time, as observed in intestinal trichomonads (Brugerolle, 1973). However, the pseudocyst forms present in *T. foetus*, *T. vaginalis* and *Trichomitus batrachorum* do not possess a cyst wall (Pereira-Neves et al., 2003).

True cysts are formed as a mechanism of survival in harsh environments for a prolonged period of time after passing out from the host. Organisms that pass quickly and remain only a short time period outside the host do not need to mobilize the carbohydrate and proteins necessary for cyst formation that leads to a pseudocyst form (Boggild et al., 2002). Several studies have described the infective nature of these pseudocysts for rodents and birds (Friedhoff et al., 1991; Stachan et al., 1984). It has been suggested that the proteinases and neuraminidases secreted by the pseudocyst facilitate their pathogenicity, but no further studies have been done to prove this hypothesis (Hussein and Atwa, 2008). Some researchers argue that the capability of *T. foetus* to generate multinucleated organisms when environmental conditions become favourable allows them to transmit to a new host as a pseudocyst rather than as a piriform cell, which could then contribute to a more efficient infection in the host (Pereira-Neves et al., 2011).

### 1.10.2 Precysts and cysts in *Histomonas*

*Histomonas meleagridis* is a protozoan parasite that parasitizes the intestinal tracts of birds causing the disease Typhlohepatitis, also known as black head disease (Zaragatzki et al., 2010a). It is an important pathogen in poultry and causes high rates of morbidity and mortality especially in turkeys (McDougald, 2005). The main mode of transmission of *H. meleagridis* is believed to occur via the ova of *Heterakis gallinarum* (Lucius and Loos-Frank, 1997; Munsch et al., 2009). Several authors believe that transmission may occur via cysts as in other protozoans such as *Acanthamoeba*, which form cysts (Mehlhorn, 2008). Some authors have described stages which resembled cysts that could survive the passage of the stomach after oral intake (Hess et al., 2006; Mehlhorn, 2008; Mielewczik et al., 2008). Several *in vitro* studies have shown that *H. meleagridis* exists as two stages; an amoeboid stage without a flagellum and a stage with a flagellum (Hess et al., 2006; Munsch et al., 2009). Recent light and transmission electron micrographs have shown double membrane spherical or ovoid cells that are assumed to

be cystlike stages in *H. meleagridis* (Munsch et al., 2009). These cystlike stages are described as round, totally filled with glycogen granules, and being characterized by an amorphous layer beneath the cell membrane (Munsch et al., 2009). Two different cystlike stages have been described: A larger spherical stage, which represents the initial stage of cyst formation known as a precyst, and the smaller very condensed version of a true cyst (Munsch et al., 2009) as seen in sections through the caeca of experimentally infected turkeys (Mehlhorn et al., 2006). Some experiments have shown that under poor culture conditions some of the amoebic stages transform into these large spherical forms and, later on into small spherical forms (Zaragatzki et al., 2010b). All these stages were free of any organelles while the interior of the cysts were filled up with glycogen and ribosomes (Munsch et al., 2009). These cysts, which are rarely seen in culture, could be an ideal means for the oral transmission of *H. meleagridis* in nature. Moreover, some authors (Liebhart and Hess, 2009) have suggested that these precyst and cyst stages would be stable enough to pass through the stomach of the infected host and would explain the successful oral transmission experiments carried out by Liebhart and Hess (2009) (Liebhart and Hess, 2009) and Huber et al. (2007). Since no further animal studies have been carried out since 2007 to analyse the infectivity of these cyst like stages, nor has research been done on the potential of a cyst stage to occur in the natural environment, further work is required to elucidate this question.

### 1.10.3 True cysts in *Giardia* sp.

*Giardia lamblia* is a parasitic trichomonad which causes the gastrointestinal disease giardiasis in humans worldwide (Benchimol, 2002). This protozoan has a biphasic life cycle that alternates between the trophozoite stage in the vertebrate host and environmental-resistant true cysts with a cyst wall made with a thick extracellular matrix (Adam, 2001). The cyst wall structure is critically important for the survival of the parasite due to two reasons; firstly, with regard to infection it is the excystation process which liberates trophozoites from the cyst into the intestine where an infection is established. Secondly, it is the encystment process that protects the trophozoites from harsh environmental conditions including exposure to antibiotics such as metronidazole (Paget et al., 1989), thereby ensuring the viability of the encysted trophozoite to allow successful transmission to a new host (Erlandsen et al., 1996). The cyst wall consists

oftwo components: a double membrane bilayer composed of an outer and inner cyst wall membrane, and a filamentous layer of 0.3-0.5µm made up of a complex array of fibrillar material laid down on the outer surface (Arguello-Garcia et al., 2002). The external meshwork consists of interconnected filaments 7-20nm in diameter, which is composed of peptides and carbohydrate moieties (Manning et al., 1992). The inner cyst wall membrane is continuous with the plasma membrane of the encysted parasite (Coggins and Schaefer, 1986). The encysted parasite either adheres closely to the cyst wall or is separated from the outer cyst wall by an open space known as the peritrophic space (Coggins and Schaefer, 1986). Mature cysts consist of three separate limiting membranes: the outermost membrane is attached to the filamentous layer of the cyst wall, while the two inner membranes enclose the peritrophic space, the innermost being closely attached to the plasma membrane of the encysted parasite as mentioned earlier (Chavez Munguia et al., 2004). The main cyst wall component in *Giardia* cysts is (1, 3)-N-acetyl-D-galactosamine polymer (Chavez-Munguia et al., 2007; Gerwig et al., 2002; Jarroll et al., 2001). Protein components termed CWP1, CWP2 (Gillin et al., 1996; Luján et al., 1997) and CWP 3(Sun et al., 2003) are associated with this polymer. Studies done using low voltage scanning electron microscopy coupled with immunogold labelling has detected an early deposition of cyst wall materials as surface cap like protrusions that were later embedded in the cyst wall meshwork (Erlandsen et al., 1996). The mechanism/composition underlying the progressive organisation of the outer fibrillar layer of the *Giardia* cyst wall still remains unknown (Arguello-Garcia et al., 2002).

### 1.11 Animal Models

Animals play an important role in the transmission of many enteric protozoan parasites that infect humans, such as *Giardia* and *Cryptosporidium* (Schlundt et al., 2004; Smith et al., 2007). Animal reservoirs such as dogs, cats and pigs also serve as potential reservoirs in human parasitic infections (Inpankaew et al., 2007; Robertson, 2009; Yoshikawa et al., 2003). Therefore, it is possible that the *D. fragilis* life cycle may involve an animal reservoir in its transmission. Few studies have explored the zoonotic potential of *D. fragilis* and of those there has not been clear cut success (Barratt et al., 2011a). A study done in 1936 reported the detection of *D. fragilis* in the stools of

captive macaques using iron haematoxylin stains, but the infection rate was very low (1/30) (Knowles and Gupta, 1936). Another study reported the presence of *D. fragilis* in a small proportion of wild monkeys (2/44) from the Philippines (Hegner and Chu, 1930). *Dientamoeba* was also reported in < 1percent of captive baboons and <2percent of wild baboons (Myers and Kuntz, 1968). A study carried out in 2008 identified *Dientamoeba* in three western lowland gorillas using iron haematoxylin stains, which was confirmed by PCR (Stark et al., 2008). A recent study reported an irritable bowel like disease in a Western lowland gorilla that was later attributed to *Dientamoeba* (Lankester et al., 2010). *Dientamoeba* trophozoites were observed in haematoxylin and Giemsa stained smears made from stools of sheep, although the prevalence was not reported (Noble and Noble, 1952). In contrast, Stark et al. 2008 did not find *Dientamoeba* in iron haematoxylin smears prepared from stools of sheep. *Dientamoeba* trophozoites were detected in the stools of 53/121 pigs using the Giemsa staining technique (Crotti and D'Annibale, 2007a). In contrast, Noble and Noble (1952) examined 30 pigs and made no mention about *D. fragilis* in the stool samples. Similarly Stark et al (2008) examined 135 pigs and found no evidence for the presence of *D. fragilis* in the stools. One study identified rabbits as a risk factor for *D. fragilis* infection (Stensvold et al., 2009). In contrast, Stark et al. 2008 examined 20 rabbits and found no evidence for the presence of *D. fragilis*.

Many attempts were made to induce experimental infections in a range of animals but without success (Dobell, 1940a; Kean and Malloch, 1966; Knoll and Howell, 1945; Wenrich, 1944). Dobell (1940) tried to infect 6 chickens rectally using cultured *D. fragilis* trophozoites. A transient infection was achieved in one chick but was cleared within one week. The histological findings revealed no pathological changes in the caeca and liver of the infected chick (Dobell, 1940a). Dobell (1940) also tried to infect himself and two macaques orally with a trophozoite culture of *D. fragilis* but failed. The rectal administration of *D. fragilis* trophozoites to one of these macaques also failed (Dobell, 1940a).

Wenrich (1944) tried to infect laboratory rats orally and rectally with *D. fragilis* trophozoites but failed. Knoll and Howell (1945) attempted to infect kittens with cultured *D. fragilis* trophozoites via oral and rectal injection, but with no success. They were able to recover *D. fragilis* trophozoites at autopsy but no pathological changes

were noted in the gastrointestinal tract (Knoll and Howell, 1945). Moreover, these researchers examined the entrails of 12 laboratory rats and found no trace of *D. fragilis* infection (Knoll and Howell, 1945). Kean and Malloch (1966) attempted to infect laboratory rats. Their preliminary observations showed that *Dientamoeba* attaches to the caecal mucosa and causes damage to the underlying cells as well as oedema in the mucosa but without producing any ulceration (Kean and Malloch, 1966).

### 1.12 Concluding remarks

*Dientamoeba fragilis* has remained a neglected parasite for years although it was discovered more than a century ago by Jepps and Dobell (1918). Despite the discovery of *D. fragilis* almost a century ago, the life cycle of this parasite has not been completely defined yet. The only known stage identified until recently was the trophozoite stage (Barratt et al., 2011a). Even though many studies were done in order to find a possible cyst stage, all those studies failed due to the inability to establish a suitable animal model to study the infection caused by *D. fragilis*. In contrast, extensive studies have been done in other parasitic protozoa closely related to *D. fragilis* that form cysts such as *Giardia*, *Histomonas*, *Trichomonas* and *Tritrichomonas*. This review provided an overview of the life cycle andproposed mode of transmission of *D. fragilis* as well as information on other cyst forming protozoa related to *D. fragilis*.

## 1.12 Aims and hypotheses

The aims of this thesis are:

- To improve the existing *in vitro* culture technique for *Dientamoeba fragilis* in order to yield high trophozoite numbers needed for further molecular studies (chapter 2).
- To establish an animal model in order to study the mode of transmission of *D. fragilis* (chapter 3)
- To investigate the morphology and ultrastructure of the *D. fragilis* cyst by transmission electron microscopy (chapter 4)

Hypothesis

- The main hypothesis in this study is that the mode of transmission of *D. fragilis* occurs via cysts.

Although *D. fragilis* was discovered a century ago (Jepps and Dobell, 1918a) its mode of transmission has remained a mystery to the scientific community throughout the past decade. This study was able to discover a new life cycle stage of the parasite and provided a detailed description of the new stage which would be a valuable resource to the research community. Moreover the identification of a cyst stage in this study will enable the researchers to identify new treatment options and strategies to overcome failures in the treatment of dientamoebiasis.

# Chapter 2:

## Improvements in the *in vitro* culture of *Dientamoeba fragilis*

## Published as

**Munasinghe, V.S.,** Stark, D., Ellis, J.T., 2012. New advances in the *in-vitro* culture of *Dientamoeba fragilis*. Parasitology 139, 864-869.

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Date: April 2016

# New advances in the *in-vitro* culture of *Dientamoeba fragilis*

VARUNI S. MUNASINGHE[1], D. STARK[1,2] *and* J. T. ELLIS[1]*

[1] *University of Technology Sydney, School of Medical and Molecular Biosciences and the Ithree Institute, Broadway, NSW, Australia*
[2] *Division of Microbiology, SydPath, St. Vincent's Hospital, Darlinghurst, NSW, Australia*

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SUMMARY

*Dientamoeba fragilis* is an intestinal protozoan in humans that is commonly associated with diarrhoea and other gastrointestinal complaints. Studies conducted to investigate the biology of this parasite are limited by methods for *in vitro* cultivation. The objective of this study was to improve a biphasic culture medium, based on the Loeffler's slope, by further supplementation in order to increase the yield of trophozoites in culture. The current *in vitro* culture of *D. fragilis* is a xenic culture with a mix of bacteria. Three different liquid overlays were evaluated including Earle's balanced salt solution (EBSS), PBS and Dulbecco's modified PBS (DPBS), for their ability to support the *in vitro* growth of *D. fragilis* trophozoites. Out of these 3 overlays EBSS gave the highest increase in the trophozoite numbers. The effect of supplementation was analysed by supplementing EBSS with ascorbic acid, ferric ammonium citrate, L-cysteine, cholesterol and alpha-lipoic acid and quantification of *in vitro* growth by cell counts. A new liquid overlay is here described based upon EBSS supplemented with cholesterol and ferric ammonium citrate that, in conjunction with the Loeffler's slope, supports the growth of *D. fragilis* trophozoites *in vitro*. This modified overlay supported a 2-fold increase in the numbers of trophozoite in culture from all 4 *D. fragilis* isolates tested, when compared to a PBS overlay. These advances enable the harvest of a larger number of trophozoites needed for further studies on this parasite.

Key words: *Dientamoeba fragilis*, *in vitro* culture, Earle's balanced salt solution, ferric ammonium citrate, cholesterol.

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# Chapter 3:

## Development of an animal model and the discovery of the cyst stage of *Dientameoba fragilis*

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Succinctus

# Cyst formation and faecal–oral transmission of *Dientamoeba fragilis* – the missing link in the life cycle of an emerging pathogen

Varuni S. Munasinghe [a], Nicole G.F. Vella [b], John T. Ellis [a,*], Peter A. Windsor [c], Damien Stark [a,d]

[a] School of Medical and Molecular Biosciences and the i3 Institute, University of Technology Sydney, Broadway, New South Wales 2007, Australia
[b] Microscopy Unit, Faculty of Science, Macquarie University, North Ryde, New South Wales 2109, Australia
[c] Faculty of Veterinary Science, University of Sydney, Camden, New South Wales 2560, Australia
[d] Division of Microbiology, Sydpath, St. Vincent's Hospital, Darlinghurst, New South Wales 2010, Australia

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ABSTRACT

*Dientamoeba fragilis* is a protozoan parasite emerging as a cause of diarrhoea and "irritable-bowel-like" gastrointestinal disease in humans with a propensity for establishing long-term, chronic infections in humans. Although *Dientamoeba* was discovered over a century ago its life cycle and mode of transmission is not known. No cyst stage has been described and no animal models are presently available for the study of this parasite. Here we describe the establishment of an animal model using laboratory rodents, the fulfilling of Koch's postulates, and the discovery of a new cyst stage in the life cycle of *D. fragilis*. Our demonstration of long-term parasite carriage by rodents and prolonged shedding of cysts, together with elevated levels of calprotectin in the stool, confirms the capacity of this organism to cause disease and indicates dientamoebiasis should be considered in the differential diagnosis of gastrointestinal diseases such as Inflammatory Bowel Syndrome (IBS). Finally, we suggest that the cyst stage described here is the vehicle that mediates faecal–oral transmission of *D. fragilis* between hosts.

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## Chapter 4

# New observations and review on the transmission of *Dientamoeba fragilis* and the cyst life cycle stage

**VARUNI S. MUNASINGHE[1], NICOLE G. F. VELLA[2], DAMIEN STARK[3] and JOHN T. ELLIS[1]**

[1] School of Medical and Molecular Biosciences, University of Technology Sydney, Broadway, NSW, Australia

[2] Macquarie University Microscopy Unit, Faculty of Science, Macquarie University, North Ryde, NSW, Australia

[3] Division of Microbiology, Sydpath, St Vincent's Hospital, Darlinghurst, NSW, Australia

*corresponding author

Email:Damian.Stark@svha.org.au (DS)

**Abstract**

Recently it was found that fecal–oral transmission of cysts may play a role in transmission of *D. fragilis* yet many of the specific details remain unknown. In this study we investigated the ability of cultured trophozoites to withstand very low pH and we provide evidence that trophozoites of *D. fragilis* are resistant for 4 hours of time to highly acidic conditions. This is an important finding as the pH in the human stomach is an important factor considering the survival of *D. fragilis* in the gut after passing through the stomach. The ultrastructure of *D. fragilis* cysts obtained from mice and rats was studied by transmission electron microscopy. Cysts possess a clear cyst wall surrounding an encysted parasite. The cyst wall was double layered with an outer fibrillar layer and an inner layer enclosing the parasite. Hydrogenosomes, endoplasmic reticulum and nuclei were present in the cysts. Pelta-axostyle structures, costa and axonemes were identifiable and internal flagellar axonemes were present. This study therefore provides additional novel details and summarises current knowledge of the ultrastructure of the cyst stage of *D. fragilis*.

Key words: *Dientamoeba fragilis*, transmission, cyst, electron microscopy

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# Chapter 5:

# General Discussion and future directions

## General Discussion and future directions

*Dientamoeba fragilis* is an intestinal trichomonad parasite that causes gastrointestinal disease in humans (Stark et al., 2010). It was first described by Jepps and Dobell in 1918 as a non pathogenic commensal (Jepps and Dobell, 1918a). Recent studies have shown its pathogenic potential thereby classifying it as a pathogenic protozoan that inhabits the gastrointestinal tract of humans (Stark et al., 2010). Despite its discovery a century ago, its life cycle hasn't been completely elucidated (Barratt et al., 2011a). Dobell in 1940 was the first to describe the trophozoite stage of *D. fragilis* (Dobell, 1940b). Although no cyst stage could be identified he postulated that *Dientamoeba* may produce cysts in an unidentified animal species (Jepps and Dobell, 1918b). Several studies has reported animal species that carry *Dientamoeba* (Crotti et al., 2007; Dobell, 1940a; Knowles and Gupta, 1936; Lankester et al., 2010; Stark et al., 2008) which further suggests the possibility of zoonotic transmission. Therefore, these studies provide evidence for the presence of a possible cyst stage that may occur in these animal species although no detailed description was given.

The main goal of this PhD study was to identify and establish a suitable animal model to study the life cycle of *D. fragilis,* especially the mode of transmission. There have been two proposed theories on transmission of *Dientamoeba*: transmission via pin worm eggs and transmission via a cyst stage. Dobell in 1940 first postulated that *D. fragilis* could be transmitted via ova of helminths such as *Trichuris trichura* and *Ascaris lumbricoides* (Barratt et al., 2011a). Burrows and Swerdlow in 1956 were the first to postulate that *Enterobius vermicularis* is a probable vector for *D. fragilis* and described tropohozoites that appeared to be *D. fragilis,* which were found in the ova of *E. vermicularis* (Barratt et al., 2011a). Moreover, they found a higher incidence of co infection with *D. fragilis* and *E. vermicularis* than *D. fragilis* infections alone further strengthening their hypothesis of *D. fragilis* transmission via *E. vermicularis* ova (Clark et al., 2014). Ockert in the 1970s further published a series of reports that supported the pinworm hypothesis suggesting that *E. vermicularis* is a possible vector of *D. fragilis* (Ockert, 1972; Ockert, 1975; Ockert and Schmidt, 1976). Ockert self-infected himself and two other subjects, using pinworm eggs derived from a patient who was infected with both pinworm and *Dientamoeba* (Ockert, 1972; Ockert, 1975). Isoelectric studies on proteins/enzymes extracted from pinworms further showed that the nuclei and

cytoplasm of amoeboid bodies occurring in *Enterobius* ova had identical isoelectric points as to those of trophozoites from Dientamoeba culture (Johnson et al., 2004; Ockert and Schmidt, 1976).

A strong association between *D. fragilis* infections and *E. vermicularis* infections was found in a survey that examined 43000 individuals further adding to the evidence for the pinworm hypothesis (Yang and Scholten, 1977). A recent study reported a relationship between the incidence of *Dientamoeba* and *Enterobius* infections (Girginkardesler et al., 2008). Another study identified *Dientamoeba*like structures within the ova of *A. lumbricoides* from stool samples obtained from patients who had mixed infections of *Ascaris* and *Dientamoeba* (Sukanahaketu, 1977). In contrast to those reports, there were several studies that did not support the pinworm hypothesis.

Kean and Malloch 1966 conducted a study using 100 patient stool samples infected with *D. fragilis* and found that these samples were free of other parasites, thus refuting the original idea of co-infection with both *D. fragilis* and *E. vermicularis* put forward by Burrows and Swerdlow in 1956 (Kean and Malloch, 1966). A study conducted using 125 subjects in Sydney suburbs found that only 2 were infected with *E. vermicularis* while 21 subjects were infected with *Dientamoeba* further showing that there is no association between *D. fragilis* and pinworms. However, the authors of that study noted that the prevalence of *E. vermicularis* could have been under-represented as only stools were examined and *E. vermicularis* ova are harder to detect in stools (Walker et al., 1985). In a study carried out in New Zealand the *D. fragilis* incidence was only 3% (Oxner et al., 1987). The helminth incidence in that study was only 1/1350 (Oxner et al., 1987), which further highlights that pinworm eggs cannot be a vector for the transmission of *D. fragilis*. Finally, a study of 25 paediatric cases of *D. fragilis* along with one carried out using multiple sticky tape tests for the detection of *E. vermicularis* ova in patients diagnosed with *D. fragilis*, found no association between *E. vermicularis* and *D. fragilis* (Cuffari et al., 1998; Stark et al., 2005a). In another study, the DNA extracted from the ova of *E. vermicularis* obtained from patients infected with *D. fragilis* failed to produce a PCR product using *Dientamoeba*-specific primers (Menghi et al., 2007). A study carried out in 2009 found no pin worm infections in two *D. fragilis* infected patients in two unrelated families from Sydney, Australia (Stark et al., 2009). Furthermore, Stark et al. 2010 were unable to detect co-infections of

*Dientamoeba* with any other helminths in a group of 19 people infected with *Dientamoeba* (Stark et al., 2010).

In contradiction to the above findings, a recent study showed the presence of *D. fragilis* DNA inside *Enterobius* eggs (Roser et al., 2013). The study used surface-sterilised *E. vermicularis* eggs from patients who were also infected with *D. fragilis* as confirmed by PCR, and found to contain *D. fragilis* DNA inside the eggs (Roser et al., 2013). The presence of *D. fragilis* DNA inside *E. vermicularis* is not sufficient to confirm that *E. vermicularis* is a possible vector of *D. fragilis* transmission. This is because the study hasn't yet found the life cycle stage present inside the nematode eggs that is responsible for the *D. fragilis* DNA found in this study. In addition, the surface sterilisation method, which used a graded concentration series of hypochlorite (HClO) solutions to wash the eggs and PCR to confirm the total removal of DNA from the surface of *Enterobius* eggs, may be insufficient to remove external DNA from the *D. fragilis* egg. A similar study from South America that used DNAse treatment of pinworm eggs failed to find *D. fragilis* DNA inside eggs. Hence, further studies are needed to resolve this question.

Therefore the main goal of this study was to find the mode of transmission of *D. fragilis* and test the hypothesis that *D. fragilis* transmission occurs via a cyst stage.

The aims that were achieved through this PhD project are discussed as follows:

**The first aim of this study was to develop a reliable method to increase the yield of trophozoites in the *in vitro* culture of *D. fragilis*.**

Previous work on *in vitro* culture for the xenic cultivation of *D. fragilis* trophozoites include liver infusion medium, inspissated horse serum or egg slopes with an overlay of egg white or serum Ringer's solution supplemented with rice starch, TYSGM-9 broth, and Robinson's modified BD medium (Barratt et al., 2010). The current study was carried out in order to improve this biphasic medium by supplementing it with essential growth nutrients. In this study, growth media supplements were tested for their ability to enhance the growth of *D. fragilis in vitro*, so that a threefold increase of trophozoites could be obtained for future studies. Three different liquid overlays were evaluated, including Earle's balanced salt solution (EBSS), PBS and Dulbecco's modified PBS (DPBS), for their ability to support the *in vitro* growth of *D. fragilis* trophozoites. All 4

overlays, PBS, EBSS, DPBS and EBSS supplemented with 20mM HEPES, were able to support the growth of *D. fragilis*. Earle's Balanced Salt Solution gave the highest ($1.2 \times 10^6$) trophozoite numbers (which was a threefold increase compared to PBS) and so was chosen for use in further studies that include supplementation with cholesterol and ferric ammonium citrate.

In the experiments reported in this study, supplementation of culture medium with cholesterol and lipids supported the growth of *D. fragilis* significantly ($1.4\times10^6$, p=0.04) and cholesterol supplementation alone resulted in the highest growth ($5.5 \times10^6$) compared to the control. Consequently, cholesterol was used in the culture medium to enhance the growth of *D. fragilis*. The modified EBSS supplemented with ferric ammonium citrate and cholesterol was able to support the growth of all 4 isolates of *D. fragilis* tested as described in Munasinghe et al. 2012. The modified EBSS increased the trophozoite numbers in all 4 isolates more than 2-fold. Therefore, the modified EBSS supplemented with ferric ammonium citrate and cholesterol was regarded as a superior overlay to PBS, which had been used before for the *in vitro* culture of *D. fragilis*, since it significantly increased ($1.4\times10^6$) the trophozoite numbers of *D. fragilis in vitro*. This is an important improvement in the *in vitro* culture of *D. fragilis* since a higher yield of trophozoites was needed for molecular and *in vivo* studies using animal models.

**The development of an animal model using mice and rats.**

The second aim of this study was to develop an animal model to study the mode of transmission of *D. fragilis*. Prior to this study the mode of transmission of *D. fragilis* was unknown and it was widely believed that a cyst stage was absent (Barratt et al., 2011a; Johnson et al., 2004). There has been many attempts to infect animals with *D. fragilis* but without any success (Barratt et al., 2011a; Dobell, 1940a; Kean and Malloch, 1966; Wenrich, 1944). The first attempt to infect rats with *D. fragilis* trophozoites given orally and rectally was done in 1944 by Wenrich which was unsuccessful (Wenrich, 1944). In another study the entrails of 12 laboratory rats were examined and failed to demonstrate any *D. fragilis* infection (Knoll and Howell, 1945). Kean and Malloch 1966 conducted a study to infect laboratory rats and the preliminary observations showed that *Dientamoeba* attaches to the caecal mucosa and causes

damage to the underlying mucosa. No successful animal model was developed prior to that study.

The current study was conducted using Sprague Dawley rats and Balb/C mice which had been confirmed as specific pathogen free. Briefly, the mice were given an oral suspension of *D. fragilis* trophozoites cultured in horse serum slopes overlaid with EBSS. Since *D. fragilis* culture was a xenic culture, a bacterial suspension from the *D. fragilis* cultures was used as a control. Post infection all mice were weighed individually daily. After day 1, all mice that were given the oral suspension were shedding binucleated cysts of *D. fragilis,* which measured 5µm in diameter as shown in iron hematoxylin stained smears. Moreover, these smears contained white blood cells, which were indicative of gut inflammation suggestive of active infection. In addition, infected mice showed a weight loss of 12% compared to the control group. Calprotectin levels, which was a marker of inflammation in the lower gastrointestinal tract (Ton et al., 2000), were measured in the stool of the infected mice by ELISA over the period of 28 days post infection. The average calprotectin levels in the infected mice were twice that of the control mice, which indicated the presence of inflammation in the gut. Subsequently, two healthy mice were infected orally with a suspension of mouse faeces containing cysts. These mice started shedding cysts from day 1 post infection as confirmed by iron haematoxylin smears. A rat model was also tested by giving an oral suspension of *D. fragilis* trophozoites obtained from the stool of infected mice. The rats became infected and started shedding cysts intermittently from day five through to day 26 post infection. These cysts were similar to those that were found in the stool of Balb/C mice with a distinct cyst wall and a diameter of 5-6 µm. This study demonstrated three criteria of Koch's postulate successfully, which was done for the first time for *D. fragilis*: Firstly, all mice that were inoculated orally became infected with *D. fragilis* compared to the controls. Secondly, mice infected with *D. fragilis* had a mild inflammatory reaction in the gut, which was demonstrated by the presence of white blood cells in the stools and a significant transient weight loss. Thirdly, a new *D. fragilis* infection could be established in healthy mice and rats when given orally. The successful establishment of three criteria of Koch's postulates, out of four, further elucidated the infectious nature of *D. fragilis,* which had not been shown previously.

**The cysts produced by mice and rats were studied by transmission electron microscopy.**

The stool samples obtained from infected mice and rats were screened for cysts using transmission electron microscopy, which was the first detailed transmission electron microscopic description of the *D. fragilis* cyst. Feces were fixed in 3% glutaraldehyde in PBS, embedded in 2% low melt agarose fixed, stained and infiltrated in osmium tetroxide, uranyl acetate, and LR white resin by standard procedures. Ultra thin sections were cut using an ultramicrotome, stained and examined using a transmission electron microscope. Cysts had a thick filamentous cyst wall similar to *Giardia intestinalis* (Chavez Munguia et al., 2004) and contained one or two nuclei. The cyst wall consists of 2 layers: an outer dense fibrillar layer and a double membrane bilayer enclosing the trophozoite as described in *Giardia* cysts (Arguello-Garcia et al., 2002). (Arguello-Garcia et al., 2002). But it was different to the precyst stage described in *Histomonas meleagridis,* which is a close relative of *D. fragilis (*Barratt et al., 2011b*),* where a fibrillar dense cyst wall was absent; instead, it contained only an outer amorphous zone (Zaragatzki et al., 2010). The outer cyst wall consisted of a meshwork of fibrillar elements. The inner cyst wall appeared more membranous and was observed directly adjacent to the fibrillar region of the wall. There was a peritrophic space between the cyst wall and the trophozoite as observed in *Giardia* cysts (Coggins and Schaefer, 1986). Immediately beneath the cyst wall there were numerous double membrane vesicles. Some of these vesicles were embedded in the cyst wall. There were numerous vesicles scattered underneath the cyst wall as well as inside the peritrophic space which are similar to the Encystation Specific vesicles (ESV) described in *Giardia* cysts (Chavez-Munguia et al., 2007). It has been suggested that in *Giardia* cysts these ESVs contain material that forms the fibrillar component of the cyst wall (Reiner et al., 1990). Therefore these vesicles observed in the *D. fragilis* cyst may closely relate to the ESVs mentioned in *Giardia* cysts. The trophozoite enclosed within the cyst wall has an amoebic appearance. In addition to glycogen granules there were small spherical particles that appeared across the cytoplasm, which were deemed to be free ribosomes. These were apparent in *Histomonas* cysts (Mielewczik et al., 2008). Therefore, it is not surprising to see them in *D. fragilis* as it is a close relative of *Histomonas* (Barratt et al., 2011b). The endoplasmic reticulum could be seen scattered throughout the cytoplasm and around the nuclear region. There were electron dense granules scattered in the

cytoplasm, which seem to be glycogen granules as described in *Histomonas meleagridis* (Munsch et al., 2009), which belongs to the same family as *D. fragilis*.

The nucleus was bound by a nuclear membrane and consist of a centrally located nucleus. The cytoplasm consists of electron dense hydrogenosomes which consist of a double membrane with a diameter of 0.15-0.35µm. The basal body structure consists of the axostyle, flagellar axonemes, pelta and costa. Flagella axonemes consist of 9+2 microtubule arrangement, which are present in the periphery of the cytoplasm. Previous phylogenetic studies showed that both the mastigont and the pelta axostyler complex were completely lost in *D. fragilis* and its inclusion in the Parabasalia was purely based on the presence of hydrogenosomes (Cepicka et al., 2010; Silberman et al., 1996). This was the first observation of internal flagella and the presence of pelta in the encysted parasite, although no external flagella were found. Although the costa and pelta structure are visible in *D. fragilis* there weren't any external flagella or axonemes seen in the peritrophic space. Therefore these structures could be remnants and they provide further ultrastructural evidence for *D. fragilis* to be a trichomonad. In the *Histomonas meleagridis* life cycle an amoeboid stage, cyst like stage and a uni flagellated stage has been identified (Munsch et al., 2009). As *D. fragilis* is a close relative of *Histomonas* sp. the presence of an internal flagella in the cyst stage suggests that it could be either a remnant of a flagellum or a new life cycle stage, which needs to be investigated further.

The concept that the *D. fragilis* cyst stage (Munasinghe et al., 2013) is the mode of transmission for this parasite contradicts the current dogma that the trophozoite stage found inside pin worm eggs is the mode of transmission. In a recent review (Clark et al., 2014) it was stated that the cyst study published by Munasinghe et al. (2013) has some flaws in it. The first flaw pointed out was that the rodents used were not confirmed to be specific pathogen free. In response, faeces from the rodents used in the study were examined by microscopy and PCR and were found to be free of *Giardia muris*, *Chilomastix mesnilli*, *Tritrichomonas muris*, *Spironucleus muris (*which are specific for rodents) as well as *Giardia intestinalis* and *Dientamoeba fragilis*. Therefore, the claim by Clark et al. 2014 saying that the cysts we identified belong to another unrelated organism is incorrect.

Clark et al. (2014) also questioned why there is no evidence of similar cysts being present in infected humans. In a subsequent study (Stark et al., 2014b), *D. fragilis* cysts were found in patient samples that were morphologically similar to the *D. fragilis* cysts found in the mice seen by Munasinghe et al. 2014. These cysts were 5µm in diameter, had a distinct cell wall and a peri-trophic space (Stark et al., 2014b) very similar to the cysts described in Munasinghe et al. 2013.Therefore the study by Stark et al. 2014 supports the fact that *D. fragilis* cysts can be found in patients infected with *D. fragilis* answering the question raised by Clark et al. 2014.

Clark et al. (2014) also argued that there were no data provided on the pattern of intermittent shedding of cysts and therefore concluded that perhaps shedding did not occur during the pre-screening process of the mice. In the study by Munasinghe et al. (2013) fifty percent of the mice started shedding cysts on day 1 post infection whereas the stool samples prior to infection were always negative during the pre-screening week. Furthermore, the pattern of intermittent shedding showed that the maximum number of cystfree stools in the mice were 4 days in a row. Therefore, it is highly unlikely that the absence of *D. fragilis* cysts or DNA for 7 days in the stool during the prescreening period is due to the intermittent nature of the shedding pattern; rather it is because they are free of *D. fragilis* infection, as stated by Clark et al. 2014.

Another point that was highlighted by Clark et al. 2014 is that the cysts were not purified. Several different purification procedures were tried, such as percoll and sucrose gradient techniques, but without success (not shown). In the first experiment, the faecal pellets that were confirmed positive for *D. fragilis* cysts via iron haematoxylin stains were suspended in 1X PBS in a fifty ml tube. The suspension was filtered through a tea strainer to remove particulate matter such as faecal debri. The filtrate was spun at 1000g for ten minutes and the pellet was resuspended in five ml of 1X PBS. The suspension was underlaid with thirty ml of Sucrose solution that has a specific gravity of 1.2 and then spun at 1050g for ten minutes. The topmost layer (one ml) was aspirated and transferred to a 5ml tube and washed three times with 1X PBS. This extract was observed via a light microscope for the presence of any *D. fragilis* cysts. There were no cysts present in the aspirate. This is because the number of cysts found in the stool samples was very low and this low number can be lost easily during the purification process.

Clark et al. 2014 posed another question as to whether experimental *D. fragilis* infections can be produced from surface-sterilised eggs or cysts? The study by Munasinghe et al. 2013reported the transmission of cysts from infected mice to healthy rats and mice, which produced infection in them by shedding cysts (Munasinghe et al., 2013). This was further confirmed by microscopy and PCR. Cases of pinworm are rare in Sydney (Stark et al., 2005b) and it was not possible to source eggs during the course of this study. However, it would be interesting to surface-sterilise pinworm eggs with DNAse and use the eggs to infect naïve mice and determine whether cysts of *D. fragilis* are detectable in mouse faeces.

**Future directions**

This PhD study led to the development of an improved xenic culture media, which increased the yield of *D. fragilis* trophozoites in culture. Future improvements in the development of an axenic culture medium would be helpful to overcome the limitations of using xenic culture media. For example, the main limitation encountered in the xenic culture media is the growth of bacteria, which compete for the nutrients in the culture environment with *D. fragilis*. This is one reason why *D. fragilis* growth decreases after 72 hours in culture as bacteria doubling time is faster than the multiplication of *D. fragilis*. An axenic culture will overcome this problem since it is sterile and the only living organism that would be grown will be *D. fragilis*. The EBSS, which was a major improvement in the *in vitro* culture of *D. fragilis,* can be further improved by the incorporation of antibiotics in order to achieve a monoxenic or axenic culture.

This study led to the development of an animal model using rodents which can maintain *D. fragilis* infection. This is an important advance in the research on *D. fragilis* enabling us to study the precise mode of transmission, which had remained a mystery for so many years in research on *D. fragilis*. It is worthwhile to determine the role of other animal species in the transmission of *D. fragilis,* such as pigs, as it can provide important clues on the potential of a zoonotic transmission.

The treatment options for *D. fragilis* are somewhat limited with high levels of resistance to antimicrobial agents such as metronidazole and benzoimidazoles (Stark et al., 2014a). Therefore, further studies on antibiotic susceptibility in *D. fragilis* will add value to the current treatment regime for dientamoebiasis. The most important aspect in terms of treatment is to find new treatment options for *D. fragilis* cysts. To date, most studies are directed towards testing treatment options of *D. fragilis* trophozoites rather than cysts. This could be another reason for the development of resistance by *D. fragilis* to most antimicrobials. Therefore, this study opens the door to future studies on susceptibility testing for *D. fragilis* cysts using in vitro culture.

The most important discovery of the study was the cyst stage of *D. fragilis,* which many scientists had attempted to identify but failed. This provides a clue to explain the recurrence of *D. fragilis* infections in humans and has implications on the best strategy for the treatment of Dientamoebiasis. In the future it would be worthwhile to explore the effect of the current treatment regime for *D. fragilis* on the cyst stage both *invit*ro and *in vi*vo using a range of antiprotozoal agents. Using the rodent model developed in this study, different antibiotic regimes can be tested *in vivo* using *D. fragilis* trophozoites and cysts as the cyst is likely to be much more resistant to treatment. This will open the possibility to find a suitable antibiotic or antiprotozoal agent to stop the recurrence of *D. fragilis* infections in the future.

This study was able to elucidate the mode of transmission of *D. fragilis* by developing an animal infection model. By using transmission electron microscopy anddetailed molecular studies, it will be possible to sequence the genome of *D. fragilis* cyst in order to confirm the phylogentic relationship of *D. fragilis* to other trichomonads. Molecular biology techniques such as Fluorescent In Situ Hybridization (FISH) can further add value to the study of the *D. fragilis* mode of transmission via confirming the cyst stage of *D. fragilis*.

This study found the presence of internalised flagella in the *D. fragilis* cyst, which suggests that it shares a close relationship with *Histomonas* as *Histomonas* has a flagellated stage in its life cycle. Therefore, further studies utilizing molecular biological techniques such as FISH and immunogold labeling would be useful to provide more information about the cyst stage and to compare it with the cyst stages of *Histomonas* and *Giardia,* which belong to the same family as *Dientamoeba*.

Overall, this thesis focused on enhancing the *in vitro* culturing and establishing an animal model to study the mode of transmission of *D. fragilis,* which is a clinically significant parasite yet has been a neglected in the world of parasitology. This extensive study made the first discovery of the cyst of *D. fragilis,* which unraveled the mystery of the mode of transmission of this parasite that has been a burning question in the world of parasitology for many years. Since this was a novel discovery further studies are warranted to examine the cyst stage and its phylogenetic relationships with other parasites. This novel discovery of the cyst stage and the improved *in vitro* culture of *D. fragilis* will enable future researchers to study its genome, trial antiprotozoal drugs and possible zoonotic implications of this parasite.

Proposed life cycle of *D. fragilis*

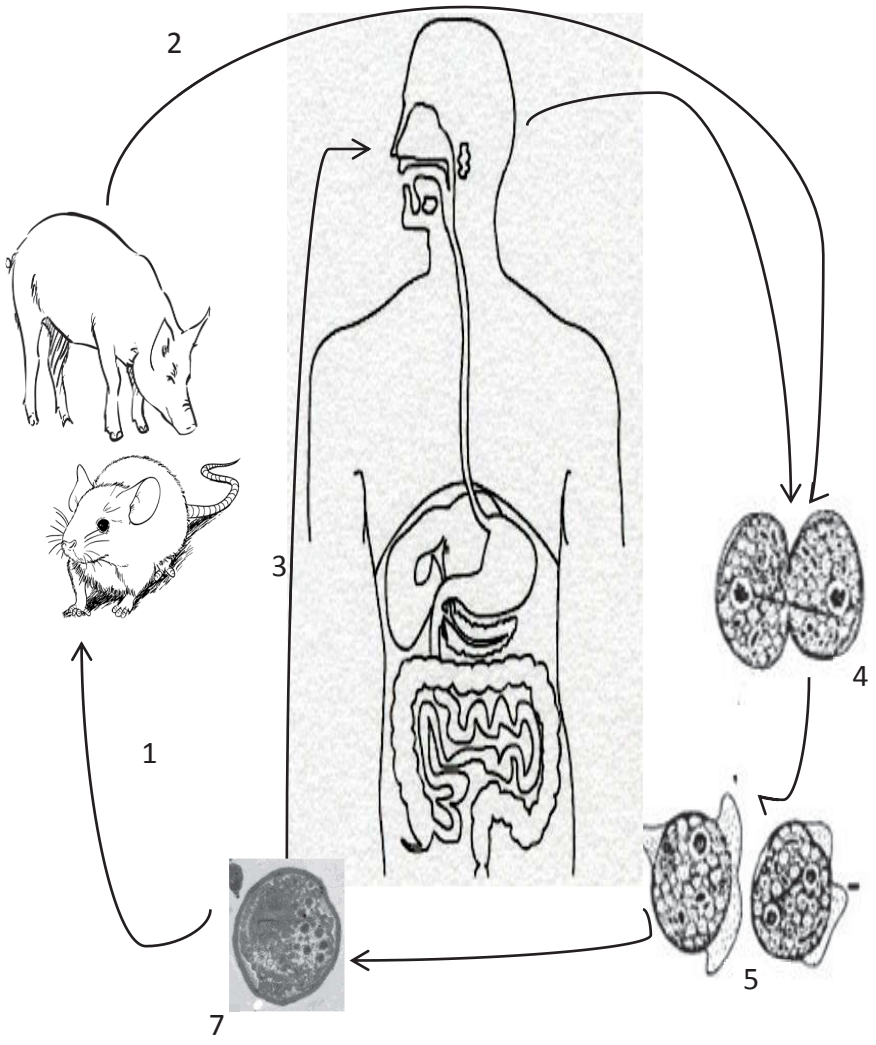


Figure 4. Porposed life cycle of D. fragilis: 1-Cysts in the environment are ingested by pigs and rodents. 2-Exystation and liberate trophozoites in the animal gut. 3-cysts ingested by human host via contaminated food and water. 4-excysted trophozoites divide by binary fission in the host intestine.5-binary fission forms two daughter trophozoites. 6-encystation as passing through the intestine due to stimuli such as bile acids. 7-cysts pass into the environment through faeces.

# Chapter 6:
# References

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