IN VITRO CULTURE AND BIOLOGICAL STUDIES ON *DIENTAMOEBA FRAGILIS*

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FACULTY OF MEDICINE
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ABSTRACT

Dientamoeba fragilis, since its discovery 87 years ago, very little is known about the parasite's prevalence, biology, life cycle and mode of transmission. The present targeted two vulnerable groups which are the orang asli and school children from the state of Selangor. Out of a total of 409 and 380 stool samples collected from Orang Asli and school children population the prevalence of those infected with *D.fragilis* were 3.9 % and 0.7% respectively. There is a challenge in identifying the parasites in stools and this could be the main contributory reason why information on this parasite is still lacking. The present study is also the first to suggest using potassium dichromate as a preservative as the results showed that the parasite remained intact and could easily be stained even after preservation of more than 12 months. The present study also reported Loeffler's medium supplemented with 70% horse serum and rice starch as well as modified Jones' medium supplemented with 10% horse serum and rice starch were ideal culture media to detect and grow *D. fragilis* in *in vitro.* The former supported growth of *D. fragilis* and showed a parasite count of $5.55X10^4$ while modified Jones' medium showed a parasite of count of $3.3X10^4$ on day 2 . The present study also was able to differentiate *D.fragilis* and *Blastocystis* sp. using a simple and effective stain i.e. Modified Fields' stain which was shown to be better and a more rapid stain than Giemsa and Iron haematoxylin especially when it comes to differentiating the two organisms when grown in cultures The study also showed that *In vitro* culture at the 18^{th} hour showed the highest parasite count and this proved to be the best time point to harvest parasites for further sub-culture. Furthermore the parasites when harvested at the 18^{th} hour remained viable for the next eight days. This finding was shown to be repeatable. The study also highlighted another mode of reproduction where the organism was seen to elongate prior to the release of a nucleated progeny leaving an empty space at the far end of the original organism. This proves that binary fission is not the only mode of reproduction reported to be seen in *D.fragilis*. *In*

vivo studies using 36 Sprague dawley rats was carried out to provide a better understanding on the life cycle and parasite transmission. Cyst-like structures which could resist the lysis of distilled water showing a thickened membrane could cause experimental infection in Spraque dawley rats. This is the first study to provide evidence that cyst-like structures do exist in the parasite's life cycle and these life cycle stages could cause experimental infection in Sprague dawley rats.

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Dientamoeba fragilis, since its discovery 87 years ago, very little is known about the parasite's prevalence, biology, life cycle and mode of transmission. The present targeted two vulnerable groups which are the orang asli and school children from the state of Selangor. Out of a total of 409 and 380 stool samples collected from Orang Asli and school children population the prevalence of those infected with *D.fragilis* were 3.9 % and 0.7% respectively. There is a challenge in identifying the parasites in stools and this could be the main contributory reason why information on this parasite is still lacking. The present study is also the first to suggest using potassium dichromate as a preservative as the results showed that the parasite remained intact and could easily be stained even after preservation of more than 12 months. The present study also reported Loeffler's medium supplemented with 70% horse serum and rice starch as well as modified Jones' medium supplemented with 10% horse serum and rice starch were ideal culture media to detect and grow *D. fragilis* in *in vitro.* The former supported growth of *D. fragilis* and showed a parasite count of $5.55X10^4$ while modified Jones' medium showed a parasite of count of $3.3X10^4$ on day 2 . The present study also was able to differentiate *D.fragilis* and *Blastocystis* sp. using a simple and effective stain i.e. Modified Fields' stain which was shown to be better and a more rapid stain than Giemsa and Iron haematoxylin especially when it comes to differentiating the two organisms when grown in cultures The study also showed that *In vitro* culture at the 18^{th} hour showed the highest parasite count and this proved to be the best time point to harvest parasites for further sub-culture. Furthermore the parasites when harvested at the 18^{th} hour remained viable for the next eight days. This finding was shown to be repeatable. The study also highlighted another mode of reproduction where the organism was seen to elongate prior to the release of a nucleated progeny leaving an empty space at the far end of the original organism. This proves that binary fission is not the only mode of reproduction reported to be seen in *D.fragilis*. *In*

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
<	Less than
>	More than
°	Degree
µl	Microliter
µm	Micrometer
AIDS	Acquired immune deficiency syndrome
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
b	number of cells at end of time period
B	number of cells at zero time
BALB	Bagg Albino
BD	Boeck and Drboholav
bp	Base pair
C	Celsius
CI	Confidence interval
dH_20	Distilled water
DNA	Deoxyribonucleic acid
DPX	DinButyl phthalate and Xylene
G	Gram
GI	Gastrointestinal
GT	generation time
H	hydrogenosome.

HIV	Human immunodeficiency virus
hr	Hour
HSP	Heat shock protein
IBS	Irritable Bowel Syndrome
Ig	Immunoglobulin
IMDM	Iscove's Modified Dulbecco's medium
JaKOA	Jabatan Kemajuan Orang Asli
KCl,	Potassium chloride
kDa	Kilo Dalton
Km	Kilometer
log	logarithm to the base 10 (common log)
m	rate of sampling
mg	Milligram
$MgCl_{2,}$	Magnesium chloride
min	Minute
ml	Milliliter
mM	Millimolar
MT-PCR	Multiplex Tandem Real-Time PCR
n	number of generations
n	sample size
N	nucleus
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
nm	Nanometer
NO	Nitric oxide
NP	New progeny

OR	Odd ratio
p	estimated rate which happened in the population
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Power of hydrogen
PVA	Poviyl alcohol
qPCR	Quantitative PCR
RFLP	Restriction fragment length polymorphism
RM	Ringgit Malaysia
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPS	Rancangan Petempatan Semula
RS	Rice starch
RT	Reverse transcription
RT-PCR	Real-time polymerase chain reaction
SAF	Sodium acetate-acetic acid formalin
Sec	Second
SEM	Scanning electron microscopy
sp	Species
SPSS	Statistical Package for Social Sciences for Windows
SSU rDNA	Small sub-unit ribosomal deoxyribonucleic acid
SSU_rRNA	Small sub-unit ribosomal ribonucleic acid
ST	Subtype
STH	soil transmitted helminthes
STS	Sequence-tagged site

t	time period
Taq	*Thermus aquaticus*
TEM	Transmission electron microscopy
Tris-Hcl	Tris hydrochloric
UMMC	University Malaya Medical Centre
UVP	Ultra-violet gel documentation system
WHO	World Health Organization
x^2	Chi-square
z	standard score
α	Alpha

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CHAPTER 1

INTRODUCTION

Dientamoeba fragilis is derived from the trichomonad group and has been responsible for causing gastrointestinal symptoms in persons infected globally (Nagata et al., 2012). The prevalence of the infection range between 0.5% to 16 % (Nagata et al., 2012). *D.fragilis* was first described as an amoeba by Jepps and Dobell a century ago (Jepps & Dobell et al., 1918) It has been more than 87 years since the discovery of *D.fragilis*, however information on its biology, life cycle, mode of transmission as well as the pathogenesis remain an enigma when compared to other parasite (Stark et al., 2006).

The understanding of the parasite's life cycle remains still in its infancy (Banik et al., 2012) and information pertaining to the biology of this parasite is still uncertain with many more aspects of the parasite remaining to be explored (Johnson et al., 2004). In addition, the understanding of the host distribution and zoonotic potential of this organism still remains uncertain (Johnson et al., 2004).

D.fragilis got its name due to the presence of its two nucleus and also due to the fragile nature of the parasite where it has been shown to degrade easily when exposed in the environment (Johnson et al., 2004). Initially this parasite was classified as an amoeba for 50 years, but due to the existence of centrodesmus and resemblance to *Histomonas meleagridis*, its taxanomy was questioned (Johnson et al., 2004). It was believed that *D.fragilis* possessed a flagella which can disintegrate when cultured or when the parasite invades a tissue (Johnson et al., 2004). Then for the next 20 years since the classification of *D.fragilis* was not confirmed till the DNA studies proved that *D.fragilis* belongs to Trichomonad (Johnson et al., 2004).

Although animals play a small role in transmitting this parasite, humans however remain the main host in transferring *D.fragilis* (Barratt et al., 2011b). The mode of transmission of *D.fragilis* remains uncertain (Clark et al., 2014). The parasite is either transmitted by human, animals or other parasites. Most studies reports that *D.fragilis* is transmitted via the fecal oral route (Barratt et al., 2011a). The possibility of *D.fragilis* transmission via the egg of an intestinal nematode have been proposed (Dobell et al., 1940).

The presence of pseudocystic, precyctic or cystic stage of this parasite have yet to be proven since the parasite is known to be fragile (Greenway, 1928; Knoll & Howell, 1946; Wenrich, 1936). The survival period of this parasite is between 6 to 24 hours (Johnson et al. 2004).

It has been reported that the prevalence of *D.fragilis* is between 0.3% and 52% (Barratt et al., 2011b). This parasite has always been known to be non-pathogenic although there are evidence to support its pathogenicity. Co-infection with other parasites such as *Entamoeba histolytica*, *Giardia* and *Cryptosporidium* have been reported (Barratt et al., 2011b). The reported prevalence of *D.fragilis* have been based mostly using light microscopy which may not accurately provide the information when compared to polymerase chain reaction (PCR) or the use of the *in vitro* culture method (Barratt et al., 2011b).

More studies need to be carried out to enhance the detection methods for *D. fragilis*. Till date, there is only one prevalence data on this organism in Malaysia. Therefore there is a need to assess if this parasite does occur in the stools of Malaysians especially in Orang Asli and school children. Furthermore, studies *in vitro* and *in vivo*

animal models need to be carried out to ensure maintenance of the parasite. This will ensure a constant supply for more molecular, biochemical, biology and mode of transmission studies so that more information of the parasite can be elucidated. With a steady supply of parasite material there will be a greater opportunity to improve the detection methods including staining and molecular methods as well as explore other fundamental aspects of this interesting organism.

1.1 Objectives of the study

1.1.1 To assess the prevalence of *D.fragilis* in Orang Asli (aborigines) villages and school children in the state of Selangor and

a) To assess its associative presence with other parasites including *Blastocystis* sp.
b) To assess the associated risk factors

1.1.2 To assess the biological features of the life cycle stages of *D.fragilis* seen in *in vitro* cultures.

1.1.3 To assess the susceptibility of mice and rats towards experimental infection with inoculation of cyst-like stages of *D.fragilis*

1.2 Research hypothesis

1.2.1 Orang asli and school children are easily infected with *D.fragilis* due to being lesser aware of good hygiene practices .

1.2.2 *D.fragilis* has another mode of reproduction apart from binary fission which possibly could account for the high parasite numbers seen in *in vitro* cultures within a short frame of time.

1.2.3 Rats and mice can be infected with cyst –like stages of *D.fragilis*.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to *D.fragilis*

D.fragilis, a trichomonad parasite is usually found in the gastrointestinal tract of humans and is known to cause gastrointestinal symptoms (Stark et al., 2005b). The parasite is globally distributed and mostly found in rural and urban areas (Stark et al., 2005b). Although *D. fragilis* has been discovered more than 87 years ago, very little is known about these parasite's biology, life cycle, mode of transmission as well as the prevalence (Stark et al., 2006). *D.fragilis* is now known as a *trichomonad* and is placed within the Phylum *Parabasalia* (Barratt et al., 2010; Banik et al., 2012).

D.fragilis causes various symptoms such as diarrhea, abdominal pain, loss of appetite, fatigue and abnormal stool (Chan et al., 1993). The parasite also causes fibrosis of the appendix, phagocytosis of erythrocytes and has been shown to be present in the biliary tract, colitis and low grade eosinophilia (Chan et al.,1993). The parasite is not only found in humans but also in non-human primates such as the macaques, baboons and gorillas (Stark et al., 2009).

Higher prevalence rates have been reported in developed countries around the world (Nagata et al., 2012; Stark et al., 2010b) between 0.3% and 52% (Barratt et al. 2011b). Many studies although have been carried out in the last decade but the understanding of this parasite is still limited. There is a greater need to understand better the parasite's morphology, life cycle, mode of transmission and diagnosis.

2.2 Taxonomy

The taxonomy of *D.fragilis* is still uncertain. The predominant form of this parasite is the bi-nucleate form (Jepps & Dobell, 1918; Johnson et al. 2004; Yang & Scholten, 1977). Primarily, *D.fragilis* is encompassed into sub- phylum *Sarcodina* (Johnson et al., 2004). The first transmission electron microscopy (TEM) study carried out as early as in 1974 showed the similarity between *D.fragilis* and trichomonads (Stark et al., 2006). The SSU rDNA sequences of *D.fragilis* was also compared to other *Trichomonads* and eukaryotes (Silberman et al., 1996a). *D.fragilis* was shown to be related to *Histomanas meleagridis* and consequent studies provided proof based on sequence 3 analysis of SSU rDNA (Delgado viscogliosi et al., 2000; Gerbod et al., 2002; Ohkuma et al., 2005). This parasite has been grouped with the *Trichomonads* based on molecular phylogenetics although it lacked flagella (Gerbod et al., 2002; Kleina et al., 2004; Lagacé-Wiens et al., 2006; Ohkuma et al., 2005).

The current classification of *D.fragilis*

(Source: http://en.wikipedia.org/wiki/*Dientamoeba_fragilis*)

Kingdom: Excavata

Phylum: Metamonada

Class: Parabasalia

Order: Trichomonadida

Family: Monocercomonadidae

Genus: *Dientamoeba*

Species: *Dientamoeba fragilis*

2.3 Biology

2.3.1 Morphology

The parasite is an enteric one with two nuclei or bi-nucleated and has the tendency to degenerate rapidly in excreted stool (Johnson et al., 2004). The fragile trophozoite stage in stool samples degenerate rapidly implying that stools need to be fixed as soon as they are excreted (Stark et al., 2005b). *D.fragilis* is characterized as pleomorphic with trophozoite's size ranging from 4 µm to 20 µm (Stark et al., 2006) with parasites occasionally showing only one nucleus under light microscopy (Banik et al., 2012). Usually *D.fragilis* is found in their bi-nucleate form and to a lesser degree uni-nucleate forms (Stark et al., 2006). In an unstained preparation, the nuclear structure of this parasite cannot be viewed (Stark et al., 2008).

D.fragilis is usually rounded when seen on a glass slide with a saline preparation without the nuclei being clearly visible. The parasite moves with the help of a thin, hyaline leaf like pseudopodia which have irregular lobes (Johnson et al., 2004). In *In vitro* culture, most of *D. fragilis* trophozoites are found to be mono- nucleated when compared to the permanent stained faecal smears (Banik et al. 2012). The nuclear membrane of this parasite has been shown to be delicate and does not show any peripheral chromatin (Stark et al., 2006). The karyosome contains four to eight chromatin granules which appear as chromatin packet (Stark et al., 2006). The cytoplasm of the parasite appears granular, vacuolated and contains food inclusion, bacteria and yeast. The parasite is usually rounded and show size variation in culture (20µm to 40µm) (Stark et al., 2006) corresponding to the sizes of *Entamoeba histolytica* and *Endolimax nana* (Johnson et al., 2004). The parasite is motile when seen

in fresh stool samples or when introduced in culture media but becomes less motile when exposed to room temperature as well as when refrigerated (Stark et al., 2006).

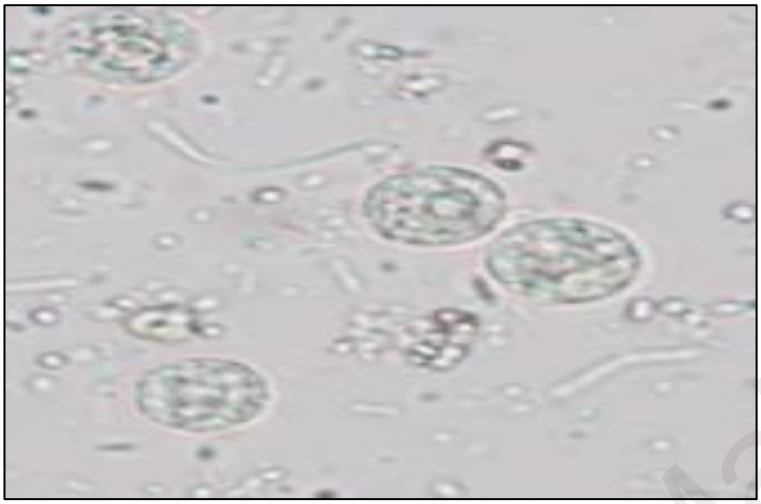

Figure 2.1 Trophozoite of *D. fragilis* trophozoite seen in vitro culture under 400x light microscopy

Transmission electron microscopy studies have shown the existence of parabasal filaments which extend laterally to attract atractrophores external surface (Johnson et al., 2004). At the filament, extensive Golgi complexes were seen with parabasal apparatus in *Trichomonads* and *Hypermastigotes* which were shown to be similar (Johnson et al., 2004) . An extra-nuclear spindle was found from the polar complexes adjacent to the one nucleus (Johnson et al., 2004). However there is little information on subtype variation. The parasite morphology isolated from animal sources have yet to be elucidated. The walls of the trophozoite have been shown to be thin which poses a doubt if this structure could really be responsible to facilitate the transmission of this parasite.

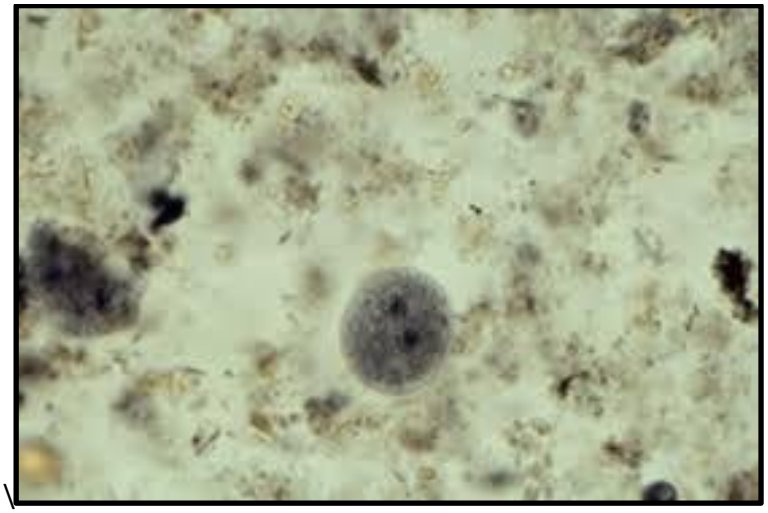

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Figure 2.2 Stained *D.fragilis* with modified iron haematoxylin observed under 400x microscopy

2.4 Life cycle and host distribution

The complete life cycle of *D.fragilis* is still not defined (Clark et al., 2014) despite studies on the host distribution and zoonotic potential of *D. fragilis* having been carried out (Johnson et al., 2004). It has been reported that human is the main host of this parasite followed by its occurrence shown in a small range of animal hosts (Barratt et al., 2011a; Cacciò et al., 2012). *D.fragilis* was found in non- human primates such as gorillas (Stark et al., 2008) pigs (Cacciò et al., 2012), swine (Crotti et al., 2007), baboons (Myers & Kuntz, 1968), macaque (Dobell, 1940) and sheep (Noble & Noble, 1952) . More animal studies need to be carried out so that a better understanding on the life cycle and host distribution of *D.fragilis* can be determined.

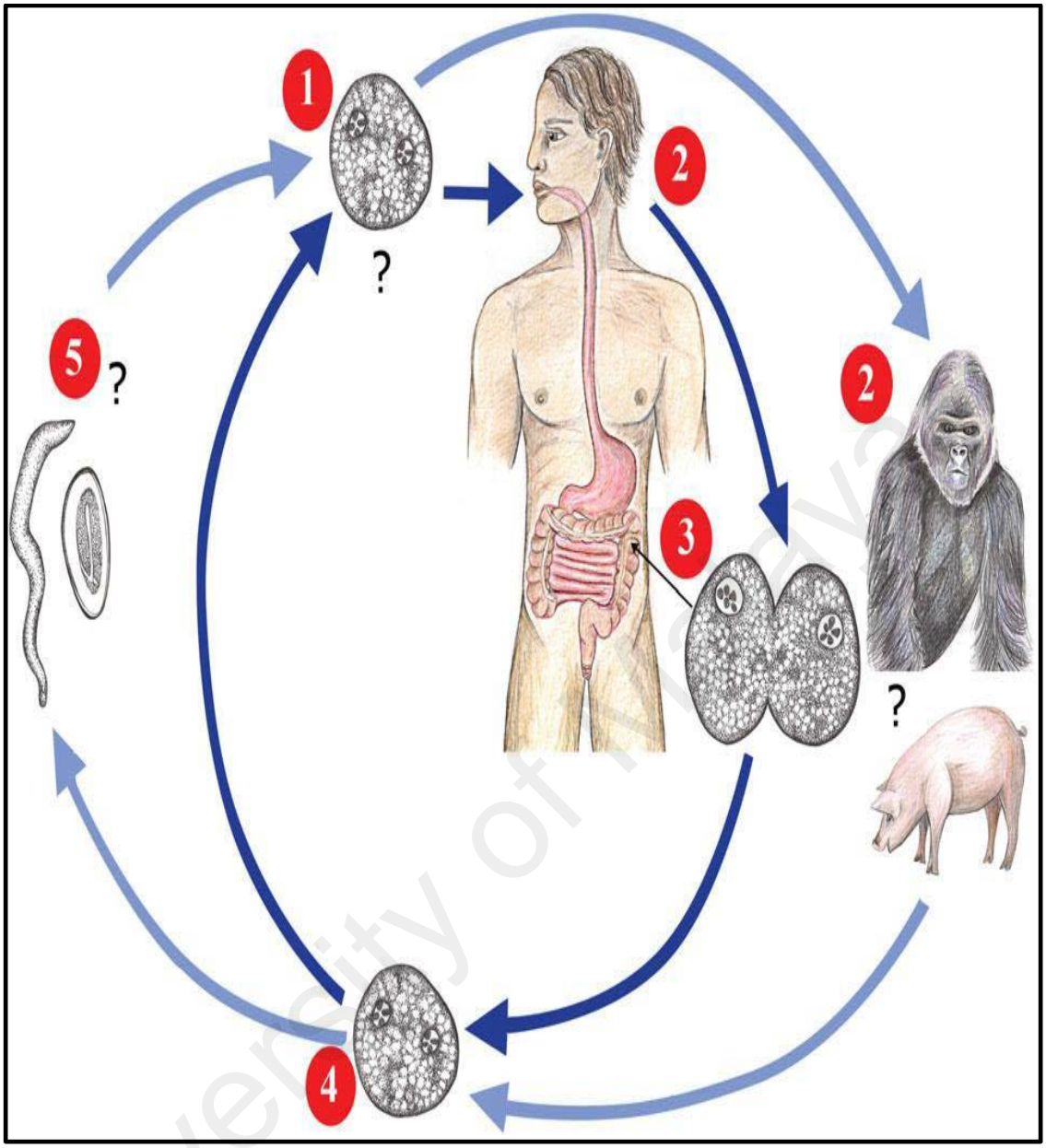

Figure 2.3 Life cycle of *D.fragilis* (1) *D.fragilis* ingested by human via the oral fecal route. (2) Primates such as gorillas and pigs are also the host of this parasite. (3) Once the parasite ingested, it undergoes binary fission in the large intestine and (4) excreted into the environment where it may infect the source of food and water. (5) The role of *Enterobius vermicularis* is still not clearly understand [Figure 2.3 adapted from Baratt et al., 2011b]

2.5 Transmission

Till date, the mode of transmission of *D.fragilis* is still unknown (Clark et al., 2014). Most of the studies believe that *D.fragilis* is transmitted via the fecal oral route (Barratt et al., 2011a). The trophozoite stage of *D.fragilis* in a stool specimen has been shown to survive in the environment from 6 to 48 hours (Johnson et al., 2004). Some studies also try to prove the presence of pseudocystic, precyctic or cystic stage of this parasite but have failed (Greenway, 1928; Knoll & Howell, 1946; Wenrich, 1936).

There also has been suggestion that eggs of nematodes such as *Trichuris* or *Ascaris* can be an intermediate host (Wenrich, 1944) with claims that similar structure to that of *D. fragilis* have been seen inside the ova of *Ascaris lumbricoides*. Then in 1956, a study suggested that human pinworm, *Enterobius vermicularis* could be a vector of *D.fragilis* (Burrows & Swerdlow, 1956). In the same report, 1518 histology of appendices when examined showed that 12 out of 22 patients positive for *D.fragilis* were co-infected with adult or eggs of E.*vermicularis* (Burrows & Swerdlow, 1956).

Subsequently, there was other reports that showed the association between *E.vermicularis* and *D. fragilis* (Ockert, 1990; Yang & Scholten., 1977) . Ockert in 1975 infected himself with *E.vermicularis* eggs and subsequently showed that he had both *Enterobius* and Dientamoebiasis (Ockert, 1974). This concurred with another study which showed the association between *E.vermicularis* and *D. fragilis* seen in both women and children (Girginkardeşler et al., 2008; Ockert, 1990). These studies became the basis for the suggestion that *E.vermicularis* does transmit *D.fragilis* (Ögren et al., 2013; Röser et al., 2013). On the other hand , another study showed that 100 patients

infected with *D.fragilis* were shown to be negative for *E.vermicularis* using the sticky tape test (Kean & Malloch, 1966).

In 1998, a research on 25 paediatric patient by Cuffari showed that there was no association between *E.vernicularis* and *D. fragilis* (Cuffari et al., 1998). The latest study carried out on 6750 patients positive for *D.fragilis* showed negative results for the presence of *E.vermicularis* but found other parasites that were transmitted via the faecal- oral route (Stark et al., 2005a). This report suggested that there was no association between *E.vernicularis* and *D.fragilis* (Stark et al., 2005a).

2.6 Genetic diversity

Two potential genotypes of *D.fragilis* have been acknowledged till date (Barratt et al., 2011b).Using the SSU rDNA, *D. fragilis* was effectively amplified using the control sample of *D.fragilis* strain Bi/PA (ATCC 309948) (Silberman et al., 1996a). In 2000, two genetic entities of this parasite was reported as genotype 1 (Johnson et al., 2004) and genotype 2 (Stark et al., 2006). Subsequently another study reported on the genetic diversity of *D.fragilis* isolated from 93 patients and 6 asymptomatic patients using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) detected only genotype 1 (Peek et al., 2004; Windsor et al., 2004). Another study showed 50 *D. fragilis* isolates using the PCR-RFLP showed the presence of genotype 1 (Stark et al., 2005b). A strong difference was shown between the two genotypes using *D.fragilis* housekeeping genes (Stensvold et al., 2013).

2.7 Diagnosis

2.7.1 Preservative, staining and microscopy analysis.

D.fragilis can only be identified by the presence of its trophozoite as the parasite is devoid of the its cystic stage (Grendon et al., 1991). As the trophozoite of *D.fragilis* degrade rapidly once excreted, it is important to preserve the sample prior to microscopy analysis (Stark et al., 2010b). The identification of *D.fragilis* require fixed or fresh unpreserved stool sample (Grendon et al., 1991). Stool samples can be preserved in polyvinyl alcohol (PVA) preservative, sodium acetate-acetic acid-formalin (SAF) preservative or Schaudinn's preservative (Grendon et al., 1991). Compared to other fixatives, sodium acetate-acetic acid formalin was seen to be easier to be prepared, was non-toxic and provided a better result using the concentration method (Johnson et al., 2004).

Merthiolate–iodine formalin was another preservative used which is a combination of preservative and a stain but this preservative was shown to be not stable and does not give optimal results when stained (Johnson et al., 2004). Another common stain used is the Schaudinn's preservative and Poviyl alcohol preservative but both these stains have been shown to be not safe as it contained mercury chloride which poses a problem when it comes to disposing such preserved fecal samples (Johnson *et al.*, 2004). Permanent staining of fixed smears have been suggested to be used in most laboratories (Dobell, 1940; Stark et al., 2005b)

There were many different stains that have been used over the years namely Mayer's haemalum, iron haematoxylin, Lawless stain, Celestine Blue and Wheatley's

trichrome (Johnson et al., 2004). The optimal contrast has been shown by combining a preservative and a stain in the right amount to provide the right clarity (Johnson et al., 2004). The common combination usually used is polyvinyl alcohol (PVA) and Schaudinn's fixative with Wheatley's trichrome and sodium acetate-acetic acid formalin (SAF) with iron haematoxylin (Johnson et al., 2004). Permanent stain however has been shown to take a longer time and may not give optimal result (Garcia, 1990; Stark et al., 2011). Furthermore, this technique may cause difficulties in differentiating *D.fragilis* from other similar protozoan such as *Endolimax nana* and *Blastocystis* sp (Sawangjaroen et al., 1993).

2.7.2 *In vitro* culture

There are only a few studies carried out on the various *in vitro* culture techniques for *D. fragilis* (Johnson et al., 2004; Stark et al., 2006). Reports highlight xenic cultivation of *D.fragilis* , however axenic cultivation is yet to be established (Barratt et al., 2010). The diagnosis of *D.fragilis* using *in vitro* culture technique is more definitive as it requires a small amount of feaces when compared to using permanent stain on fecal smears (Sawangjaroen et al., 1993; Windsor et al., 2003a).

The first biphasic xenic *in vitro* culture medium was developed by Dobell and consisted of horse serum which was overlaid with a liquid phase of egg white diluted in Ringer's solution (Barratt et al., 2010). Cleveland and Collier's medium, modified Boeck and Drbohlav (BD) medium, and Robinson media were some of the examples of biphasic medium used to cultivate *D.fragilis* (Cleveland & Collier, 1930; Rayan et al., 2007; Windsor et al., 2003a).There were many studies carried out using axenic cultures of *D.fragilis* but many failed to create an axenic culture for this parasite

(Johnson et al., 2004). There was also an attempt to create a monoculture but this too failed because the parasite was unable to grow in the presence of bacteria heated at 60-65^{o}C for an hour (Johnson et al., 2004). Subsequently, Jacob in 1953 attempted to cultivate *D.fragilis* in an axenic culture treated with antibiotic penicillin, streptomycin and also sulphadiaziane (Johnson et al., 2004) and the cultures were maintained for seven days with the parasites growing with *Clostridium perfringens* (Johnson et al., 2004). Nevertheless, despite continuous attempts to axenize the cultures, the results have been a failure (Nagata et al., 2011).

There were also a monophasic medium such as the TYGM-9 broth which was developed. The culture medium for the cultivation for trichomonads such as Medium 199 and *Tritrichomonas foetus* medium were also used for the cultivation of *D.fragilis* (Barratt et al., 2010). The growth of *D.fragilis* have been shown to be better in Loeffler's medium compared to modified BD and Robinson media (Barratt et al., 2010). Other culture media such as modified BD Loeffler TYGM-9 modified Cleveland and Collier's, Robinson,, Trishosel, Medium 199 and *Tritrichomonas foetus* medium were used (Barratt et al., 2010). The TYGM-9, Robinson, Loeffler's and modified BD medium showed a positive result in cultivating the growth of *D.fragilis* while the other two media, Trishosel, and *Tritrichomonas foetus* medium failed to grow *D.fragilis* (Barratt et al., 2010). *D.fragilis* was shown to grow better in a microaerophilic (6% O_2, 7.2 % CO_2, 3.6 % H_2, 83.3% N_2) and aerobic condition (0.2% O_2, 9.9 % CO_2, 5 % H_2, 84.9% N_2) than in atmospheric levels of oxygen (Barratt et al., 2010). Furthermore, a higher cell density was achieved at 42^{o}C than at 40 ^{o}C and 37 ^{o}C (Barratt et al., 2010)

In another study Loeffler's medium supplemented with EBSS (Earle's Balanced Salt Solution) proved to cultivate higher growth of *D. fragilis* (Munasinghe et al., 2012)

2.8 Ultrastructural description of *D. fragilis*

Two population of *D.fragilis i.e.* namely the smooth and ruffled surface wall were shown using the scanning electron microscopy (Banik et al., 2012). The size of the trophozoites were found to be varied while the shape was shown to be spherical to ovoidal. Bacteria was also seen to attach to the trophozoites. Flagella, undulating membrane as well as axostyle-pelta were not observed (Banik et al., 2012).

2.9 Molecular diagnosis

Immunofluorescence assay has been shown to produce anti-*D.fragilis* anti-serum in rabbit by using dixenic *D.fragilis* stain. These assays were designed to identify the presence of *D.fragilis* in preserved fecal sample (Johnson et al., 2004). The lower prevalence of *D.fragilis* reported in the earlier studies were due to insufficient tools for diagnosis (Spencer et al., 1982). There have been two studies that have described the development of Polymerase Chain Reaction (PCR) with one study showing specificity of 100% and 93.5 % respectively by targeting the SSU rRNA gene of *D. fragilis* (Stark et al., 2006). The procedure can be performed in a day and has been shown to be quick, simple and effective.

The conventional and real-time polymerase chain reaction (RT-PCR) targeting the 18 S rDNA have been designed (Peek et al., 2004; Stark et al., 2006; Verweij et al,. 2007). In the beginning, a PCR method was established to identify *D.fragilis* from human stool samples without culturing it but the sensitivity of the PCR method was not mentioned (Peek et al., 2004). Another PCR assay was designed that show 100% specificity and 93.5% sensitivity because there were no cross reactivity with other

parasites (Stark et al., 2005a). In addition, the same author designed a 5' nuclease (Tagman)- based real time PCR that targets the SSU rRNA gene that show 100% sensitivity and specificity (Stark et al., 2006). Then in 2010, another real time PCR was designed that show sensitivity to the detection of *D.fragilis* from human stool sample (Stark et al., 2010a). Nested PCR and Multiplex Tandem Real-Time PCR (MT-PCR) have also been described as a possible detection method for *D.fragilis* (Sarafraz et al., 2013; Stark et al., 2010a).

2.10 Symptoms

The association of *D.fragilis* with other clinical symptom such as diarrhea, abdominal pain, vomiting, nausea and fatigue have been reported by many studies from every part of the world (Barratt et al., 2011b; Stark et al., 2010b). There were other studies that showed the association between *D.fragilis* and urticaria (Yang & Scholten, 1977), biliary infection (Talis et al., 1971), diarrhea in human immunodeficiency virus patient (Lainson & Da Silva 1999) and Irritable Bowel Syndrome (IBS) (Borody et al., 2002). The most common symptom associated with children are diarrhea, constipation, flatulence, abdominal pain, nausea as well as fatigue, anorexia and peripheral eosinophilia (Banik et al., 2011; Norberg et al., 2003; Ter Schure et al., 2013).

2.11 Treatment

Antimicrobial agents such as the iodoquinol, metronidazole, tetracycline, secnidazole, ornidazole, and paramomycin have been used to eliminate the presence of *D.fragilis* in human (Stark et al., 2010b). The use of these antimicrobial have proven to relieve patients with clinical symptoms but there are not many studies which have shown the best treatment regime or drug against *D.fragilis* (Stark et al., 2010b). The frequently used treatment to treat children are iodoquinol and metronidazole (Banik et al., 2011; Vandenberg et al. 2007). Lately, a study reported that clioquinol was proven to be more effective than metronidazole in treating children infected with *D.fragilis* (Ter Schure et al. 2013).

2.12 Prevalence

There have been several studies which describe the high number of *D.fragilis* infection in certain groups (Fathallah et al., 2004; Yang & Scholten, 1977). The highest prevalence rate reported in one community was 52% (Spencer et al., 1983). Furthermore, prevalence studies of *D.fragilis* is usually based on light microscopy which have lower sensitivity compared to PCR or *in vitro* cultivation (Stark et al., 2010b).

In the earlier studies , *D.fragilis* was usually reported to be having a lower prevalence than *Entamoeba coli, Entamoeba histolytica* or *Entomoeba nana* but recent reports reveal that prevalence range between 6.3% and 29% in people infected with other intestinal parasite who usually have *D.fragilis* (Barratt et al., 2011b; Stark et al., 2010b; Vandenberg et al., 2007). The high fragile condition of *D.fragilis* is the single

most important reason for the lower prevalence reported in the earlier studies (Kean & Malloch, 1966; Schuster & Jackson, 2009). However, due to the increasingly established methods of detection, the rate of prevalence reported of *D. fragilis* has increased and is described to be higher than prevalence of *Giardia lamblia* (Barratt et al., 2011b). Thus, the latest prevalence of *D. fragilis* is between 0.3% and 52% according to the method of detection (Barratt et al., 2011b).

D. fragilis occurs more in children than in adult (Barratt et al., 2011b). However another report showed that *D. fragilis* infection was highest in persons of ages between 40 to 59 years old although this has not been proven statistically (Kean & Malloch, 1966). Crotti and Annibale also showed reports of higher prevalence rate in adult compared to children (Crotti et al., 2005). The highest prevalence rate of infection was seen between 5 and 14 years old (De wit et al., 2001; Norberg et al., 2003) and between 16 to 20 years old (Lagacé-Wiens et al., 2006; Stensvold et al., 2013).

The results appeared to vary with another report showing the parasite is seen in children between ages 0 and 10 years and adults between 41 to 60 years old (Stark et al., 2010b). The infection between children and adults may be due to the close contact between them (Ayadi & Bahri, 1999; Grendon et al., 1991). The transmission of this parasite is through the fecal oral route often facilitated by the lack of hygiene and poor living conditions (Millet et al., 1983).

Several studies report that females are more likely to be infected with *D.fragilis* compared to the males (Ayadi & Bahri, 1999; Crotti & D'annibale, 2007a; Crotti & D'annibale 2007b; González-Moreno et al., 2011; Grendon et al., 1991; Rayan et al., 2007; Yang & Scholten, 1977). This intimate contact between mother and child more

than with the fathers could possibly account for this (Barratt et al., 2011b) however there are studies which claim that the difference is only small (Kean & Malloch, 1966; Stark et al., 2010b; Vandenberg et al., 2007). Hence the role of males and females can influence transmission within a community (Barratt et al., 2011b) .

The relationship of *D.fragilis* and *Entamoeba histolytica* was seen when *D. fragilis* was found in 9.6% of stool sample having *E.histolytica* (Sargeaunt et al., 1980) . There was also studies associating the parasite with *Blastocystis* sp. (Ayadi & Bahri, 1999; Lagacé-Wiens et al., 2006; Ozcakir et al., 2007) with studies showing high association between them (Norberg et al., 2003). 34.8 % *Blastocystis* sp. infected patient have been shown previously to harbor *D.fragilis* (Stensvold et al., 2013). The relationship between *D.fragilis* and other enteric parasite propose that these parasites share similar mode of transmission (Barratt et al., 2011b).

Table 2.1 Prevalence of *D.fragilis* in clinical samples [adapted from Baratt et al., 2011]

No	Sample type	Percentage (%)	Total number	Country
1	Fecal specimen from inmates in mental asylum	36.25	60	Holland
2	Fecal specimen from parasitology laboratory	2.4	14203	USA
3	Fecal specimen from gastrointestinal outpatient	20.1	1114	Israel
4	Fecal specimen containing *Entamoeba dispar*	9.6	125	Mexico
5	Fecal specimen submitted for parasitological examination	4.2	43029	Canada
6	Fecal specimen from children attending dental	21.1	104	USA
7	Fecal specimen from patient with GI disorder	3	1350	New Zealand
8	Fecal specimen from children infected with any GI protozoa	82.9	123	Germany
9	Fecal specimen from dehydration and diarrhea	2	100	Dominic Republic
10	Fecal specimen from patients with diarrhea	1.5	260	Brisbane
11	Fecal specimen from HIV infected patient without diarrhea	25.6	82	Argentina
12	Fecal specimen from pediatric refugees	2.3	87	USA
13	Fecal specimen from HIV negative patient	2.1	48	Hondurus
14	Fecal specimen submitted for routine microbiological analysis	5.1	857	Oman
15	Fecal specimen submitted to a university hospital	5.5	27053	Tunisia

16	Fecal specimen from HIV positive patient	3	34	North Brazil
17	Fecal specimen from patient with various GI complaint	11.3	151	Italy
18	Fecal specimen from patient admitted to hospital	8.8	400	Turkey
19	Fecal specimen from patient with various GI complaints	3.7	3139	Italy
20	Fecal specimen from patient with various GI complaints	3.4	1141	Italy
21	Fecal specimen from patient with various GI complaints	4.1	1989	Italy
22	Fecal specimen from children from IBN Sina hospital	2	350	Libya
23	Fecal specimen from hospital patient	2.7	770	Turkey
24	Fecal specimen from patient infected with intestinal parasite	8.9	168	Egypt
25	Fecal specimen from patient infected with intestinal parasite.	29.8	168	Egypt
26	Fecal specimen from HIV negative man who have sex with men	0.8	628	Australia
27	Fecal specimen from HIV infected MSM	0.3	618	Australia
28	Fecal specimen from non MSM patient	1.1	622	Australia
29	Fecal specimen from patient with diarrhea	0.9	6750	Australia
30	Fecal specimen from sanitary workers	0.82	241	Turkey
31	Fecal from patient with parasitic bowel infection	6.3	448	Belgium

32	Fecal specimen from patient with IBS associated disease	3.5	171	Karachi
33	Fecal specimen from patient with IBS associated disease	4	171	Karachi
34	Fecal specimen from patient suspected harboring intestinal parasite	11.7	103	Denmark
35	Fecal specimen from patient with various GI complaints	32	397	Netherland
36	Fecal specimen from patient with various GI complaints	5.2	750	Sydney
37	Fecal specimen from people attending complimentary health practices between 2002-2004	14.6	3719	British Isle
38	Fecal specimen from people attending complimentary health practices between 2002-2004	16.9	2491	British Isle
39	Fecal submitted to the Department of Microbiology at St Vincent's hospital	5.5	472	Sydney
40	Fecal specimen from patient with digestive disorder	1.6	8313	Catalonia
41	Fecal specimen from patient with clinical suspicion of intestinal parasitosis	21.4	491	Parma
42	Fecal specimen from patient with IBS associated disease	4	171	Karachi

Table 2.2 Prevalence of *D.fragilis* in non-clinical sample. (Baratt et al .,2011)

No	Sample type	Percentage	Total number	Country
1	Fecal specimen from school children	1.1	94	South Africa
2	Fecal specimen from adult members in a semi-communal group	52	81	USA
3	Fecal specimen from children from day care center	8.6	900	Canada
4	Fecal specimen from adult staff from day care center	4	146	Canada
5	Fecal specimen from homosexual man	1.3	150	USA
6	Fecal specimen from residential community	16.8	125	Australia
7	Fecal specimen from homosexual man with diarrhea	1.1	274	USA
8	Fecal specimen from indigenous people	21	242	Irian Jaya,Indonesia
9	Fecal specimen from aborigine community	2.7	112	Argentina
10	Fecal specimen from school children	0.21	2975	Turkey
11	Fecal specimen from children in rural communities	3	266	Hondurus

In Malaysia there has been no study carried out on this organism. There is a need to establish a national prevalence data and correlate these to the various vulnerable communities. There is a need to establish *in vitro* and *in vivo* animal models to sustain the maintenance of the parasite so that molecular, biochemical, biology and mode of transmission of the parasite can be studied in detail.

Susceptibility studies need to be carried out with laboratory animals using both intra-caecal and fecal oral routes. The most recent detailed description of the organism using electron microscopy was revealed only in 2012 (Banik et al., 2012). This implies that much of the information regarding the organism continues to remain only in its infancy.

CHAPTER 3

METHODOLOGY

The experiments in this study have been divided based on three parts, i) the prevalence, ii) the biology and iii) the *in vivo* experimental infection studies. The overall methodology used has been simplified in the flow chart below. A description of each method is subsequently provided.

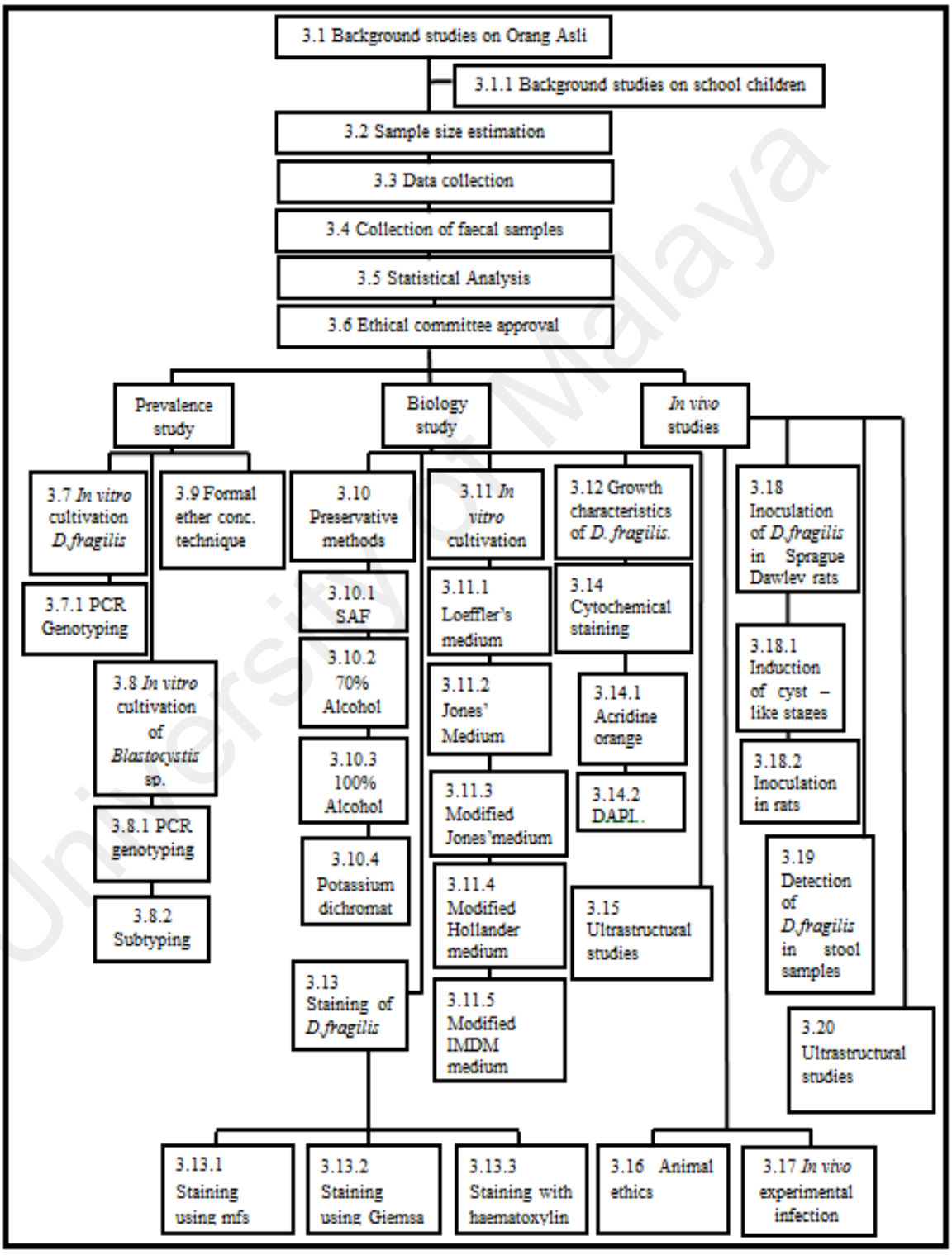

Figure 3.1 Schematic representation of the overall methodology

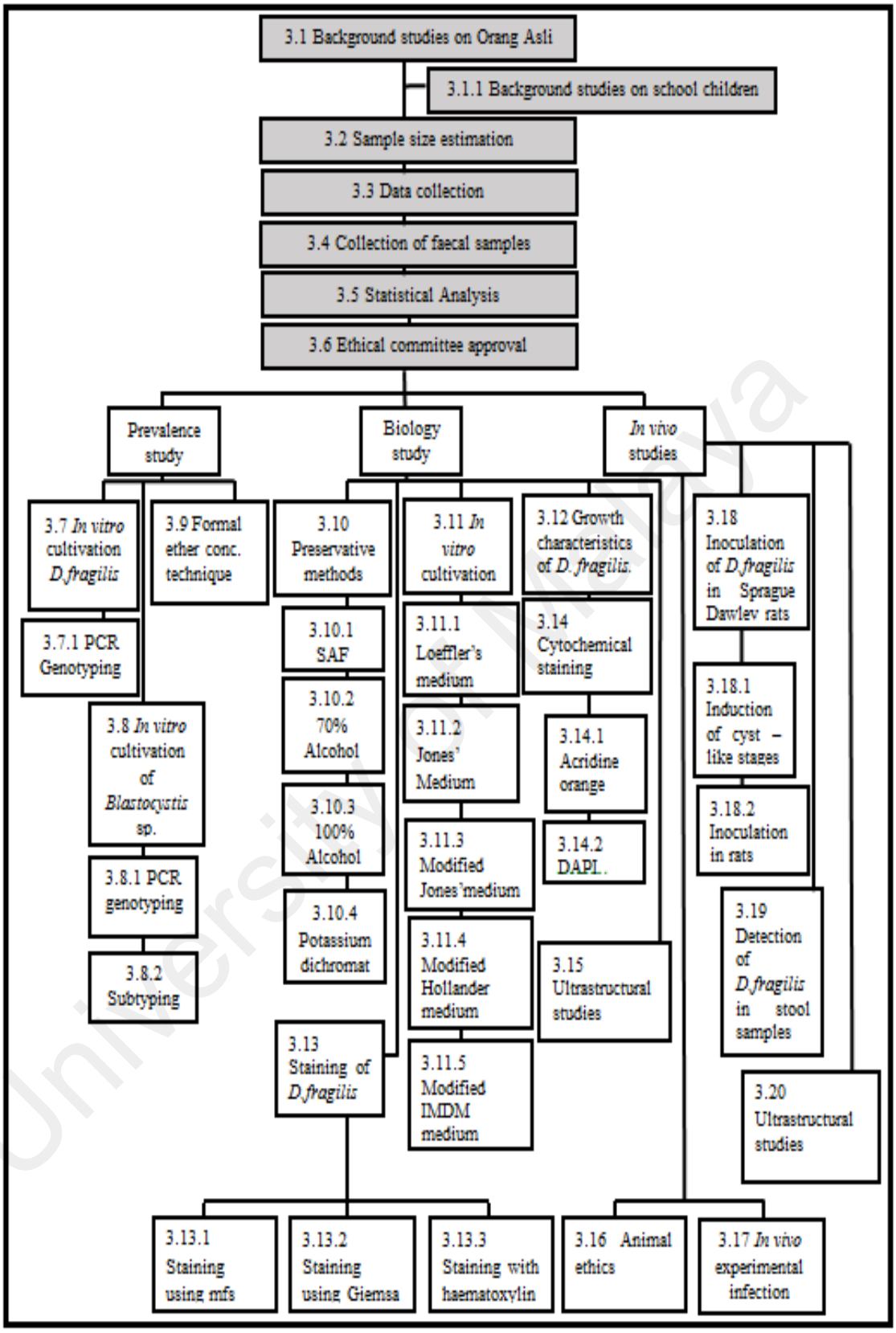

Figure 3.2 Schematic representation of the overall methodology emphasizing on the sample collections for the prevalence aspect of the study.

3.1 Background on the Orang Asli and sample collection sites

A cross-sectional study was carried out from December 2013 to April 2015 amongst the various subgroups of Orang Asli from the state of Selangor with the collaboration of Jabatan Kemajuan Orang Asli (JAKOA). In the present study, a total of 8 villages participated with the communities involved divided into two different subgroups namely the Suku Temuan and Suku Mahmeri (Table 3.1). Kampung Orang Asli Bukit Cheeding is under the Rancangan Petempatan Semula (RPS) scheme under the government where basic facilities such as electric supply, clean water, main road, school, clinic and other facilities have been provided to improve the quality of life for the Orang Asli community members. Villages in the Hulu Langat, Tanjung Sepat area have houses built within a typical traditional villages with basic facilities provided by the government. However there are communities that are devoid of these amenities and continue to live in conditions that spell poverty. Poverty being a major problem, Orang Asli community members find it a challenge when it comes to paying their bills. They get their water from a flowing stream while their main source of income comes from their work in the forest.

3.1.1 Background on the school children and study areas

The schools in Selangor chosen for this study are categorized into two types namely rural and urban schools. A total of 7 urban and 5 rural schools participated in this study. The urban and rural schools are categorized based on the number of people in the area. The minimum number of people for a location to be considered as urban or rural is 10000 and for the urban is more than 10000 people respectively. In addition, a urban location is classified as when 60 % of its people work with a job not related to agriculture. The rural schools in Selangor have been shown to have a lower number of students compared to urban schools. The facilities were seen to be better in urban school than facilities seen in rural school.

Table 3.1: Information on the site of the sample collection

Location	Subgroup	Approximate distances from Kuala Lumpur (km)(GPS)
Kampung Orang Asli Pangsun	Suku Temuan	39.7 (3.210037,101.882715)
Kampung Orang Asli Bukit Cheeding, Jenjarom	Suku Temuan	62.2 (2.898936,101.574000)
Kampung Orang Asli Sungai Lalang Baru	Suku Temuan	42.7 (3.052892,101.870861)
Kampung Orang Asli Serendah Rawang	Suku Temuan	50.4 (3.367189,101.6114859)
Kampung Orang Asli Broga	Suku Temuan	41.9 (3.018254,101.904570)
Kampung Orang Asli Kemensah	Suku Temuan	23.2 (3.219237,101.779538)
Kampung Orang Asli Batu 12, Gombak	Suku Temuan	29.5 (3.267447,101.572125)
Kampung Orang Asli Tanjung Sepat	Suku Mahmeri	87.3 (2.676831,101.550540)

Table 3.2: List of rural and urban school

Rural school	**Approximate distances from Kuala Lumpur (km)(GPS)**
Sekolah Rendah Pangsun, Hulu Langat	44 (3.109210,101.856023)
Sekolah Rendah Lebuk Kelubi, Hulu Langat	20 (3.086167,101.790012)
Sekolah Jenis Kebangsaan (T) Highland, Klang	42 (2.992425,101.441479)
Sekolah Kebangsaan Sg Serai, Hulu Langat	45 (3.085770,101.790466)
Sekolah Kebangsaan Dato Abu Bakar Baginda,	38 (2.959104,101.729125)
Sekolah Kebangsaan Bukit Tandom,	60 (2.805363,101..622279)
Sekolah Kebangsaan Bukit Changgang,Banting	55 (2.830133,101.626976)
Urban school	
Sekolah Rendah Jenis Kebangsaan Vivekananda	8 (3.131096,101.6886728)
Sekolah Rendah Jenis Kebangsaan Batu Caves	25 (3.235584,101.682904)
Sekolah Kebangsaan Methodist, Petaling Jaya.	42 (3.110412,101.656201)
Sekolah Kebangsaan Bandar Rahman Putra	31 (3.207915,101.568366)
Sekolah Kebangsaan Sinaran Budi Rawang	42 (3.316476,101.568366)

Figure 3.3 Location of the study area, state of Selangor in Peninsular Malaysia Map

3.2 Sample size estimation

The sample size essential for this study was calculated according to the anticipated and latest prevalence of soil transmitted helminthes (STH) in the orang asli population. The calculation for the sample size was made according to the following formula (Leedy, 1993).

$$n \geq (z/m)^2 \times p\,(1-p)$$

n= sample size

z=standard score (1.96)

m=rate of sampling (5%)

p=estimated rate or case which happened in the population

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3.3 Data collection

3.3.1 Questionnaire

This cross sectional study was carried out across 8 orang Asli villages and 12 schools (7 from rural and 5 from urban) in Selangor. The demographic and personal Survey Questionnaire (Appendix A) was pre-designed to gather information on the demographic, socioeconomic, lifestyle, environment and health status of the participants both in the Orang Asli villages and the schools. Verbal consent was obtained from everyone participating in the study before filling up the questionnaire. Permission was sought from the Jabatan Kemajuan Orang Asli (JAKOA) (Appendix B) and Ministry of Education respectively (Appendix C & Appendix D).

For the Orang Asli community, a meeting was held between the villagers and the head of the village to explain the purpose of the study .They were also told that the participation would be on a voluntary basis and they could withdraw from the study at any point of the time without giving any reason. Oral and written consent was obtained from the participant prior to the handing over the questionnaire to those who were interested and these were collected prior to the collection of fecal samples. The participants were not rewarded and the study was on a voluntary basis.

For the school children, approval was first obtained from the Ministry of Education before seeking approval from the Selangor Education Ministry. The schools were then approached and a briefing was made to the respective headmaster. Students were given a consent form to be filled by parents. Parents and their children were then informed that their participation in the survey was voluntary and therefore they could withdraw from the study at any point of time without giving any reason.

3.4 Collection of Fecal samples

Labeled wide mouth, screw capped fecal containers with attached scoop was distributed to each participant together with a plastic bag. This was done after the one to one interview session with participants using the questionnaires. The participant was instructed to scoop a thumb sized fecal sample using the provided scoop and subsequently this was introduced into the container. If the fecal quantity collected was less than a thumb size, samples will then be excluded as the amount would not be enough for the study. Fecal samples contaminated with urine were also excluded. The fecal containers containing the samples were then brought back to the Department of Parasitology, Faculty of Medicine ,University of Malaya for further analysis.

3.5 Statistical analysis

Data were analysed using Statistical Package for Social Sciences for Windows (SPSS) (Version 17.0). Descriptive analysis was mainly used to describe the characteristic of the studies population, including the prevalence of *D. fragilis* and *Blastocystis* sp. Chi-square (x^2) was used to test the differences in proportions. A significant level of 0.05 was used for all tests. Univariate and multivariate analysis were used to identify the significant risk factors.

3.6 Ethical committee approval

The study protocol (Reference Number 848.28.) was approved by the Ethics Committee of the University Malaya Medical Centre (UMMC), Malaysia before the commencement of the study.

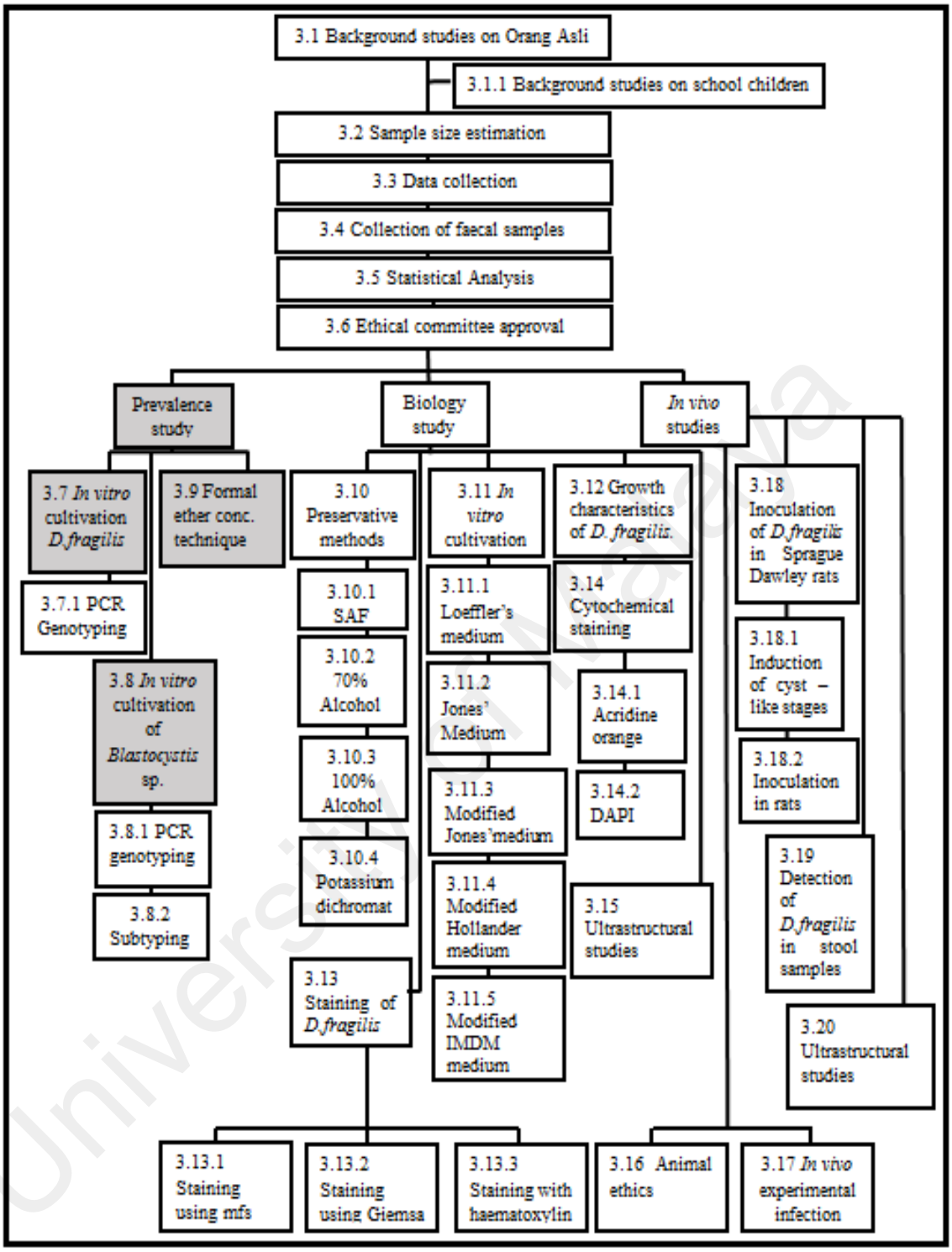

Figure 3.4 Schematic representation of the overall methodology emphasizing on the *in vitro* cultivation of *D.fragilis* and *Blastocystis* sp.

3.7 *In vitro* cultivation of *D. fragilis*

Fresh stool samples were cultured in Loeffler's medium. The medium was prepared to have glucose (Merck,Germany) (2.5g/L), nutrient broth (Merck, Germany) (6.25 g/L) and heat inactivated horse serum (Gibco, New Zealand) (700ml/L) in distilled water. In a 15ml falcon tube, 5ml of this mixture are added, sloped and insipissated at 80^{o}C. About 2 to 5 mg rice starch (Sigma, USA) will be added at the bottom of the slope. The slope was then covered with 5ml of Phosphate buffer saline (PBS).

3.7.1 Genotyping by polymerase chain reaction (PCR) of *D.fragilis*

A PCR mastermix using the pure *Taq* Ready To Go was prepared. The mater mix of 25µl contained 1.5 unit of *Taq* Polymerase, 10Mm Tris-Hcl at pH 9, 50mM KCl, 1.5mM $MgCl_{2,}$ 200mM dNTP, BSA and 1.0µl genomic DNA extract. The thermos-cycling profile was carried out as follows:- 3 minutes of denaturation, hold at 94^{o}c followed by 30 cycle of 1 minute at 94^{o}c, 1 to 5 minute at 57^{o}c and finally 2 minute at 72^{o}c. The amplification products were electrophoresed in 1.0% agarose gel (Promega, USA) Tris-Borate-EDTA buffer. Gels were stained with ethidium bromide and photographed using ultra-violet gel documentation system (Uvitec, United Kingdom). The fragment sizes were confirmed with bands of a DNA length standard (100–1,000 bp DNA markers, BioBasic). The PCR products were then purified using the QIAquick^{TM}PCR purification kit (QIAGEN) and sent for sequencing.

3.8 *In vitro* cultivation of *Blastocystis* sp.

Blastocystis sp. was isolated from the stool samples of school children and local communities by *in vitro* cultivation using Jones' medium (Jones, 1946) supplemented with 10% horse serum and incubated at 37^0C (Zaman et al. 1997). *In vitro* cultures were done by inoculating approximately 50 mg of stool sample in 3ml of Jones' medium. Isolated parasites were maintained by sub-culturing once every 3 to 4 days in Jones' medium for at least 1 month prior to the phenotypic analysis (Appendix E).

3.8.1 Genomic DNA Preparation of *Blastocystis* sp.

Blastocystis sp. isolates grown in Jones' medium were collected by centrifugation at 1000g for 5 min and washed twice using sterile phosphate buffered saline (PBS) (pH 7.4). Using the QIAamp DNA Stool Mini Kit (Qiagen, Australia) the harvested *Blastocystis* sp. was subjected to DNA extraction according to the manufacturer's protocol. The concentration and purity of DNA was measured using Nanodrop 2000 (Thermo Scientific, USA).

3.8.2 Subtyping of *Blastocystis* sp.

All genomic DNA of *Blastocystis* sp. was amplified using polymerase chain reaction (PCR) using seven sets of sequenced-tagged site (STS) primers (Yoshikawa et al. 2003). Amplification of 2µl genomic DNA was carried out in 20µl reaction containing 2.5µl of 10X Taq Buffer with KCL, 3.125mM of $MgCl_{2,}$ 0.5mM of deoxyribonucleotide triphosphates, 0.25mM of forward and reverse primer and 1U Taq DNA polymerase (recombinant) (Fermentas, USA). The thermal profile was programmed with one cycle of initial denaturation at 95^0C for 5min; followed by 30 cycles of denaturation at 95^0C for 1min, annealing at 56.3^0C for 1min 30sec and extension at 72^0C for 1min; one cycle of final extension at 72^0C for 10min and final infinite holding at 10^0C (Thermal Cycler Bio-rad, USA). The amplified products were observed by electrophoresis using 1.5% agarose gels (Pronadisa, Spain) in Tris-Borate-EDTA buffer and stained with ethidium bromide. Gels were visualized and photographed using ultra-violet gel documentation system (UVP, Germany).

Table 3.3 List of sequenced-tagged site primers (Yoshikawa et al., 2003)

* Isolate B was axenic culture purchased from the Department of Microbiology, National University of Singapore, Singapore and was used as the positive control in all the PCR reactions.

Subtypes	STS primer sets	Product size (bp)	Sequence of forward (F) and reverse (R) primers (5'-3')	Source of primer
1	SB83	351	F:GAAGGACTCTCTGACGATGA R:GTCCAAATGAAAGGCAGC	Nand II
2	SB155	650	F:ATCAGCCTACAATCTCCTC R:ATCGCCACTTCTCCAAT	B*
3	SB227	526	F:TAGGATTTGGTGTTTGGAGA R:TTAGAAGTGAAGGAGATGGAAG	HV93-13
4	SB332	338	F:GCATCCAGACTACTATCAACATT R:CCATTTTCAGACAACCACTTA	HJ96AS-1
5	SB340	704	F:TGTTCTTGTGTCTTCTCAGCTC R:TTCTTTCACACTCCCGTCAT	HJ96-1
6	SB336	317	F:GTGGGTAGAGGAAGGAAAACA R:AGAACAAGTCGATGAAGTGAGAT	SY94-3
7	SB337	487	F:GTCTTTCCCTGTCTATTCTGCA R:AATTCGGTCTGCTTCTTCTG	RN94-9

3.9 Formal ether concentration technique of other intestinal parasites.

A pea size stool was mixed with 7ml formal saline in a 15ml falcon tube. The mixture was filtered into a new falcon tube using a gauze and formal saline was added until 7ml. Then 3ml of diethyl ether was layered on the formal saline and was inverted for 30 second. The mixture was then placed in a centrifuge machine and spun at 2000rpm for 10 minute. After 10 minute, the supernatant was discarded. The sediment was then mixed with a drop of iodine and viewed microscopically.

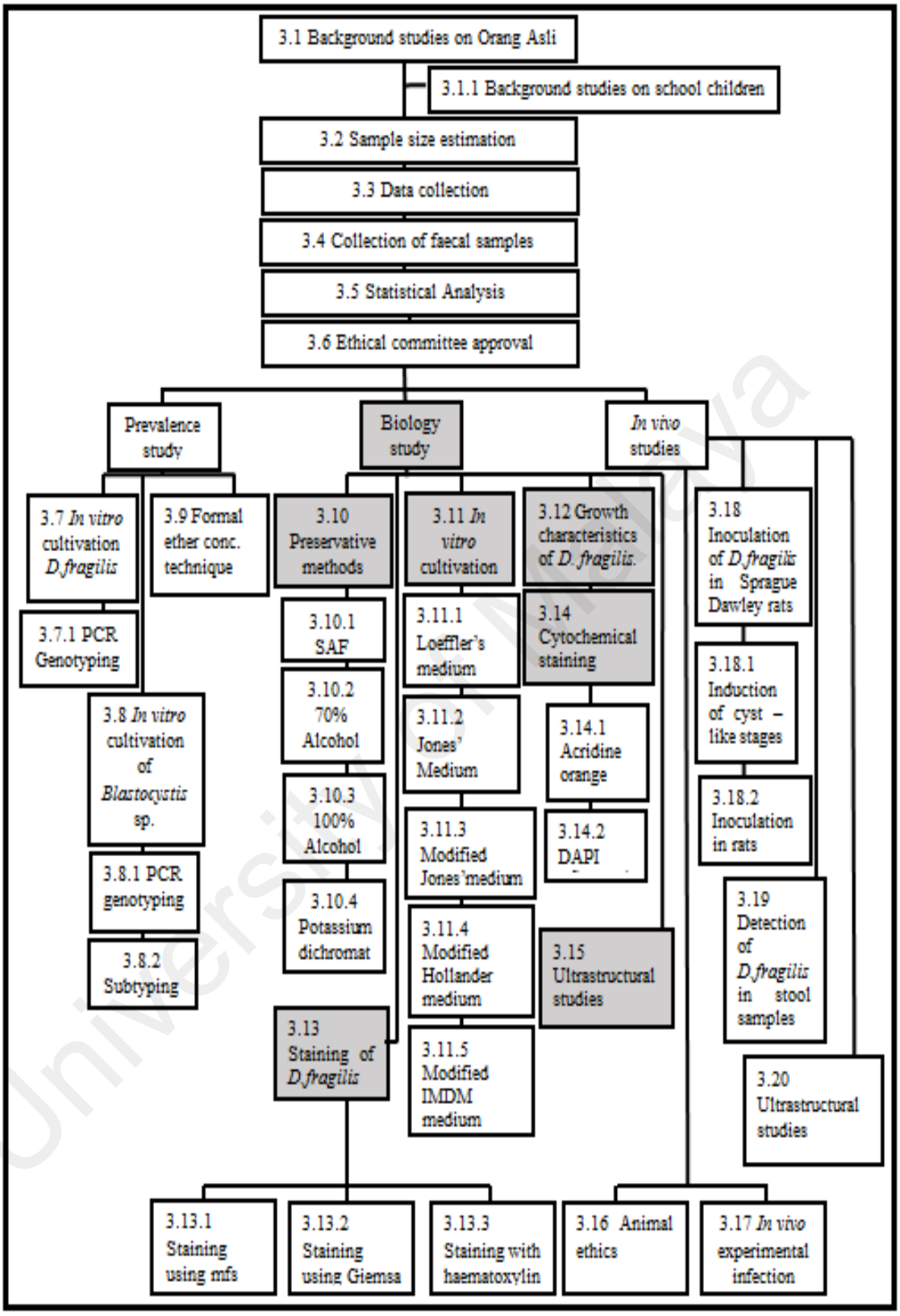

Figure 3.5 Schematic representation of the overall methodology to elucidate the biology of *D.fragilis.*

3.10 Preservative methods for *D.fragilis*

3.10.1 Sodium acetate-acetic acid formalin (SAF)

1.5g sodium acetate, 2.0ml glacial acetic acid and 7.4ml 20% formalin was added to 88.6ml distilled water and was mixed.

3.10.2 70% Alcohol

70ml ethanol was added to 30ml distilled water to prepare a 70% solution

3.10.3 100% alcohol

90ml ethanol was added to 10ml ethanol to prepare a 90% solution.

3.10.4 Potassium dichromate

2.0g potassium dichromate was dissolved in 40.0ml distilled water and 5M NaOH was added and adjust to pH 7.2 for the preparation of the potassium dichromate.

3.11 *In vitro* cultivation of *D.fragilis*

3.11.1 Loeffler's medium supplemented with 70% horse serum

(as described previously in 3.7).

3.11.2 Jones' medium supplemented with 10% Horse serum

Jones' medium supplemented with 10% Horse serum prepared as described previously in 3.8.

3.11.3 Modified Jones' medium supplemented with 10% Horse serum

Rice starch was added in the Jones' medium supplemented with 10% Horse serum prepared as described previously in 3.8.

3.11.4 Modified Hollander medium supplemented with 10% horse serum

Fresh stool samples was cultured in Hollander medium supplemented with 10% heat-inactivated horse serum (Gibco Laboratories, Life Technologies) at 37ºC and sub-cultured once every three days.

3.11.5 Modified Iscove's Modified Dulbecco's medium (IMDM) supplemented with 10% horse serum

Fresh stool samples were culture in IMDM medium supplemented with 10% horse serum. The medium was added with rice starch at 37^0c and sub-cultured once every three days.

3.12 Growth characteristics of *D.fragilis.*

3 parasite isolate were chosen for the study and for each isolate, experiment were done in triplicate. Parasites from the triplicates were pooled together from day 3 cultures to make a final concentration of $1x10^4$cells/ml in 15 ml falcon tube containing Loeffler's medium supplemented with 10 % horse serum, Jones' medium supplemented with 10% horse serum, Hollander medium supplemented with 10% horse serum and Iscove's Modified Dulbecco's medium (IMDM) supplemented with 10%horse serum. All cultures were incubated at 37ºC for up to 10 days. All experiments were done in triplicates. The *D.fragilis* count was carried out using haemocytometer chamber (Improved Neubauer, Hausser Scientific) with 0.4 % trypan blue dye exclusion (Sigma-Aldrich Corp. USA) as viability indicator. The parasite count was determined daily in cultures until it became non-viable. Only viable cells that did not take up trypan blue stain were counted.

3.13 Staining of *D.fragilis*

3.13.1 Staining using modified Fields' stain (Afzan et al., 2010)

Smeared slides taken from fresh stool sample and culture medium were air dried for 15 to 20 minutes at room temperature. Then, 8 drops of Fields' stain B (0.2% solution of eosin in methanol) were added and then 15 drops of Fields stain A were immediately added and slightly agitated for 20 second. A golden scum appeared once the slides were agitated. The slides were then rinsed under a stream of water for two second, air dried and mounted with DPX. The slides were then viewed under 400 x magnifications.

3.13.2 Staining using Giemsa stain (Afzan et al.,2010)

Smeared slides taken from fresh stool sample and culture medium were air dried at room temperature for 15 to 20 minutes .The slides were fixed with methanol for 1 minute and then stained with 10% Giemsa (Merck, Germany) in distilled water for 20 minute. The slides were rinsed under a stream of water for two second, air dried and mounted with DPX. The slides were then viewed under 400 x magnifications.

3.13.3 Staining with Iron haematoxylin

Samples taken from the culture medium were fixed with sodium acetate-acetic acid formalin (SAF) and was fixed on slide with a drop of Mayer albumin and was left to air dry for 30 minutes followed by routine staining with iron haematoxylin according to the staining protocol of World Health Organization (WHO) (Mondiale de la Santé, 1998). Slides were then mounted with DinButyl phthalate and Xylene (DPX) and viewed under 400x magnification.

3.14 Cytochemical staining of *D.fragilis*

3.14.1 Acridine orange staining method (Afzan et al., 2010)

Trophozoites of *D.fragilis* were stained with acridine orange solution. 5 ml of 0.1% acridine orange stock solution was diluted with 45 ml of phosphate buffered saline (PBS) pH 7.4 before use. Then, a drop of culture sediment containing trophozoites and a drop of diluted acridine orange was mixed carefully on a clean glass slide. The slide was viewed with a fluorescence microscope (Leitz Wetzlar, Germany) at 400X magnification.

3.14.2 DAPI (4',6-diamidino-2-phenylindole) staining method (Afzan et al., 2010)

A drop of the trophozoites was mixed in 0.5 µg/ml of DAPI (4',6-diamidino-2-phenylindole, Sigma) for 3 minutes at room temperature in the dark and observed under a fluorescence microscope and images were captured.

3.15 Ultrastructural studies (Transmission electron microscopy)

Culture containing trophozoite was collected from day 2 culture, washed three times using PBS pH 7.4 and centrifuged at 2000× g, for 5 min. The pelleted cells were re-suspended overnight in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer **,** pH 7.3 at 4^{o}C, washed thoroughly with cacodylate buffer and post-fixed for 30 min in 1% osmium tetroxide in cacodylate buffer. The fixed cells were dehydrated for 5 minutes in ascending series of ethanol (30%, 50%, 70%, 80%, 90% and 100%) and embedded in epoxy resin. Semithin sections were stained with toluidine blue. Ultrathin sections were cut using an ultramicrotome, contrasted with uranyl acetate and lead citrate and viewed using a transmission electron microscope (LEO Libra120).

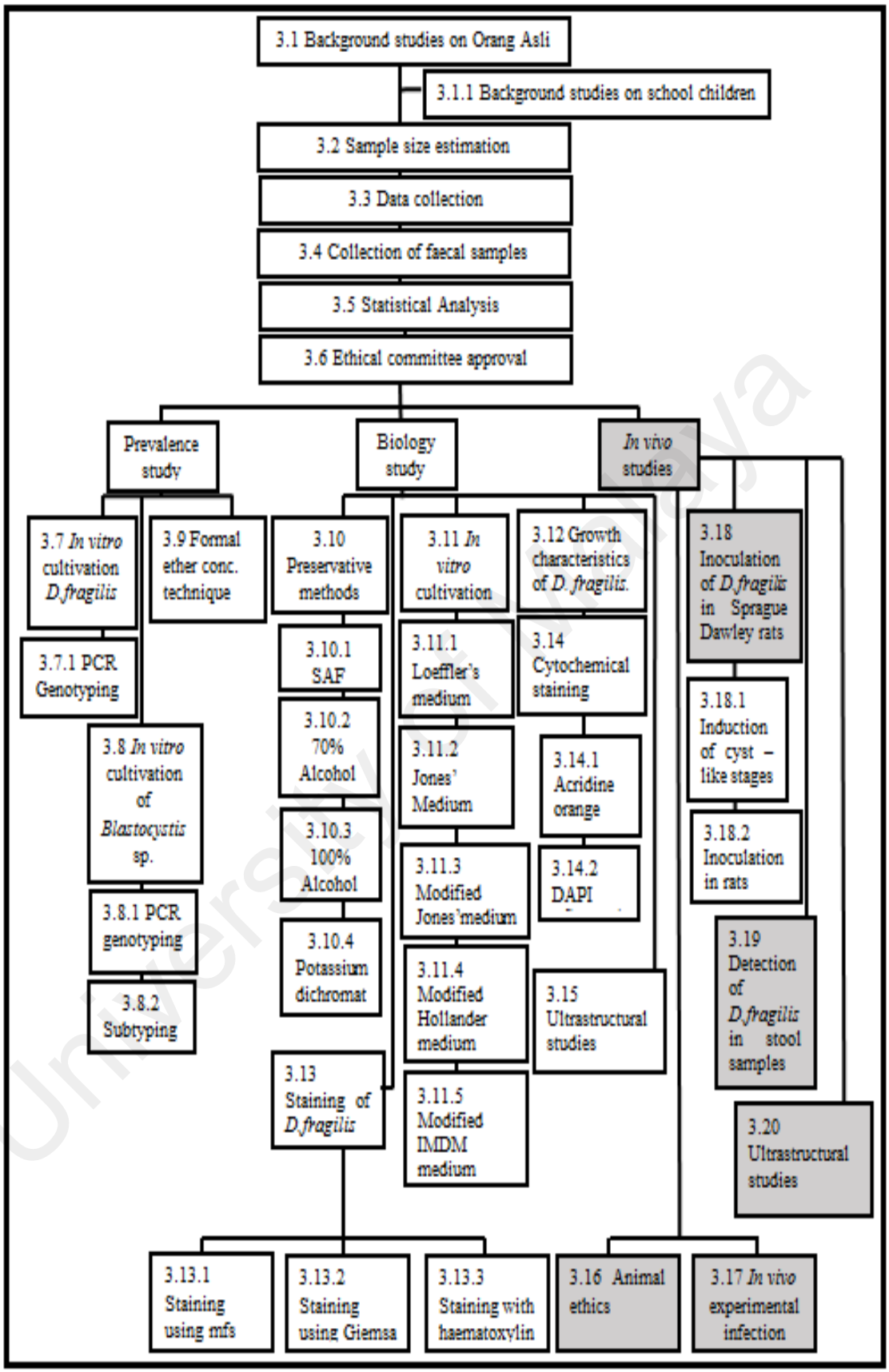

Figure 3.6 Schematic representation of the overall methodology highlighting aspects of the *in vivo* studies.

3.16 Animal ethics

The animal ethics was obtained from Institutional Animal Care and Use Committee (FOM IACUC). The approval number is 2014-04-01/PARA/R/ADR. (Appendix F).

3.17 *In vivo* experimental infection

A total of 36 rats previously confirmed negative for *D. fragilis* were divided into four groups. A cyst-like stage concentration was made up to three concentrations ie. $1X10^4$ cysts/ml, $1X10^5$ cysts/ml and $1X10^6$ cysts/ml respectively and was orally inoculated to each rat in the respective group. Control rats were fed with 1ml of PBS. The animals were maintained in the experimental room at the Department of Parasitology at room temperature and were fed with a normal diet of commercial pellets and water ad libitum.

3.18. Inoculation of *D.fragilis* in Sprague Dawley rats

3.18.1 Induction of cyst –like stages

Trophozoites of *D. fragilis* were collected from Loeffler's medium. The collected parasites were washed with distilled water to lyse trophozoites. The trophozoites that survived the distil water treatment were stained with Modified Fields' stain. These life cycle stages that survived the lysis were termed cyst-like stages.

3.18.2 Inoculation in rats

The cyst-like stages that survived the distilled water treatment were counted in varying doses between $1X10^4$ and $1X10^6$ trophozoites/ml and were inoculated using a 1.5 inch 20 G feeding needle and a ball diameter of 2.25 mm into three batches of rats. One batch of three rats were kept as control and were orally inoculated with phosphate buffer saline.

3.19 Detection of *D.fragilis* in rat fecal samples

3.19.1 Detection of *D.fragilis* using Loeffler's medium

Stool samples from all rats were checked for the parasite. Fecal samples were collected every 6 hour and cultured in Loeffler's medium supplemented with 10% horse serum. Loeffler medium supplemented with 70% horse serum were prepared as 3.7. The samples were microscopically examined.

3.19.2 Detection of *D.fragilis* using PCR

The stool samples of infected rats were subjected to PCR. All fecal sample of the rat are taken at every 6 hours and its DNA are extracted using the same method mention in 3.7.1 and continued with genotyping with PCR using pure *Taq* ready to go. The amplification products were electrophoresed in 1.0% agarose gels (Promega, USA) Tris-Borate-EDTA buffer. Gels were stained with ethidium bromide and photographed using ultra-violet gel documentation system (Uvitec, United Kingdom). The fragment sizes were confirmed with bands of a DNA length standard (100–1,000 bp DNA

markers, BioBasic). The PCR products were then purified using the QIAquick^{TM}PCR purification kit (QIAGEN) and sent for sequencing.

3.19.3 Counting of *D.fragilis* trophozoite

The trophozoite from fecal sample positive for *D.fragilis* were counted. The *D.fragilis* count was carried out using haemocytometer chamber (Improved Neubauer, Hausser Scientific) with 0.4 % trypan blue dye exclusion (Sigma-Aldrich Corp. USA) as viability indicator. The parasite count was done every 6 hour interval. All experiments were done in triplicates.

3.19.4 Staining of *D.fragilis* trophozoites

3.19.4.1 Staining of smears from rat fecal sample using Modified Fields' stain

The fecal sample of the rats taken at every 6 hours were stain with modified Fields' stain. The procedure of the stain are the same as 3.13.1

3.19.4.2 Staining of smears from rat fecal sample using Acridine orange

Using Acridine orange, the rat fecal sample were observed using acridine orange following the procedure as described in 3.14.1

3.19.4.3 Staining of smears rat fecal sample using DAPI stain

The fecal sample of rat at 6 hour interval was also viewed using the DAPI satin following the procedure as described in 3.14.2.

3.20 Ultrastructural studies on rat fecal sample

The fecal sample from rats positive for *D.fragilis* were collected and subjected to utrastructural studies following the procedure as stated in 3.15.

The experiment were repeated using Balb/c mice

CHAPTER 4

RESULT

4.1 To assess the prevalence of *D.fragilis* in Orang Asli communities.

4.1.1 Demographic characteristics

This comprehensive study involved 8 Orang asli villages from Selangor. The villages were Kampung Orang Asli Pangsun, Kampung Orang Asli Bukit Cheeding, Kampung Orang Asli Sungai Lalang Baru, Kampung Orang Asli Serendah, Rawang, Kampung Orang Asli Broga, Kampung Orang Asli Kemensah, Kampung Orang Asli Batu 12, Gombak and Kampung Orang Asli Tanjung Sepat. Although 832 questionnaire and fecal containers were distributed during the study, only 409 (49.2 %) participant turned up with their fecal samples on the following day. The response rate of the respective Orang Asli villages was as follows :- Kampung Orang Asli Bukit Cheeding (65.1%), Kampung Orang Asli Pangsun (56.3%), Kampung Orang Asli Serendah (55.3%), Kampung Orang Asli Tanjung Sepat (52.8 %), Kampung Orang Asli Batu 12 (46.7%), Kampung Orang Asli Sungai Lalang Baru, (35.6%) Kampung Orang Asli Broga (35.2 %), and Kampung Orang Asli Kemensah (30.3%) (Table 4.1). With regards to the age groups, 259 children (age between 1 to 18 years old) and 150 adult (age between 18 to 68 years old) participated in this study. The participants consisted of 168 (41.1 %) male and 241 (59 %) female (Table 4.2).

4.1.2 Socioeconomic characteristics

A total of 81 (31.3%) children out of 259 children obtained primary education while 61 (23.6 %) had secondary education. In the adult group, 67 (44.7%) had primary education while 36 (24.0 %) had secondary education with 39 (26.0%) did not go to school at all (Table 4.3). 75 (50.0 %) ladies were housewives while 27 (18.0 %) worked as farmers. A total of 22 (14.7%) were factory workers (Table 4.3).

The household income of 347 participants (84.8%) were more than RM1000 while the 62 (15.2%) showed less than RM1000 (Table 4.4). The number household for more than five people per house is higher (266, 65%) than less than five people per household (143, 35%).There were more people using piped water 314,(76.8%) than river water 91(22.2%) (Table 4.6). 399 (97.6%) houses were seen to have toilet whilst 383 (93.6%) of the people interviewed said that they defecated in the toilet. 94 persons (23.0%) had pets and 121 (29.6%) have close contact with pets which means that they frequently play and have close proximity with their pets. 19 (4.6 %) said they wash and clean their own respective pet animals. 295 (72.1%) stated that they eat with their hand. A total of 115 (28.1%) stated that they do not wear slippers. 292 (71.3 %) stated that they do wash hand after playing with soil, however most of them, 371 (90.7%) confessed that they do not wash their hands after playing or patting their pet animals (Table 4.6). A total of 119 (29.6%) have symptoms (Table 4.7) while 215 (52.6%) frequented the toilet between one to twice a day (Table 4.8).

4.1.3 Prevalence of Orang Asli infected with *D.fragilis*

16 (3.9 %) of the Orang Asli participants were positive for *D.fragilis* (Table 4.9). Multivariate analysis (Table 4.10) demonstrated that participants whose ages were less than 18 years (OR=9.360, 95% CI=1.224,71.580), showed association with life stock (OR=0.868,CI=0.809-0.930), did not wash hands after handling animals (OR=6.769, CI=2.311-19.827), showed gastrointestinal symptoms (OR=3.308,CI=1.202-9.100) and showed greater frequency of visits to the toilet per day (OR=0.288, CI=0.091-0.907) were identified as significant ($p<0.05$) risk factors for acquiring *D. fragilis* in these communities (Table 4.10)

Symptoms associated with *D.fragilis* in the study were shown to be abdominal pain (5, 1.2%), diarrhoea (2, 0.5%) and a mixed symptoms of abdominal pain with weight loss (2, 0.5%) (Table 4.10). *D.fragilis* was significantly associated with the presence of *Blastocystis* sp. (16, 100%) (Table 4.11)

202 (49.4%) of the Orang Asli participants were positive for *Blastocystis* sp. Subtype 3 with total number of 96 (23.5%) was seen to be the most predominant compared to subtype 1 with a total of 92 (22.5%) and subtype 5 with total number of 14 (3.4%).

Persons infected with *Trichuris trichuira* (134, 32.8 %) showed a higher number when compared with infection of other parasites such as *Giardia lamblia* (15,3.7%), *Entamoeba histolytica* (67,16.4%), *Ascaris lumbricoides* (89,21.8 %) and *hookworm* (42,10.3%) infections . 22, 5.4% of persons infected showed multiple infections (Table 4.11).

Table 4.1: Response rate of the Orang Asli villagers who participated.

Orang Asli villages	**Number of distributed questionnaire and stool container**	**Individual who responded**	
		No.	**%**
Kampung Orang Asli Pangsun	158	89	56.3
Kmpung Orang Asli Bukit Cheeding	109	71	65.1
Kampung Orang Asli Tanjung Sepat	125	66	52.8
Kampung Orang Asli Serendah	94	52	55.3
Kampung Orang Asli Batu 12	105	49	46.7
Kampung Orang Asli Sungai Lalang Baru	90	32	35.6
Kampung Orang Asli Broga	85	30	35.2
Kampung Orang Asli Kemensah	66	20	30.3
Total	832	409	49.2

Table 4.2: Distribution of age and gender of those who participated.

Age group	Age (year)	Gender		Total	
		Male	**Female**	**No.**	**%**
Children	1-4	15	14	29	7.1
	5-6	40	48	88	21.5
	7-12	19	37	56	13.7
	13-17	32	54	86	21.0
	Subtotal	106	153	259	63.3
Adult	>18	62	88	152	36.7
	Subtotal	62	88	150	36.7
Total		**168 (41.1%)**	**241 (59%)**	**409**	

Table 4.3: Distribution of levels of education and occupation in children and adults

Variables	Children N= 259		Adults N= 150	
	No.	**%**	**No.**	**%**
Level of education				
Informal	117	45.2	39	26.0
Primary school	81	31.3	67	44.7
Secondary school	61	23.6	36	24.0
Tertiary education	0	0	8	5.3
Occupational status				
Toddler/pre-school	116	44.8	0	0
Student	143	55.2	9	6.0
Housewife/not working	0	0	75	50.0
Rubber plantation/farmer	0	0	27	18.0
Unskilled labourers (Factory worker)	0	0	22	14.7
Government employees	0	0	3	2.0
Others	0	0	14	9.3

N: number examined

Table 4.4: Household income (RM per month) of the Orang Asli

Variables	**Total**	
	No.	**%**
Household income (RM/month)		
< RM 1000	347	84.8
>RM 1000	62	15.2

Table 4.5: Number of household of the Orang Asli

Variables	**Total**	
	No.	**%**
Number of household		
< 5	143	35
>5	266	65

Table 4.6: Environmental sanitation and personal hygiene habits of Orang Asli members

Variables	**Total**	
	No.	**%**
Source of water supply		
Water pipe	314	76.8
River	91	22.2
Well	4	1.0
Presence of toilet		
Yes	399	97.6
No	10	2.4
Defecation places		
Toilet	383	93.6
Others (bush, pit, river)	26	6.4
Having pets/livestock		
Yes	94	23.0
No	315	77.0
Close contact with pets/livestock		
Yes	121	29.6
No	288	70.4
Animal cleaning		
Yes	19	4.6
No	390	95.4

Table 4.6 (continued)

Variables	**Total**	
	No.	**%**
Eating using hand		
Yes	295	72.1
No	114	27.9
Wear slippers/ shoe outdoor		
Yes	294	72.0
No	115	28.1
Wash hand before/ after play with soil		
Yes	292	71.3
No	117	28.6
Wash hand after play with animal		
Yes	38	9.3
No	371	90.7

Table 4.7: Symptom associated with the Orang Asli

Variables	**Total**	
	No.	**%**
Symptoms		
Yes	119	29.1
No	290	70.9

Table 4.8: Frequency to the toilet for the Orang Asli

Variables	**Total**	
	No.	**%**
Symptoms		
1-2	215	52.6
3-4	194	47.4

Table 4.9: Prevalance of *D.fragilis* infection to the respective Orang Asli villagers

Orang Asli villages	**N**	***D.fragilis* infection**	
		No.	**%**
Kampung Orang Asli Pangsun	89	6	6.7
Kampung Orang Asli Bukit Cheeding	71	0	0
Kampung Orang Asli Tanjung Sepat	66	3	4.5
Kampung Orang Asli Serendah	52	3	5.7
Kampung Orang Asli Batu 12	49	2	4.1
Kampung Orang Asli Sungai Lalang Baru	32	0	0
Kampung Orang Asli Broga	30	0	0
Kampung Orang Asli Kemensah	20	2	10
Total	409	16	3.9

N: number examined

Table 4.10: Univariate analysis of the potential risk factors with *D.fragilis* Infections

Variables	N	*D. fragilis* infection		OR (95% CI)	*p*-value
		n	%		
Gender					
Male	168	4	25.0	0.465(0.147,1.469)	0.182
Female	241	12	75.0		
Age group					
<18 years old	257	15	93.8	9.360(1.224,71.580)	0.009
>18 years old	152	1	6.3		
Education level					
Informal education	158	7	43.8	1.247(0.455,3.417)	0.668
Formal education	251	9	56.3		
Monthly income					
< RM 1000	347	16	100	0.954(0.932,0.976)	0.085
>RM 1000	62	0	0		
Number of households					
< 5 persons	143	2	12.5	0.255(0.057,1.140)	0.055
> 5 persons	266	14	87.5		
Water sources					
Filtered	311	16	100	0.949(0.924,0.973)	0.022
Not filtered	98	0	0		

Table 4.10 (continued)

Variables	N	*D. fragilis* infection		OR (95% CI)	*p*-value
		n	%		
Defecation					
Toilet	399	14	87.5	0.145 (0.028,0.749)	0.008
Others	10	2	12.5		
Bath					
River	19	0	0	1.043 (1.022,1.064)	0.368
Bathroom	390	16	100		
Any livestock					
Yes	94	4	25.0	1.122 (0.353,3.565)	0.845
No	315	12	75.0		
Livestock association					
Yes	121	16	100	0.868 (0.809,0.930)	0.000
No	288	0	0		
Animal cleaning					
Yes	19	1	6.3	1.389 (0.174,1.103)	1.389
No	390	15	93.8		
Eat using hands					
Yes	295	11	68.8	0.844 (0.287,2.486)	0.759
No	114	5	31.3		

Table 4.10 (continued)

Variables	N	*D.fragilis* infection		OR (95% CI)	*p*-value
		n	%		
Wear slippers/shoes outside					
Yes	294	11	68.8	0.855 (0.290,2.518)	0.776
No	115	5	31.3		
Wash hands before/after play with soil					
Yes	292	12	75.0	1.211 (0.382,3.833)	1.211
No	117	4	25.0		
Wash hands after play with animals					
Yes	38	6	37.5	6.769 (2.311,19.827)	0.000
No	371	10	62.5		
Symptom					
Yes	119	9	56.3	3.308 (1.202,9.100)	0.015
No	290	7	43.8		
Frequency of going to toilet					
1-2	215	4	25.0	0.288 (0.091,0.907)	0.024
3-4	194	12	75.0		

Table 4.11: Association of *D.fragilis* with other intestinal parasites.

Name of intestinal parasite	Percentage of positive sample	
	N	Percentage (%)
Blastocystis sp.	16	100
Entamoeba histolytica	6	37.5
Trichurius trichiura	5	31.3
Ascaris lumbricoides	3	18.8
Hookworm	2	12.5

N: number examined

4.1.4 Gel electrophoresis

The genomic DNA of *D.fragilis* was isolated from fresh stool samples and subjected for PCR amplification using Pure *Taq* Ready To Go to determine the positive sample. Out of 409 samples, 16 were found to be positive as evidenced by the agarose gel picture (Figure 4.1) and (Figure 4.2)

Figure 4.1 Agarose gel image of *D.fragilis* Lane M= DNA markers of 100bp plus DNA ladder (Fermentas, USA); lane 1 till lane 9 show 9 positive band at 1000bp.

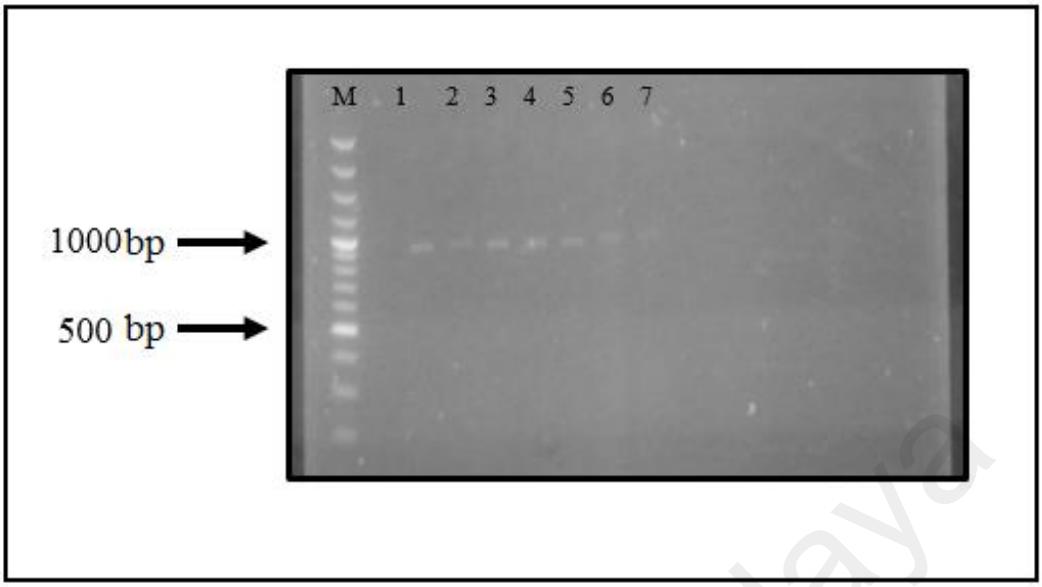

Figure 4.2 Agarose gel image of *D.fragilis* Lane M= DNA markers of 100bp plus DNA ladder (Fermentas, USA); lane 1 till lane 7 show 7 positive band at 1000bp

4.1.5 Demographic and socioeconomic characteristics for school children

A total of 1784 stool container was distributed to 12 schools i.e seven rural and five urban schools. Out of 1784 stool cup distributed, 907 stool cups was returned (Table 4.12). Based on table 4.13, in the age group between 7 and 9, 132 and 105 of the children were males and females respectively. Between the age group 10 and 12, 380 and 290 of the children were males and females respectively. Parents of children from the rural schools revealed that the fathers' level of education, 152 (28.8 %) had only primary school education while 375 (71.2%) had secondary education. Parents from the urban schools however showed 365 fathers, (96.1%) had secondary education with only 15 (3.9 %) of them with primary education (Table 4.13).

354 of the mothers from the rural schools, had secondary education (67.2%) while 173 (32.8%) mothers had primary school education. 363 mothers from the urban schools (95.5%) had secondary education whilst 17 (4.5%) mothers had only primary education.

429 (47.3%) parents hade household income of more than RM 2000, while 478 (52.7%) stated that their household income was less than RM2000 (Table 4.15). Based on table 4.16, 761 persons (83.9%) showed no gastrointestinal symptom while 146 (16.1%) had symptoms such as stomach ache, stomach bloating and diarrhoea.

4.1.6 Prevalance of *D.fragilis* infection among school children

7 (0.7%) school children from rural schools were found positive for *D.fragilis* (Table 4.17). None of the urban children were found positive for *D.fragilis*. Multivariate analysis demonstrated that female (OR= 0.127,CI=0.015-1.058), ages less than 9 years old (OR=7.198, CI=1.387-37.354), mother's education level up to primary school (OR=5.188,CI=1.151-23.385), Rural area (OR=0.987,CI=0.977-0.997), type of stools (OR=1.039,CI= I.010-1.068) and gastrointestinal symptoms OR=1.050,CI=1.031-1.089) were identified as significant ($p<0.05$) risk factors for acquiring *D.fragilis* among the school children. Symptoms associated with *D.fragilis* were abdominal pain (6, 0.7%) and diarrhoea (1, 0.1%) (Table 4.18). *D.fragilis* showed higher association with *Blastocystis* sp. (7,100%) (Table 4.19).

84 (9.26%) of the school children positive for *Blastocystis* sp showed subtype 3 (54 (6.0%)) to be predominant ones with followed by subtype 1 (16, 1.8%), subtype 2 (7, 0.8%), subtype 4 (4,0.4) and subtype 5 (3,0.3%).

School children infected with *Trichuris trichuira* (67, 7.4%) showed higher number when compared with infection of other parasites such as the *Giardia lamblia* (8,0.9%), *Entamoeba histolytica* (34,3.7 %), *Ascaris lumbricoides* (44,4.9 %), and hookworm (20,2.2%).

Table 4.12: Response rate of the School children who participated.

Area	List of schools	Number of distributed questionnaire and stool container	Individual who responded	
			No.	**%**
Rural	SK Pangsun, Hulu Langat	155	92	17.45
	SK Lebuk Kelubi, Hulu Langat	187	86	16.31
	SJK(T) Highland, Klang	120	85	16.13
	SK Sg Serai, Hulu Langat	112	61	11.60
	SK Dato Abu Bakar Baginda,Kajang	140	90	17.10
	SK Bukit Tandom	150	53	10.10
	SK Bukit Changgang	135	60	11.38
	Total		527	
Urban	Sekolah Rendah Jenis Kebangsaan Vivekananda	150	79	20.78
	Sekolah Rendah Jenis Kebangsaan Batu Caves	165	82	21.57
	SK Methodist, PJ	125	66	17.36
	SK Bandar Rahman Putra, Sg Buloh	160	76	20.00
	SK Sri Garing	185	77	20.26
	Total	1784	380	

Table 4.13: Distribution of age and gender amongst school children who participated.

Age group	Age (year)	Gender		Total	
		Male	**Female**	**No.**	**%**
Children	7-9	132	105	237	26.1
	10-12	380	290	670	73.9
Total		**512(56.4%)**	**395 (43.6%)**	**907**	

Table 4.14: Distribution highlighting the educational level of Father and Mother of school children.

Variables	Rural N= 527		Urban N= 380	
	No.	**%**	**No.**	**%**
Level of father's education				
Primary school	152	28.8	15	3.9
Secondary school	375	71.2	365	96.1
Total	**527**		**380**	
Level of mother 's education				
Primary education	173	32.8	17	4.5
Secondary education	354	67.2	363	95.5
Total	**527**		**380**	

N: number examined

Table 4.15: Household income (RM per month) of parents of the school children

Variables	**Total**	
	No.	**%**
Household income (RM/month)		
< RM 2000	429	47.3
>RM 2000	478	52.7

Table 4.16: Complaints of stomach ache in school children

Variables	**Total**	
	No.	**%**
Symptoms		
Yes	146	16.1
No	761	83.9

Table 4.17: Prevalence of *D. fragilis* infection in school children.

Area	List of schools	N	*D. fragilis* infection	
			No.	%
Rural	SK Pangsun, Hulu Langat	92	4	4.3
	SK Lebuk Kelubi, Hulu Langat	86	3	3.4
	SJK(T) Highland, Klang	85	-	-
	SK Sg Serai, Hulu Langat	61	-	-
	SK Dato Abu Bakar	90	-	-
	SK Bukit Tandom	53	-	-
	SK Bukit Changgang	60	-	-
	Total	527	7	0.7
Urban	Sekolah Rendah Jenis Kebangsaan Vivekananda	79	-	-
	Sekolah Rendah Jenis Kebangsaan Batu Caves	82	-	-
	SK Methodist, PJ	66	-	-
	SK Bandar Rahman Putra, Sg Buloh	76	-	-
	SK Sri Garing	77	-	-
	Total	380		

Table 4.18: Univariate analysis of the potential risk factors with *D. fragilis* infections

Variables	N	*D.fragilis* infection		OR (95% CI)	p-value
		n	%		
Gender					
Male	512	1	14.3	0.127(0.015,1.058)	0.024
Female	395	6	85.7		
Age group					
7-9	237	5	71.4	7.198(1.387,37.354)	0.006
10-12	670	2	28.6		
Father's Education level					
Primary school	167	3	42.9	3.366(0.746,15.183)	0.094
Secondary school	740	4	57.1		
Mother's Education level					
Primary school	188	4	57.1	5.188(1.151,23.385)	0.017
Secondary school	719	3	42.9		
Household income					
< RM 2000	429	5	71.4	2.807(0.542,14.542)	0.199
>RM 2000	479	2	28.6		

Table 4.18 (continued)

Variables	N	*D.fragilis* infection		OR (95% CI)	p-value
		n	%		
Area					
Rural	527	7	100	0.987(0.977,0.997)	0.024
Urban	380	0	0		
Stool type					
Normal	719	0	0	1.039(1.010,1.068)	0.000
Abnormal	188	7	100		
Symptoms					
Yes	146	7	100	1.050(1.031,1.089)	0.000
No	761	0			

Table 4.19: Association of *D.fragilis* with other intestinal parasites.

Name of intestinal parasite	Percentage of positive sample	
	N	Percentage (%)
Blastocystis sp.	7	100
Entamoeba histolytica	4	57
Trichuris trichiura	1	14.3
Ascaris lumbricoides	1	14.3
Hookworm	1	14.3

N: number examined

4.1.7 Gel electrophoresis

The genomic DNA of *D.fragilis* was isolated from fresh stool sample and subjected for PCR amplification using Pure *Taq* Ready To Go to determine the positive sample. Out of 409 sample, 16 sample was positive. This is depicted clearly through the agarose gel picture (Figure 4.3)

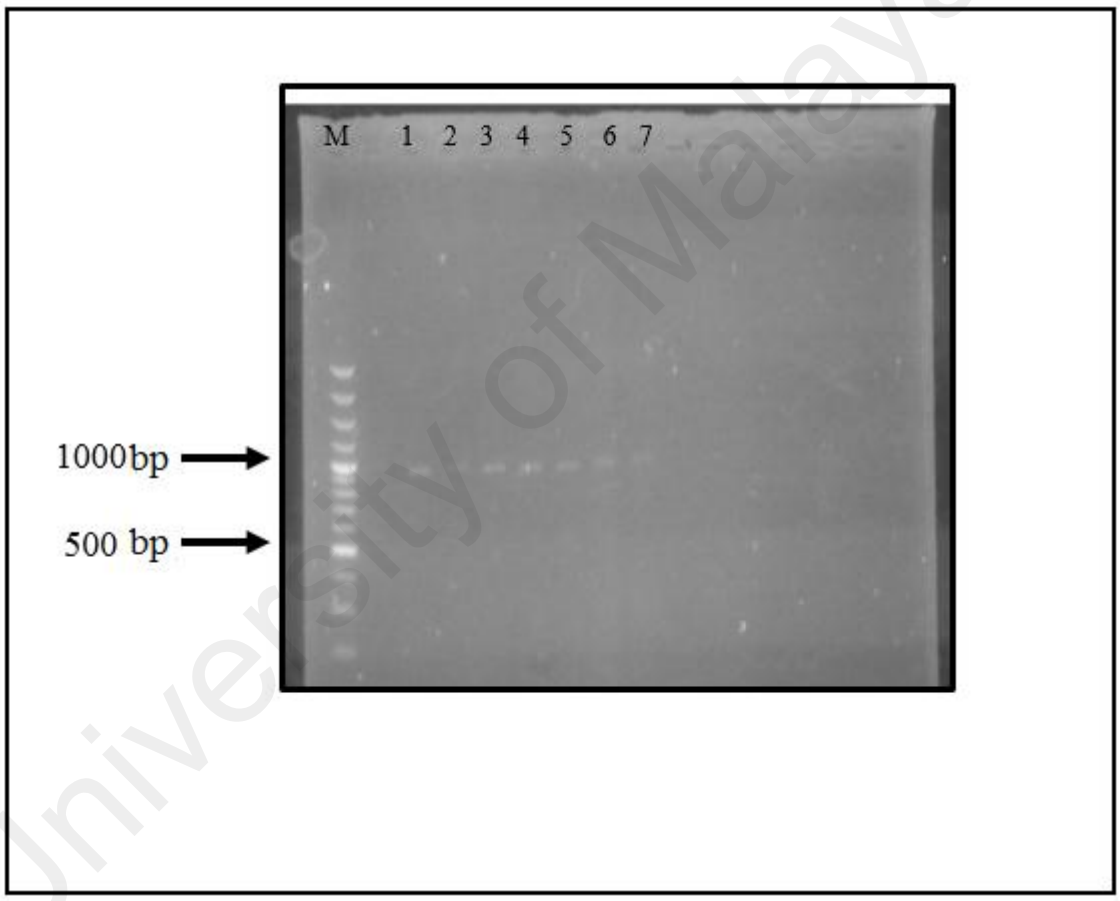

Figure 4.3 Agarose gel image of *D. fragilis* Lane M= DNA markers of 100bp plus DNA ladder (Fermentas, USA); lane 1 till lane 7 show 7 positive band at 1000bp.

4.2.1 Various types of preservatives to fix *D.fragilis*

Four types of preservatives were used to fix *D.fragilis*. The four preservatives used were sodium acetate-acetic acid formalin (SAF), 70 % alcohol, 100 % alcohol and potassium dichromate. However, only two preservative were able to fix *D.fragilis* which is the SAF (Figure 4.4) and potassium dichromate (Figure 4.5). The trophozoites in both preservatives were found intact and did not degrade even after 12 months showing that both the preservative were suitable to fix *D. fragilis* for long term.

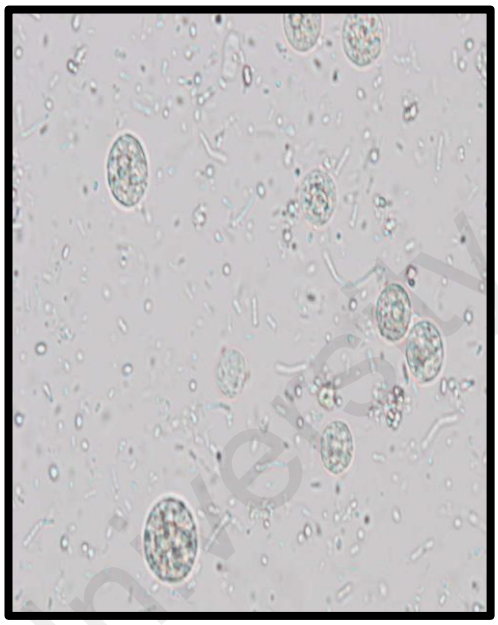

Figure 4.4: Trophozoites *D.fragilis* in SAF preservative. The size and shape of the trophozoites were shown to be still intact.

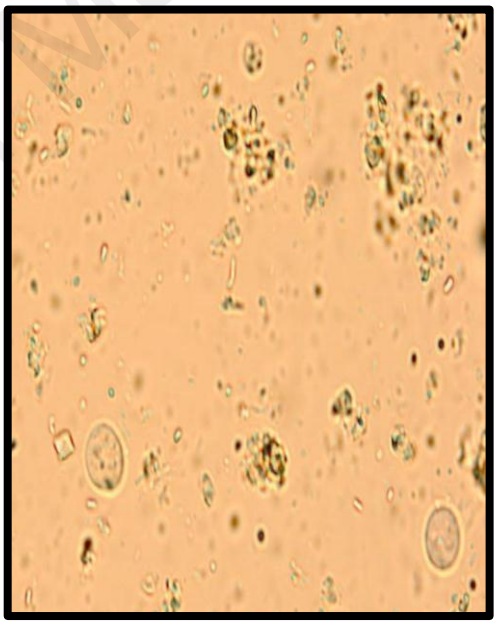

Figure 4.5: Trophozoites of *D. fragilis* in potassium dichromate preservative. The size and shape of the trophozoites were shown to be intact

4.2.2 Cultivation of *D. fragilis* using various *in vitro* culture medium.

A total of four culture media were used to culture *D.fragilis* i.e. Loeffler's medium supplemented with 10% horse serum and rice starch, Hollander medium supplemented with 10% horse serum and rice starch, IMDM medium supplemented with 10% horse serum and rice starch and Jones' medium supplemented with 10% horse serum and rice starch. The medium that supported the growth of *D. fragilis* were Loeffler's medium supplemented with 70% horse serum and rice starch as well as Jones' medium supplemented with 10% horse serum and rice starch (Figure 4.6).

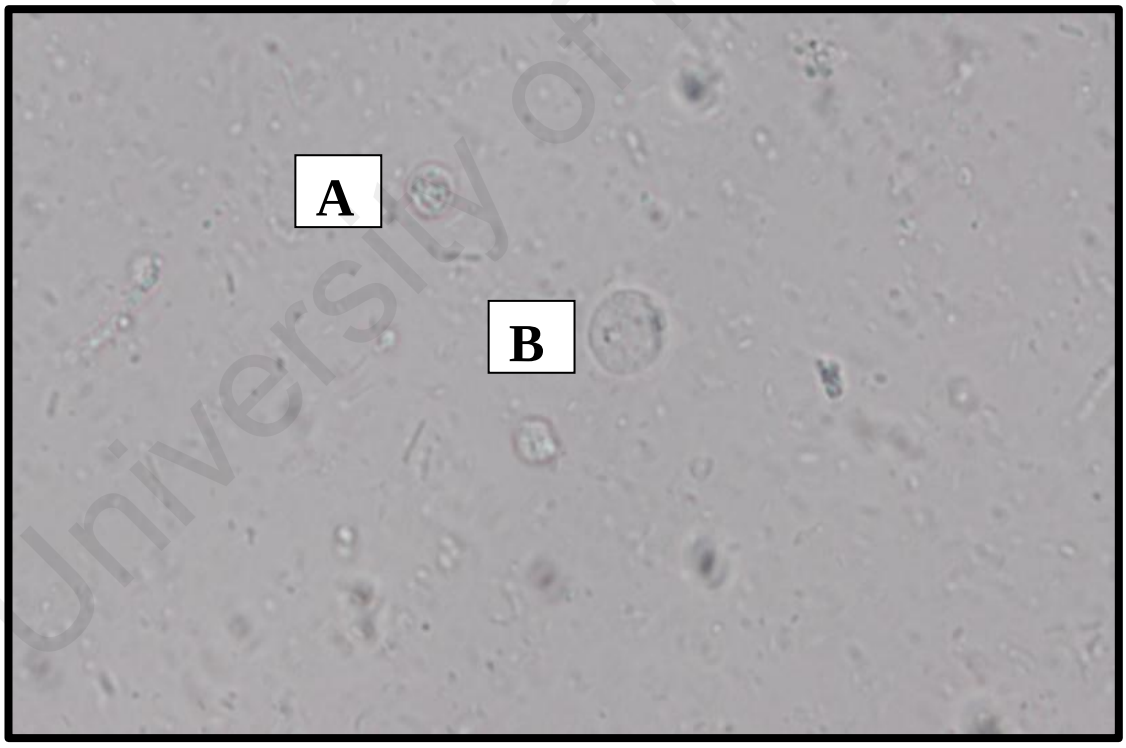

Figure 4.6: (A) *D. fragilis* and (B) *Blastocystis* sp. in Jones' medium supplemented with 10 % horse serum and rice starch.

4.2.2.1 Growth profile of *D.fragilis* in Loeffler's and Jones' medium

The growth profile was carried out using both this media and the results showed that Loeffler's medium had an average count of $5.55X10^4$ while Jones' medium showed a parasite of count of $3.3X10^4$ on day 2 culture (Figure 4.7). Trophozoites of *D.fragilis* did not grow in Hollander medium and in IMDM medium.

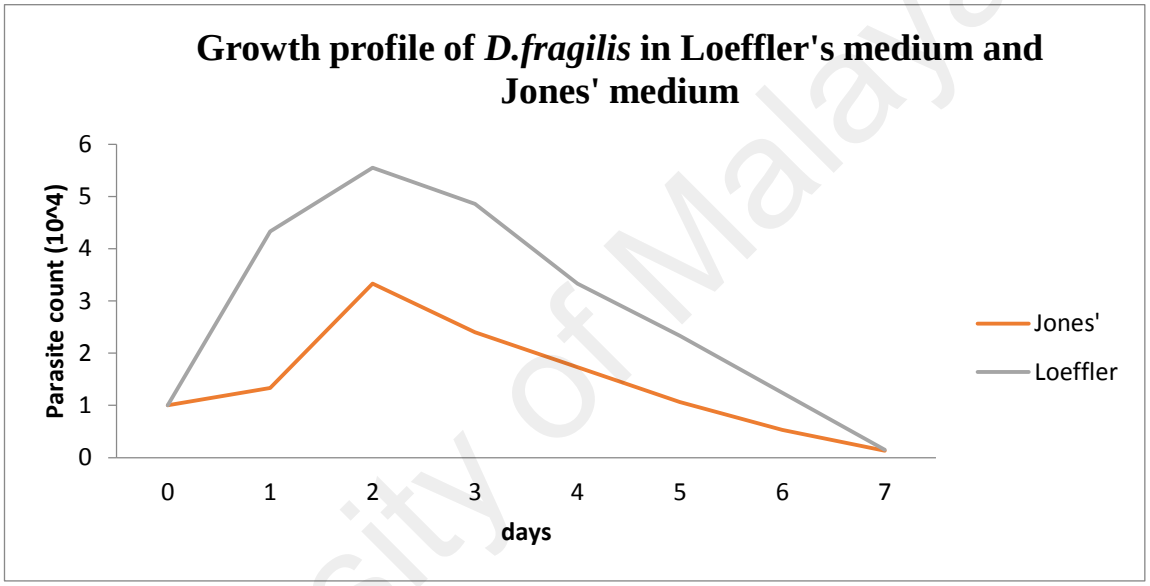

Figure 4.7: Growth profile of *D. fragilis* in Loeffler's medium and Jones' medium

4.2.3 Differentiating *D. fragilis* and *Blastocystis* sp. using staining method.

4.2.3.1 Differentiating *D.fragilis* and *Blastocystis* sp. using Iron haematoxylin, Modified Fields' stain and Giemsa stain

Iron haematoxylin stain which is the recommended stain to stain *D.fragilis* provides clear contrast of the nucleus and the cytoplasm (Figure 4.8E). However, the staining characteristics of *Blastocystis* sp. especially the peripheral rim of cytoplasm containing the nuclei was not that obvious using this stain (Figure 4.8F). Giemsa stained the cytoplasm of both parasites darkish purple; however, the contrast was not evident, sometimes misleading the identification with debris (Figure 4.8C and D). Modified Fields' stain revealed in *D.fragilis* clear bi-nucleated form as in Figure 4.8A. The nucleus was seen to be more prominent whilst the cytoplasm reflected a better contrast when compared to Giemsa staining. The peripheral rim of the cytoplasm containing the nucleus and the outer membrane were clearly stained with modified Fields' stain as in the case of *Blastocystis* sp. (Figure 4.8B)

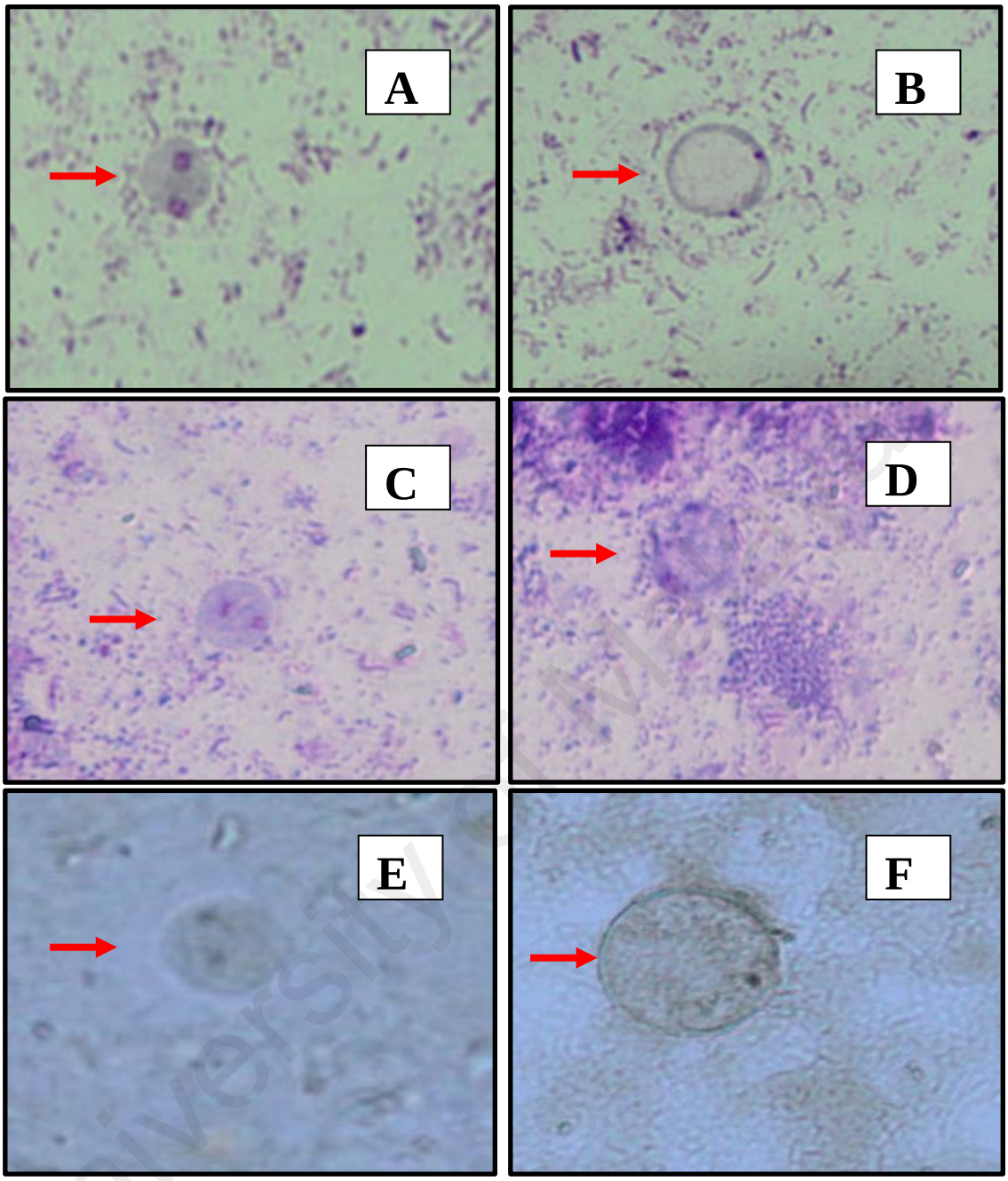

Figure 4.8: Trophozoite of *D.fragilis* seen with different staining. A trophozoite of *D.fragilis* stain with modified Fields' stain viewed under oil immersion microscopy (A) and, a *Blastocystis* sp. which clearly demonstrating its peripheral membrane stained with modified Fields' stain (B). A trophozoite of *D. fragilis* stain with Giemsa stain viewed under oil immersion microscopy (C) and, a *Blastocystis* sp. stain with Giemsa stain (D). (E) and (F) both show *D.fragilis* and *Blastocystis* sp. stain with iron haematoxylin.

4.2.3.2 Differentiating *D.fragilis* and *Blastocystis* sp. using cytochemical stain ,4'6,-diamidino-2-phenylindole (DAPI) and Acridine orange.

DAPI (Figure 4.9) and Acridine orange stain (Figure 4.10) were used to differentiate *D. fragilis* and *Blastocystis* sp. *Dientamoeba fragilis* and *Blastocystis sp.* could be differentiated in a mixed culture as the number of nucleus in both parasites became a contrasting feature to form a basis for this differentiation. Nucleus of *D. fragilis* were seen to be nearer to each other and located in the middle of the cytoplasm (Figure 4.9B & 4.10B) while the nucleus of *Blastocystis* sp. was seen to be far apart and located at the peripheral end of membrane (Figure 4.9D & 4.10D).

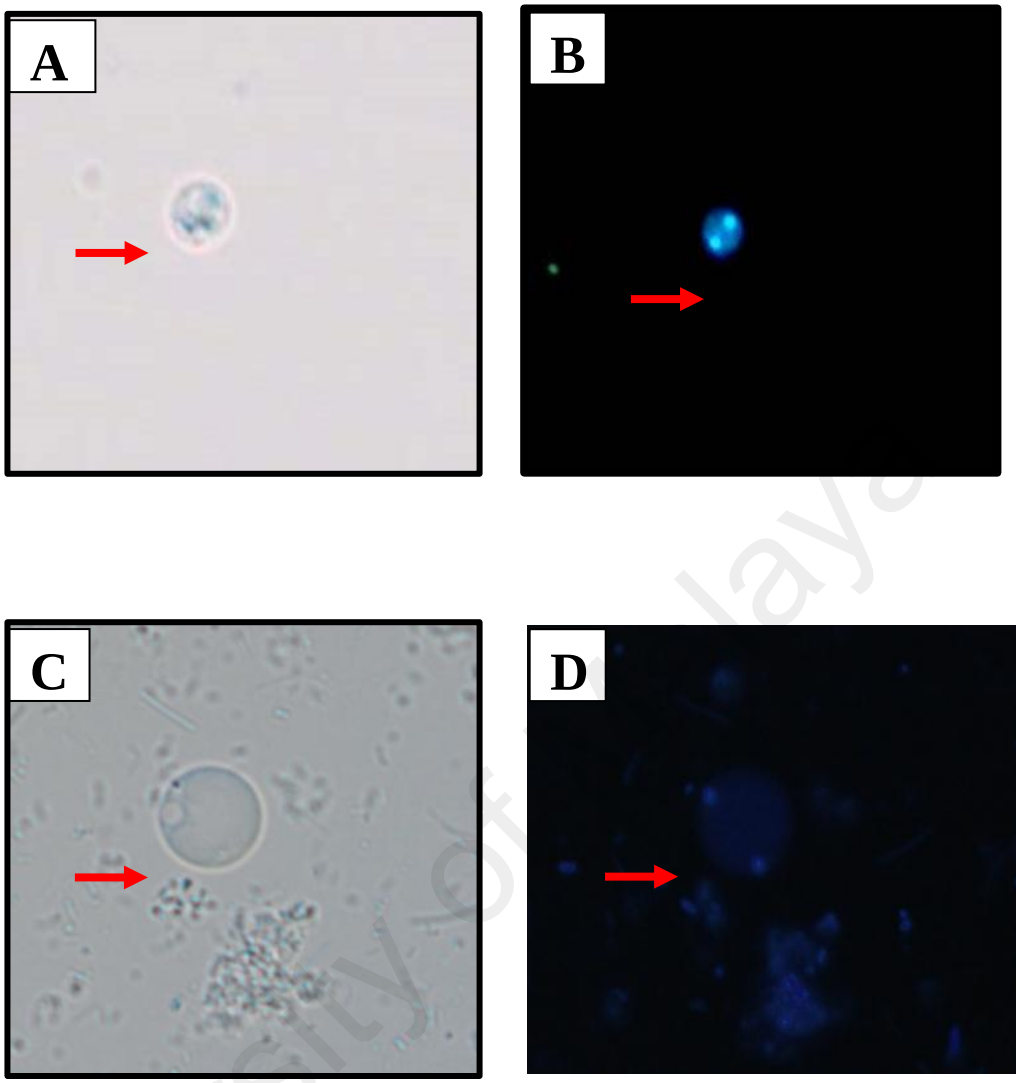

Figure 4.9: *D.fragilis* and *Blastocystsis* sp. stained with DAPI. (A)A trophozoite of *D. fragilis* observed under bright field and,(B) a *D. fragilis stain* with DAPI which clearly demonstrating its nucleus in the middle .(C) *Blastocystis* sp. observed under bright field and (D) in nucleus of *Blastocystis* sp. seen at the both end of the peripheral.

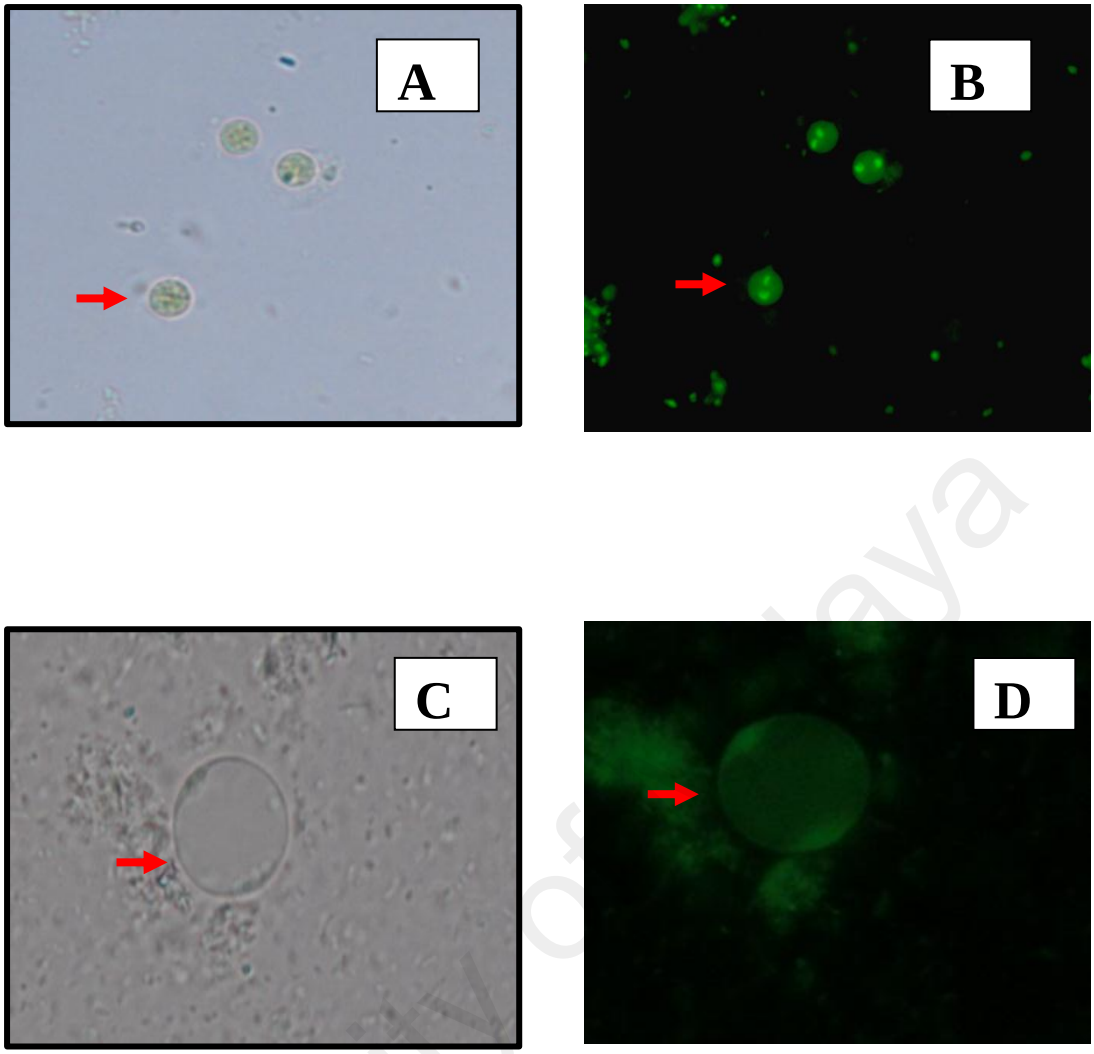

Figure 4.10: *D.fragilis* and *Blastocystsis* sp. stained with Acridine orange (x400). (A) Trophozoite of *D. fragilis* observed under bright field and (B) *D.fragilis* stained with acridine orange which clearly demonstrates its nucleus in the middle. (C) *Blastocystis* sp. observed under bright field and (D) nucleus of *Blastocystis* sp. seen at the both end of the peripheral**.**

4.2.4 Life cycle of *D. fragilis*

4.2.4.*1 In vitro* growth profile

A growth profile study was carried out on *D. fragilis* at 6 hours interval for 6 days (144 hours). The highest peak was seen at the 18^{th} hour which showed a parasite count of $6.93X10^4$ /ml. It showed a 6- fold growth (Figure 4.11) .The experiment was repeated where parasites from cultures were harvested on the 12^{th}, 18^{th} and 24^{th} hour and a parasite count of $1X10^4$ /ml was respectively made from each of the three time period. A continuous growth count was made every day to assess which time of harvest yielded the highest sustainable parasite count. The harvest again proved that at the 18^{th} hour, viable growth of parasites lasted for 8 days with a peak recorded at $9.60X10^4$/ml followed by harvest from 12^{th} hour which lasted for 4 days with a peak $2.67X10^4$ /ml. Harvest from 24^{th} hour culture lasted for 6 days with a highest parasite count of $4.80X10^4$/ml as seen in figure 4.12. The generation time was calculated to be 7.00 hour. The trophozoites were counted according to the following formula;

Cell concentration (Cells/ml) =Mean cell count in 5 squares x dilution factor $x10^4$

=Mean cell count x $2x\ 10^4$

Generation time was calculated during the most rapid growth occurred according to the following formula as described by Chaudhari and Singh, (2010):

$$GT = \frac{t}{n} = \frac{t}{3.3 \log (b/B)}, \text{ where}$$

B = number of cells at zero time

b = number of cells at end of time period

t = time period

GT = generation time

n = number of generations

log = logarithm to the base 10 (common log)

Staining on the parasites obtained at the 18^{th} hour using Modified Fields' stain (Figure 4.13), DAPI (Figure 4.14) and acridine orange stain (Figure 4.15) showed clumping of the organism. A closer observation and a detailed scrutiny of the morphological details on the different stained forms of the parasite revealed that *D.fragilis* showed apart from binary fission another reproductive process which involved the release of the progeny from the mother *D.fragilis* (Figure 4.16). This finding was further confirmed using the transmission electron microscopy (TEM). Based on TEM findings, the nucleus of the progeny within the mother cell was evident (Figure 4.17)

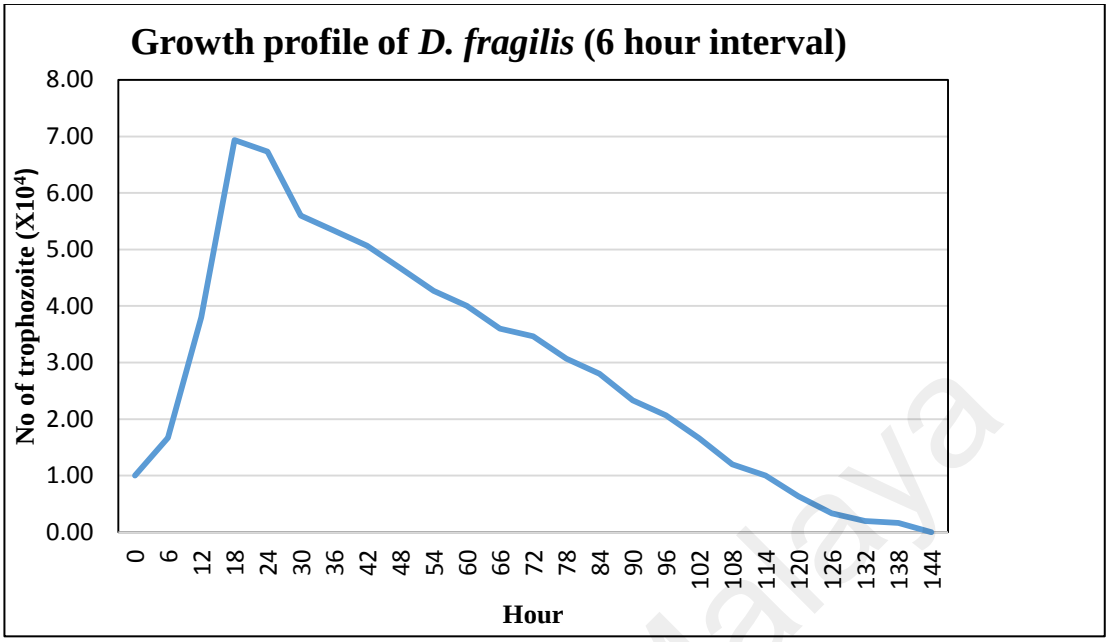

Figure 4.11: The initial growth profile of *D.fragilis* taken at 6 hour interval

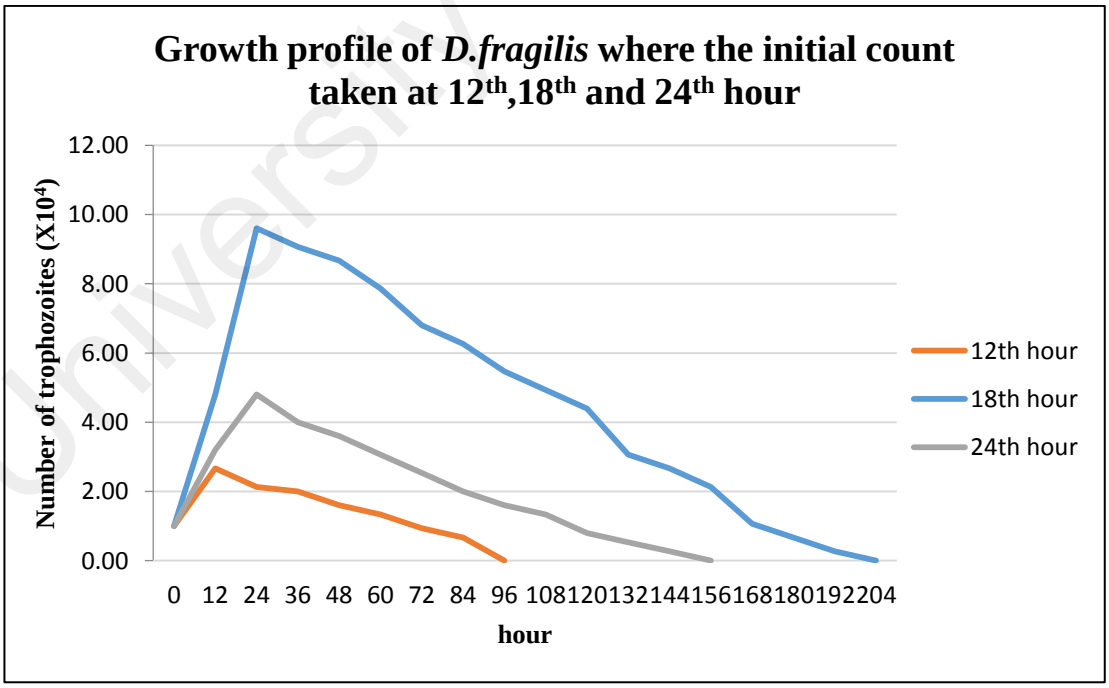

Figure 4.12: Growth profile of *D. fragilis* using initial inoculum harvested at 12th, 18th and 24th hour

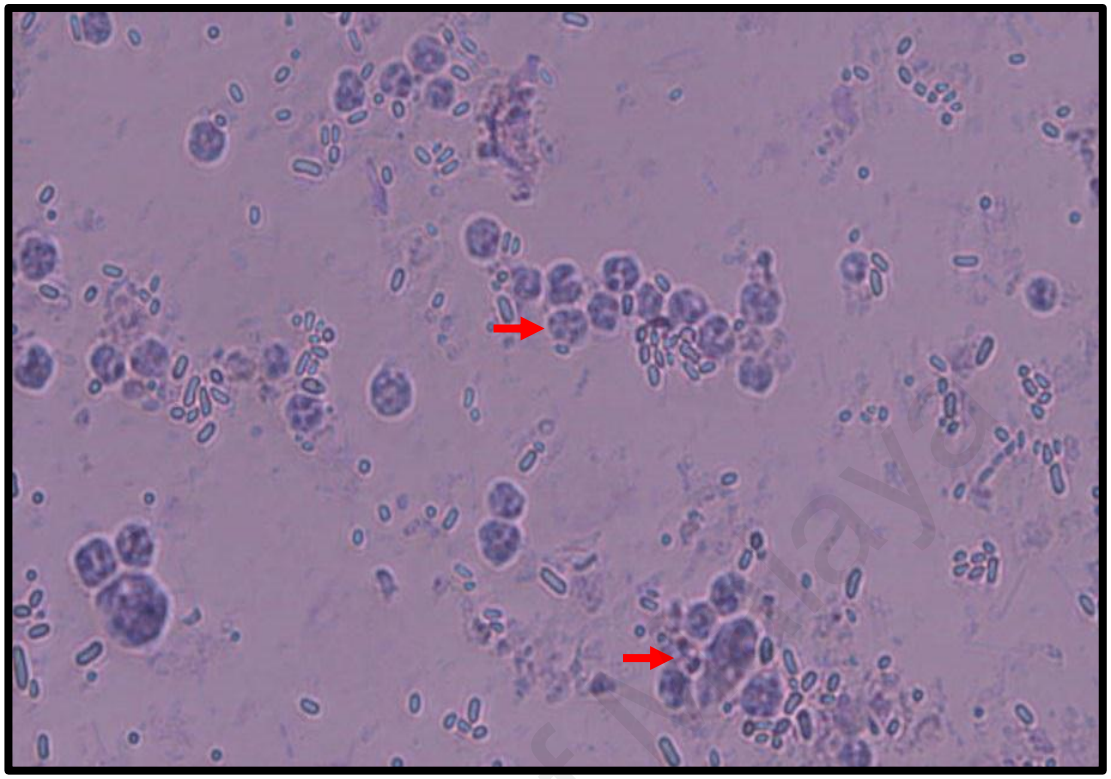

Figure 4.13: The clumping of *D.fragilis* seen at the 18th hours when stained with Modified Fields' Stain

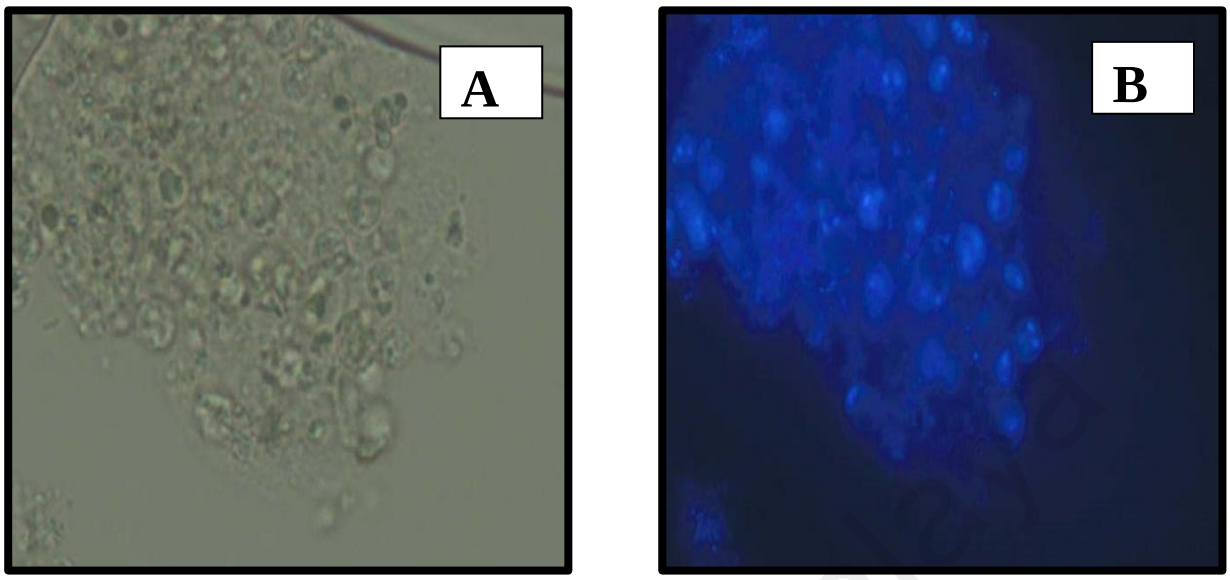

Figure 4.14: The clumping of *D.fragilis* seen at the 18th hour under bright field (A) and in (B) clumping seen when stained with DAPI stain

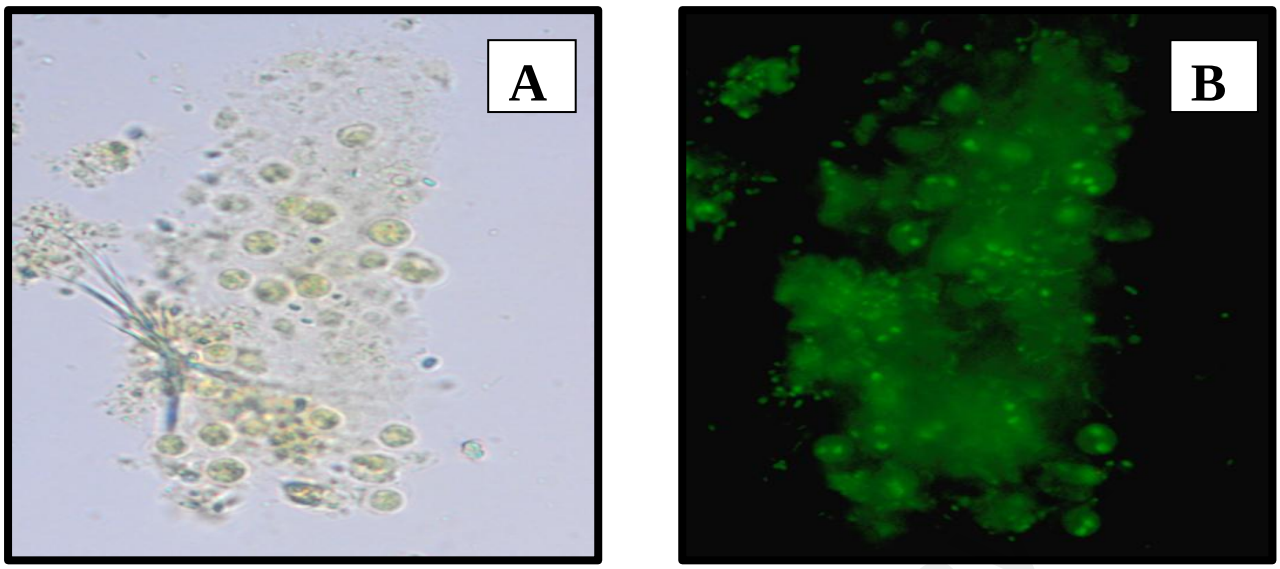

Figure 4.15: Staining of *D. fragilis* using Acridine orange. The clumping of *D. fragilis* seen at the 18th hour under bright field (A) and in (B) clumping seen when stained with acridine orange.

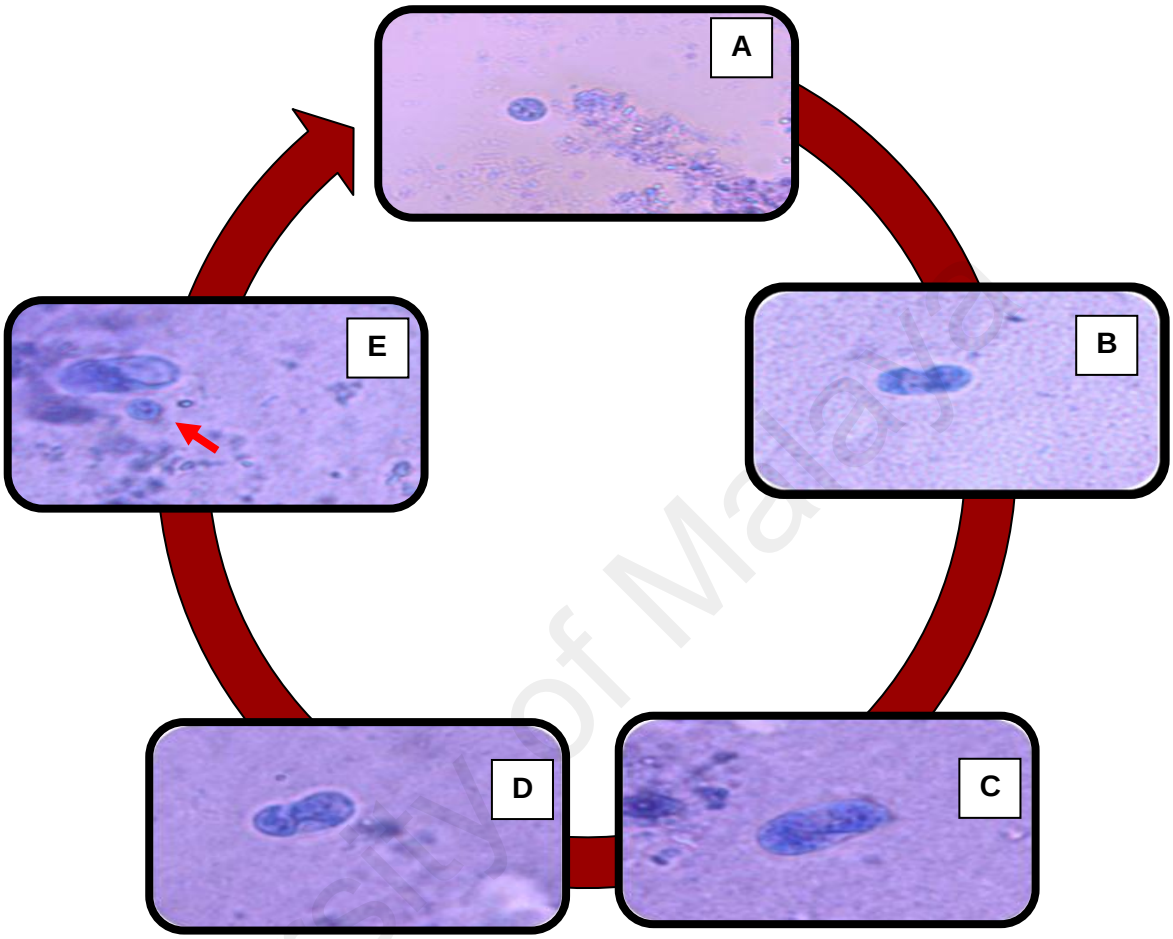

Figure 4.16: Life cycle of *D.fragilis* stained with Modified Fields' stain

(A)Rounded trophozoite of *D.fragilis* showing two nucleus.(B) Parasite is seen to elongate with nuclei seen at both ends .(C) Constriction in the cytoplasm is seen .(D) The constriction in the cytoplasm becomes obvious (E) Release of progeny leaving an empty space . Note the arrow pointing to the released progeny.

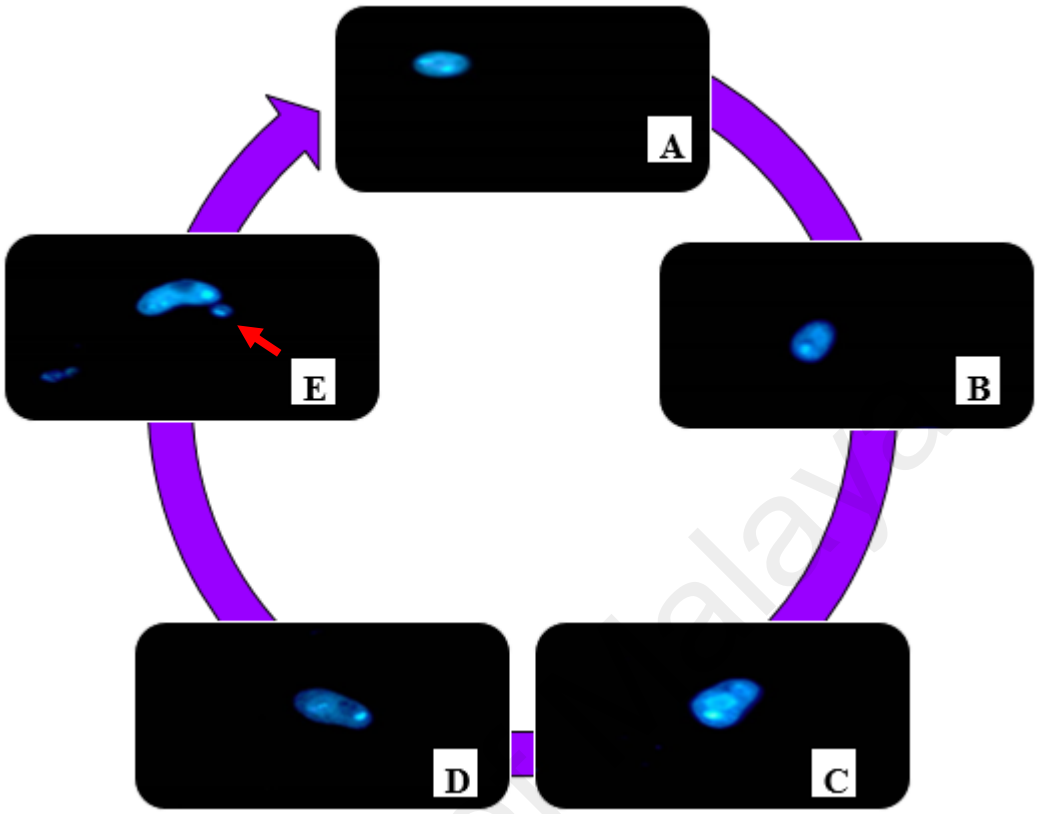

Figure 4.17: Life cycle of *D.fragilis* stained with DAPI stain.

(A)Rounded trophozoite of *D. fragilis* with two nuclei .(B) Elongation of the parasite with one obvious prominent nucleus on one end and the other showing a more diffused blue stained nucleus .(C) Constriction in the cytoplasm with division of nuclei clearly seen at both ends .(D) The constriction seen in the cytoplasm is more obvious with a distinct prominent nucleus at one end .(E) The release of progeny with one nucleus leaving an empty space in the original mother cell.

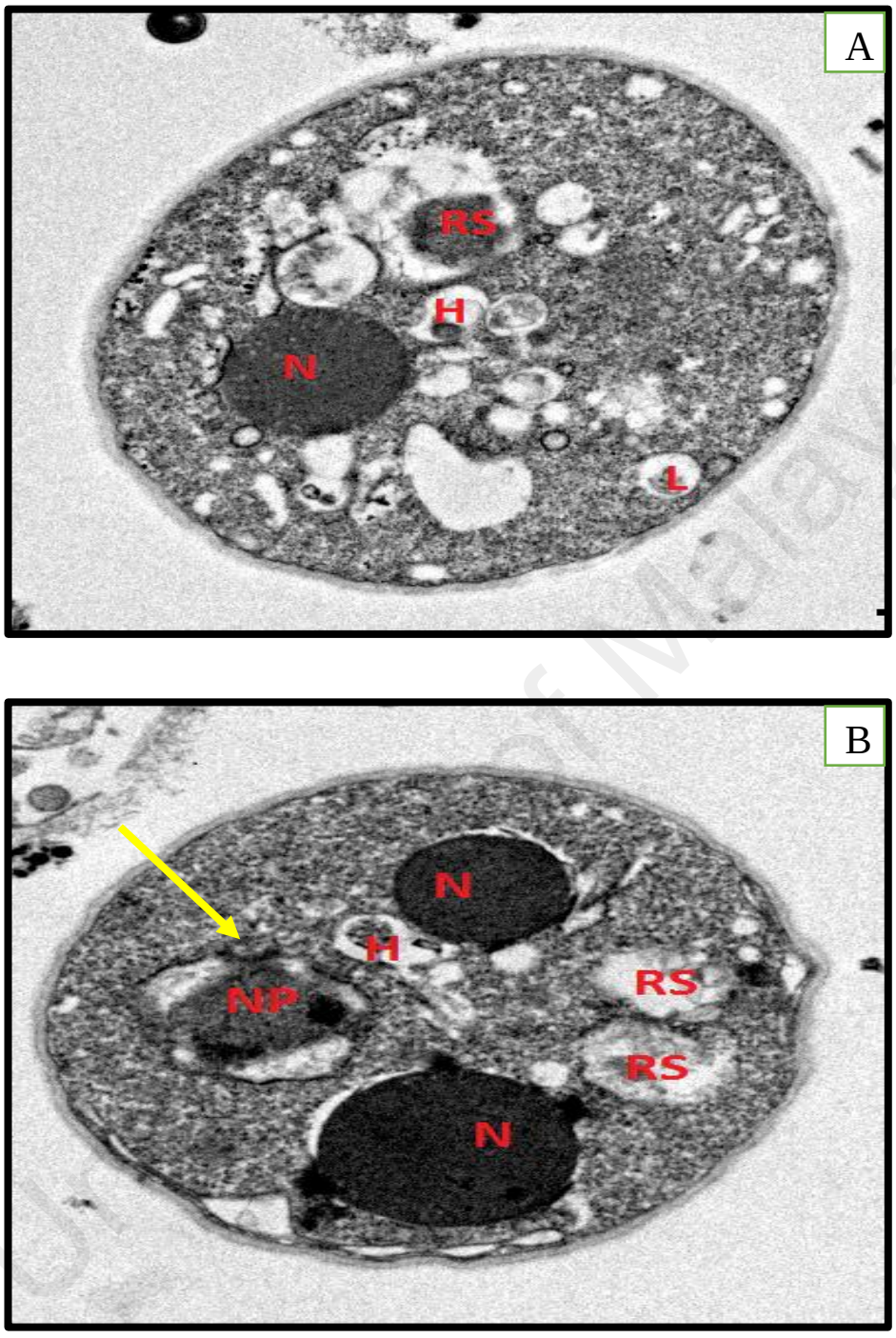

Figure 4.18 Transmission electron micrographs of *D. fragilis* showing the presence of new progeny. Figure (A) shows a mono-nucleated trophozoite of *D. fragilis* while in figure (B) the presence of new progeny can be seen within the trophozoite. Note: N nucleus, RS rice starch, NP new progeny, H hydrogenosome. Note: the new progeny shown by the arrow.

4.2.4.2 A distinct form of trophozoite seen in the 18th hr cultures

Another part of this study is to enumerate the different forms of trophozoites in cultures. A random count over 20 fields on parasites harvested at 3- hour intervals until the 36th hour culture (Figure 4.13) was carried out. It was evident that at the 18th hour, three forms of trophozoites were seen clearly visible. A rounded form with the typical two nuclei, an elongated trophozoite, with an empty space occupying at the far end of the organism as well as empty rounded forms were seen. A growth study was carried out to show the existence of these three population of life cycle stages seen in the culture of *D.fragilis* trophozoite. As seen in figure 4.13, the rounded form and the form with empty space showed a peak at the 18th hour.

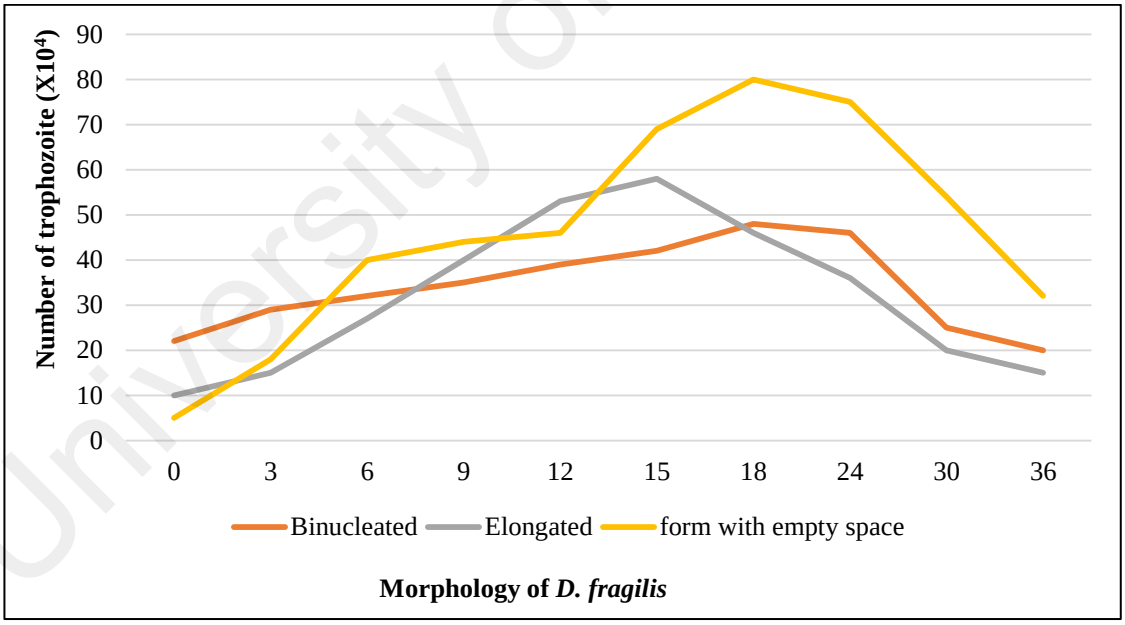

Figure 4.19: *In vitro* growth profile showing the three different life cycle stages of trophozoites.

4.3 *In vivo* experimental infection

4.3.1 Sample source (Assessing cyst-like structure)

When cultures containing *D. fragilis* were washed with distilled water, many parasites lysed and trypan blue staining revealed that these cells were not viable (Figure 4.20). The surviving viable trophozoites when stained with Modified Fields' Stain showed a thicker wall (Figure 4.21). This could be the cyst-like structure of *D.fragilis.*

These forms were then counted made up to $1X10^4$/ml, $1X10^5$/ml and $1X10^6$/ml and inoculated into Spraque dawley rats .

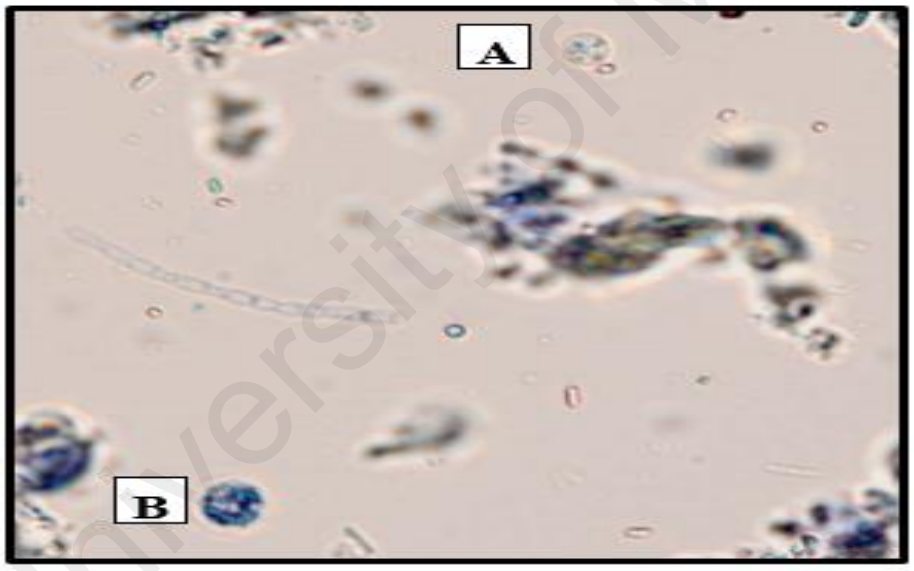

Figure 4.20: Trophozoites of *D. fragilis* washed with distilled water. Figure (A) shows the viable trophozoite of *D.fragilis* when stain with trypan blue and figure (B) shows the non-viable trophozoite.

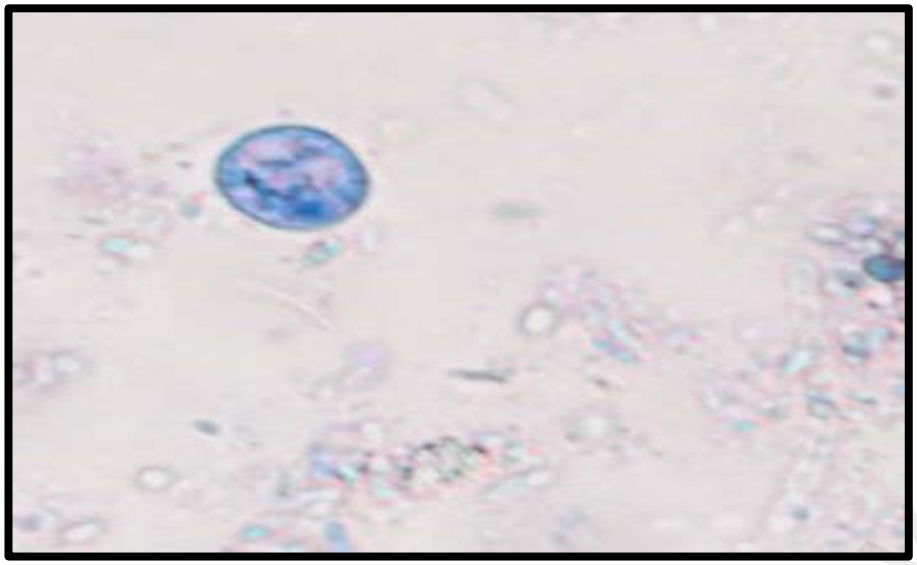

Figure 4.21 Trophozoite of *D. fragilis* stained with Modified Fields' stain. The presence of thickened cell wall can be seen after washing with distilled water

4.3.2 Examination of stools of infected rats every 6 hour post inoculation

Out of the 12, 6 rats with concentrations of $1X10^5$/ml and $1X10^6$/ml were positive for the parasite .

Trophozoites of *D.fragilis* were collected in preservative and counted over 20 random fields at x 40 under light microscopy. The results showed that the highest mean number of trophozoite was found to be at the 24th hour (Figure 4.22). Stool samples collected at every 6th hour interval till the 30th hour showed positive when PCR was carried out. However, when infected stool samples were cultured, the trophozoites did not survive.

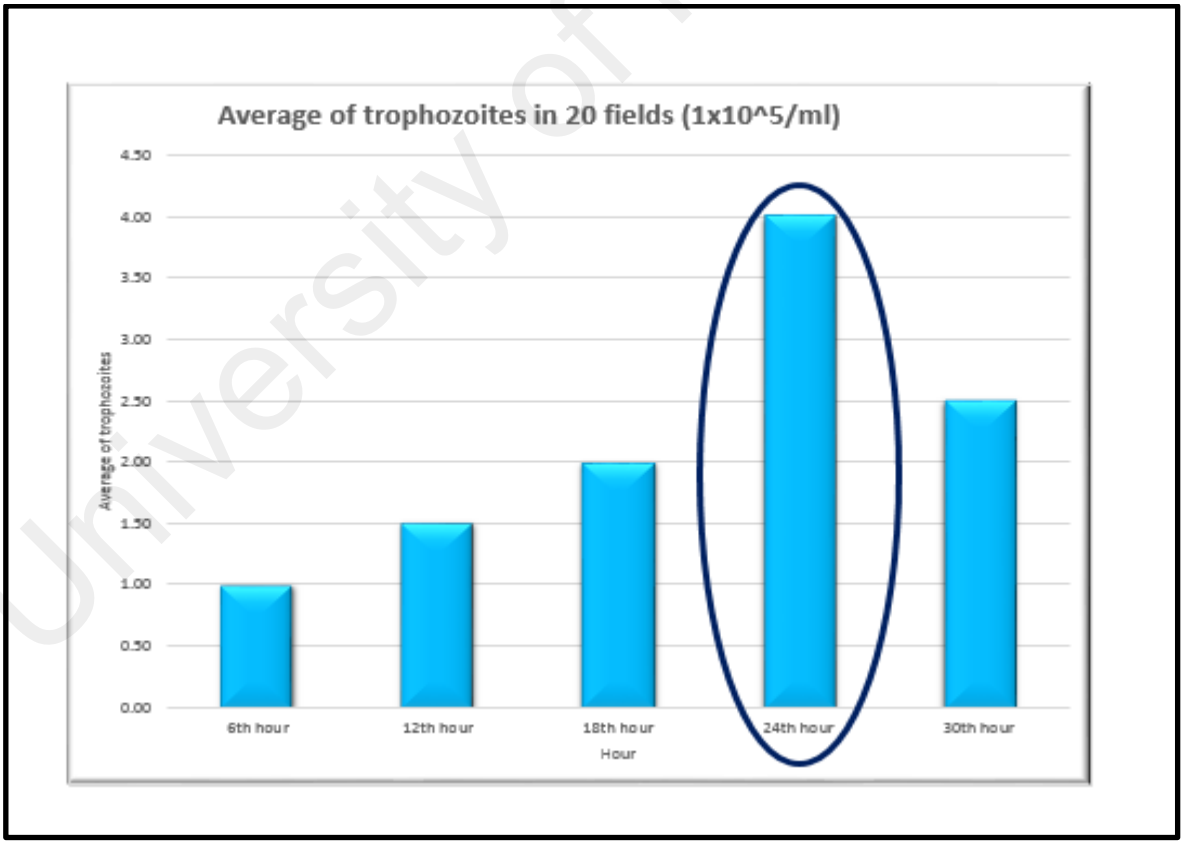

Figure 4.22: Average count of trophozoites with initial concentration of $1X10^5$/ml..

Figure shows that at 24 hour the average count of trophozoite is the highest therefore suggesting that at the 24 hour the infection is at the peak.

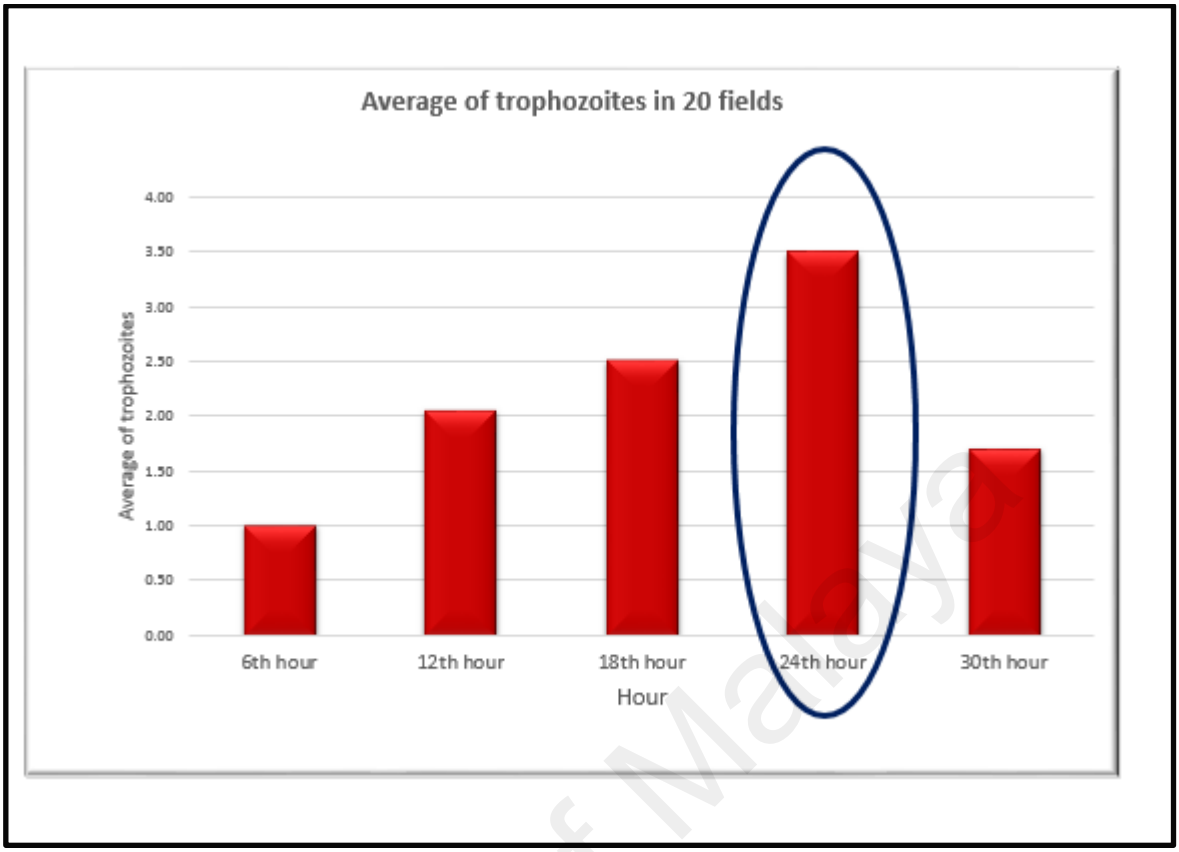

Figure 4.23: Average count of trophozoites taken from concentration 1x10^{6}/ml.

Figure shows that at 24 hour the average count of trophozoites is the highest therefore suggesting that at the 24 hour the infection is at the peak thus confirming that at the 24th hour, the trophozoites were at the peak.

4.3.3 Dissection on the 24th hour post-inoculation

The study was repeated with same number of 3-4 week old rats. After infection, stool of the rats were collected at 6 hour interval until the 24th hour. Out of the 12 rats infected, 6 rats with the concentration of 1X 10^5/ml and 1X10^6 /ml were found to be positive. Stool samples collected at every 6th hour interval till the 24th hour showed positive PCR result (Figure 4.24). Based on parasite count made from 20 random fields on fecal smears obtained from infected rats under light microscopy, 24 hours post infection showed the highest parasite count and this became the basis for the rats to be dissected at the 24th hour. However, when stool samples were cultured the results were negative for the parasites.

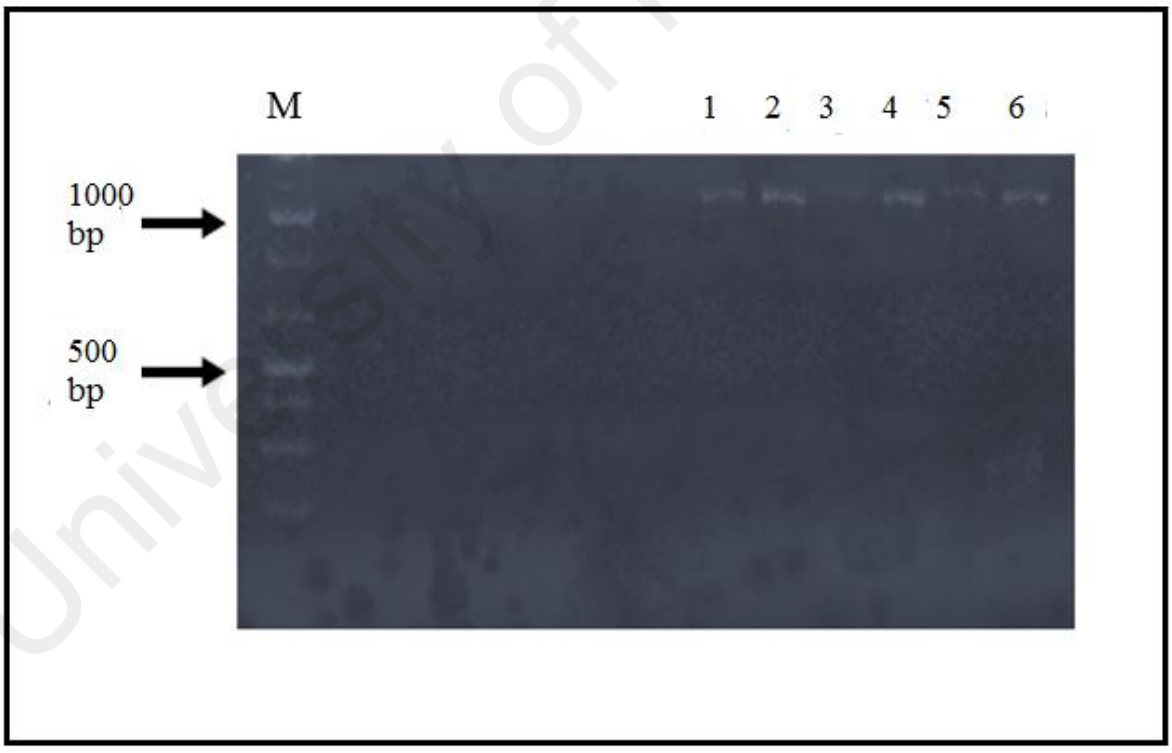

Figure 4.24 Agarose gel image of *D.fragilis* Lane M= DNA markers of 100bp plus DNA ladder (Fermentas, USA); lane 1 till lane 3 show tpositive band for concentration of 1X 10^5/ml; lane 4 till lane 6 show positive band for concentration of 1X 10^6/ml trophozoites.

4.3.4 Dissection on the 4th week post-inoculation

12 rats were infected with $1X10^4$/ml, $1X10^5$/ml and $1X10^6$/ml with cyst –like structures of *Dientamoeba fragilis*. The stools of rats from day 5 showed negative results when examined directly, PCR and when cultured.

The experiment was repeated using 12 Balb/C mice, however all 12 were not able to be infected.

4.3.5 Direct microscopy examination

Stool samples collected from the infected rats were observed under 400x microscopy as seen in figure 4.25. When observed, the trophozoite from the infected rats showed a thicker cell wall which indicate that trophozoites could have changed its structure to survive in the intestine of the rate.

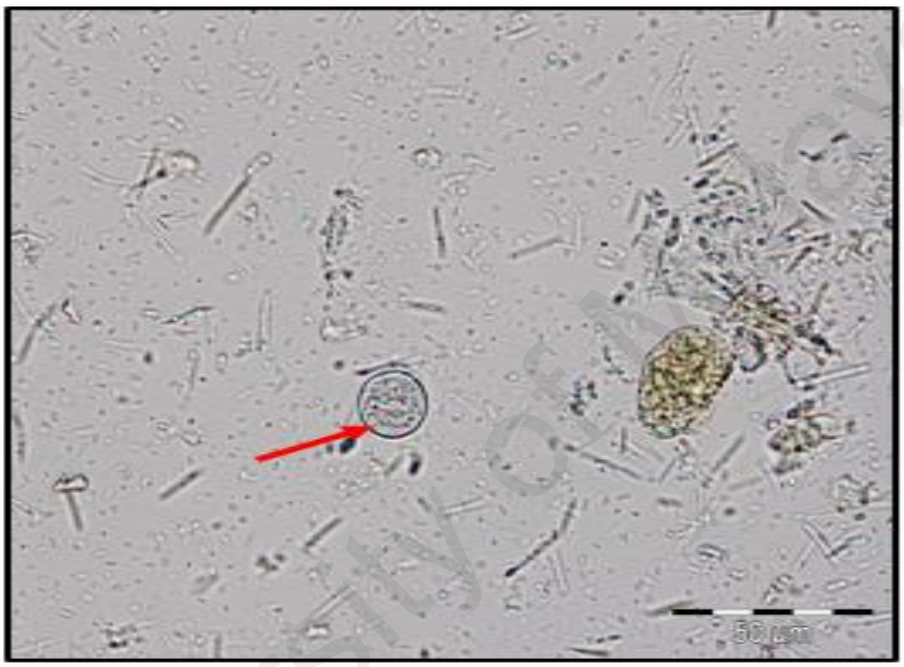

Figure 4.25: Trophozoite of *D.fragilis* seen in infected rat fecal sample under bright field microscopy at 40x A thicker cell wall of trophozoite can be seen under the light microscopy.

4.3.6 Staining of *D. fragilis* using Modified Fields' stain

Stool samples collected from fecal samples of infected rats were stained using the Modified Fields'. The trophozoite when stained with modified Fields' stain showed a thicker cell wall (Figure 4.26)

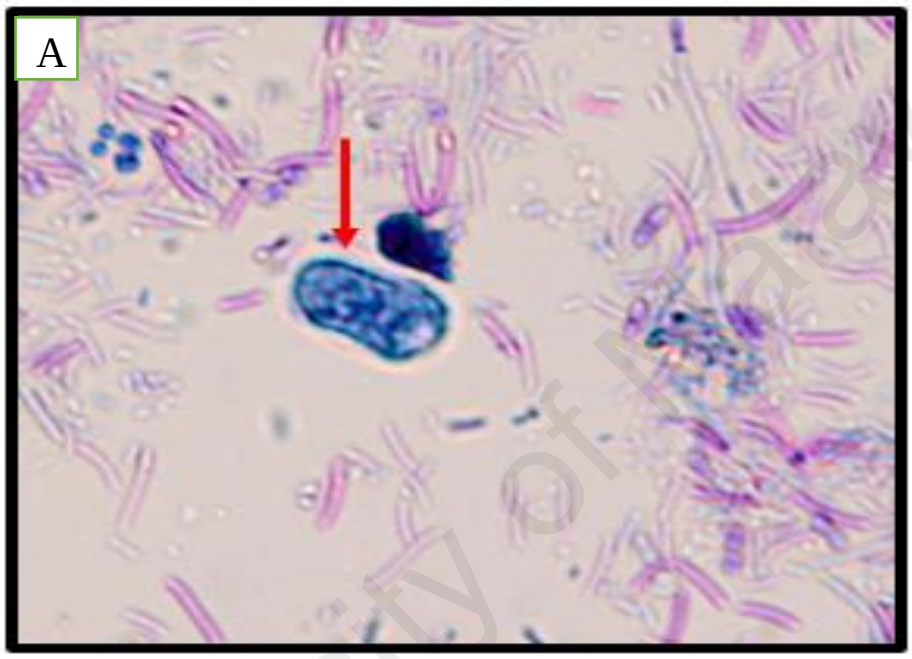

Figure 4.26*:* Trophozoite of *D.fragilis* from infected rats stain using Modified Fields' stain at 40x.

4.3.7 Staining of *D. fragilis* from infected rats stain using DAPI and Acridine orange

DAPI and Acridine orange stains as seen in figure 4.27 and figure 4.28.Trophozoite from fecal sample of infected rat showed the parasites to have a thicker membrane which implies that these could be the cyst-like structures.

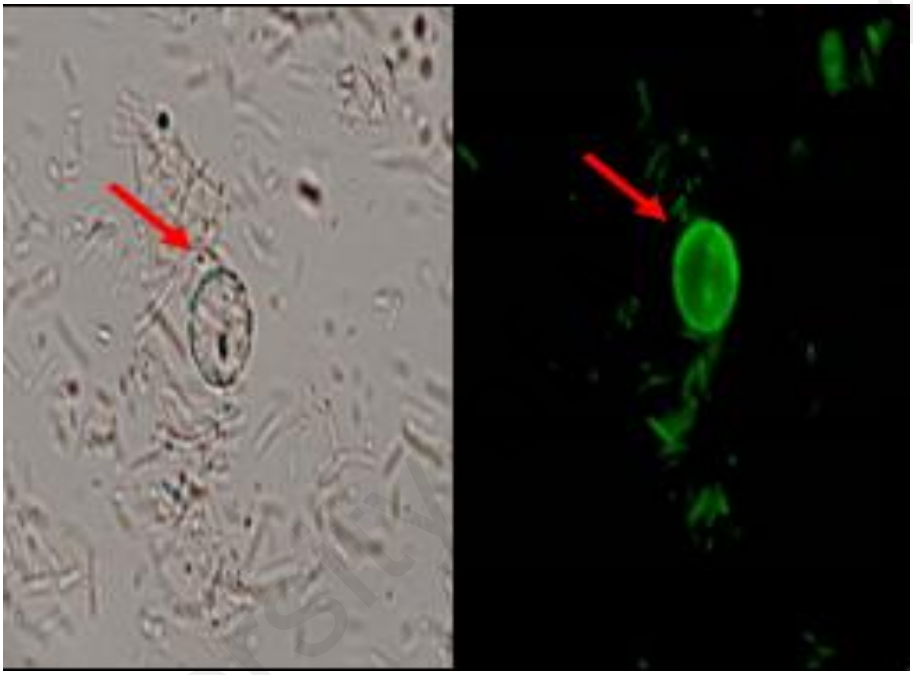

Figure 4.27 : Staining of *D.fragilis* from infected rats stained using Acridine orange at 40x. The trophozoite from fecal rat samples show a thickened cell wall.

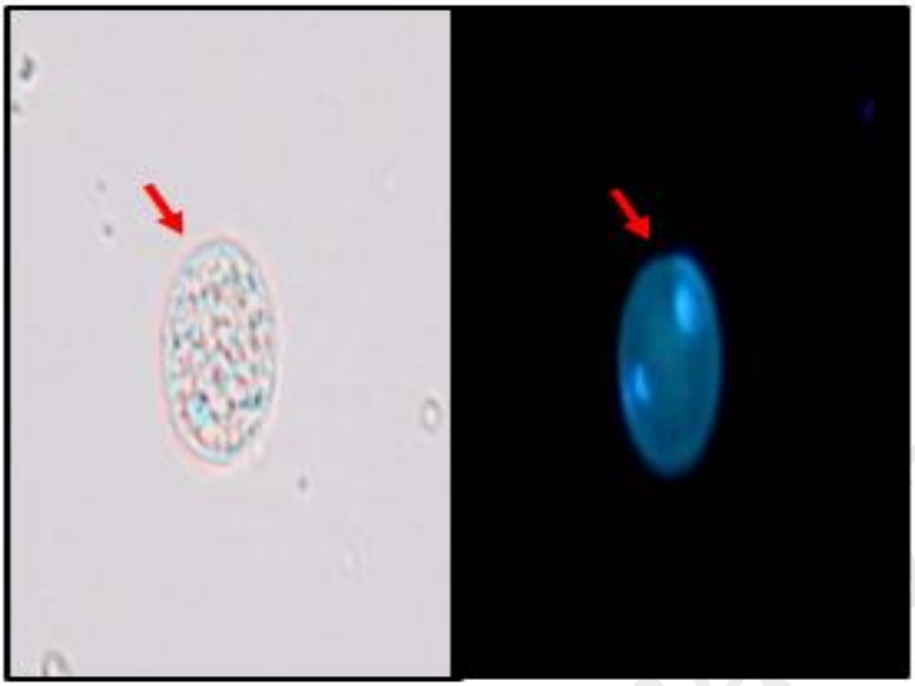

Figure 4.28: Staining of *D.fragilis* from infected rat using DAPI stain at 40x. The trophozoite from fecal sample from the infected rat showing a thickened cell wall.

4.3.8 Ultrastructural studies.

D.fragilis was obtained from the fecal samples of *D.fragilis* infected rats and was fixed in 4 % glutaraldehyde. Trophozoite from the fecal samples of infected rat showed an increase of thickness between 91.80nm and 147.13nm (Figure 4.29)

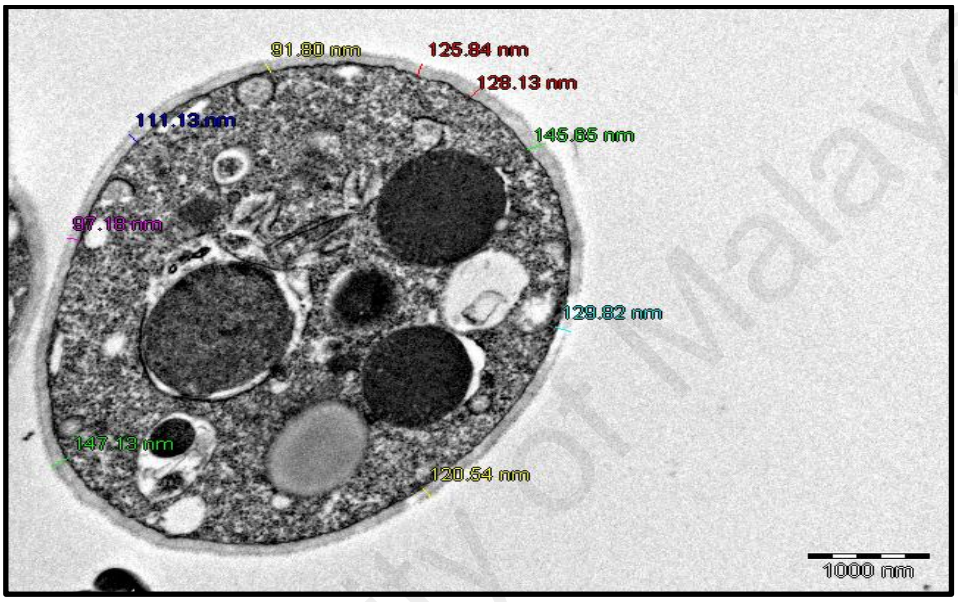

Figure 4.29: **Ultrastructure of *D.fragilis.*** Trophozoite taken from faecal samples from infected rats at concentration 1x 10^6/ml.

CHAPTER 5

DISCUSSION

5.1 Intestinal parasitic infection among Orang Asli namely *D. fragilis*

The study is the first prevalence study carried out on *D.fragilis* in Malaysia. 3.9% out of 409 Orang Asli were found to be infected with *D.fragilis*. Till date, only four studies have been carried out involving the aborigine communities globally (Baratt et al.,2004). So far the highest prevalence reported in the aborigine group was 21% and the lowest is 2.7% (Baratt et al.,2004), hygiene being the main reason reported for the transmission to occur among members of this community (Baratt et al.,2004).

There is a possibility that the lack of studies on *D.fragilis* in Malaysia could be attributed to the challenge in identifying these parasites in stools as the fragile trophozites in stool samples are known to disintegrate after 6 hours (Johnson et al., 2004; Kean et al., 1966 ;Schuster et al., 2009) as well as the lack of the conclusive evidence showing this parasite is a pathogen.

The significant risk factors associated with this parasite were age below 18, association with livestock, washing hand after handling livestock, gastrointestinal symptoms and the increased frequency of visits to the toilet. Based on our result, the age below 18 years would be the active age group where children and youth would be spending most of their time in outdoor activities and playing by the stream that was seen to flow past the Orang Asli villages. Previous studies have shown the association of *D. fragilis* to children aged 5 to 14 years old implying that children appear to be the vulnerable group to acquire this infection. The highest infection was found in children aged between 0 and 10 years old but also shown to be common in adults between 41 to 60 years old. The close association between the infected children and adults could be one of the factor for this transmission (Ayadi & Bahri, 1999; Grendon et al., 1991). It is

also reported that infection rate of *D.fragilis* is higher in children than in adults (Girginkardesler et al.,2008).

The transmission of *D. fragilis* via the fecal oral route, lack of hygiene and sanitation may be factors responsible for transmission irrespective of the age (Millet et al., 1983).

Although the government has helped in providing basic amenities to overcome living in poverty conditions, members of the Orang Asli community still have been found not to afford electricity and water forcing them to use the nearby flowing river for all their daily activities. It is highly possible that the usage of river could have facilitated the transmission of the parasite through contaminated water. Furthermore association with livestock also implies that close proximity with animals and not washing hands after handling animals may have contributed to the risk of acquiring this infection.

It has been also reported that the gastrointestinal symptoms which could have promoted the increase of frequency to the toilet visits could be the risk factors. This concurs with the finding that abdominal pain and diarrhea were commonly seen in *D. fragilis* infected person (Barratt, et al., 2011) which implicates this association to be an indicator of pathogenicity for *D.fragilis* (Adnan et al.,2013).

In the present study a strong association of *D.fragilis* have been shown with *Blastocystis* sp although studies have shown other parasites namely helminthes to have an association with *D.fragilis* (Olga Gonzalaz et al.,2010; Norberg et al., 2003). Faecal oral route appears to be the mode of transmission (Timothy et al., 2013).

The present study concurs with previous findings that children and youth below 18 years are have a higher risk of acquiring the infection. Their more active life, greater intimacy with livestock which they handle, the lack of hygiene consciousness especially when it comes to washing hands after handling livestock could be the reasons why this group is at a greater risk.

5.2 *D.fragilis* infection among school children

The present study showed that in Selangor 0.7 % of school children was infected with *D. fragilis*. The risk factors appears to be higher in the female group children less than 9 years old, lower educational status of mothers, the village locality they come from, type of stool and symptoms.

In the present study, female school children show a higher prevalence rate when compared to male school children which concur with other studies that have reported gender association with *D. fragilis* (Barratt et al., 2011; Norberg et al., 2003). The close bonding between females and their mothers (Baratt et al.,2011) as well as crowding and close contact with peers (Barratt et al., 2011; Norberg et al., 2003) may have facilitated the transmission.

The infection appears to be more seen in younger children whose hygiene practices may be compromised especially when children after washing their anal area with bare hand after defecation use them for eating as well (Barratt et al., 2011). It is also been reported that child to child transmission may be another cause of transmission among school children from the rural area (Girginkardesler et al., 2008). Another significant finding that was associated with the risk of *D. fragilis* infection is the level of

education of mothers. The lack of education in mothers has been shown to be a factor that influences the hygiene level of children as mothers play an important role in educating the children. The prevalence rate was also seen to be higher in the rural than urban school children possibly due to poverty, low hygiene and low socioeconomic level (Ngui et al., 2011).

The present study has shown a prevalence of 3.9% in Orang Asli and 0.7% in school children. The present study has highlighted a few concerns. The fact that the parasite exists in the Malaysian population implies that this parasite must be included in the routine differential diagnosis for persons showing gastrointestinal symptoms. The more important aspect of this finding is to question its relationship to the presence of symptoms. In the Orang Asli population it would be impossible to ascribe any symptoms to this parasite alone as the stools were also infected with other parasites mostly *Blastocystis* sp. This then brings to the point of ascribing pathogenicity to *Blastocystis* sp. as most prevalent studies do not screen for *D. fragilis* and the results therefore cannot be conclusive. In the present study, all persons infected with *Blastocystis* sp. also had *D.fragilis*. Whether this association is incidental or truly a symbiotic relationship exists between these two organisms need further investigation, but based on the results in the present study all seven school children infected with *D.fragilis* showed symptoms such as abdominal pain with soft stool.

5.3 Influence of preservative

Preservative is useful when stool samples cannot be observed within a time period. The fragility of *D.fragilis*, further compounds the need for a preservative as the parasite is known to disintegrate easily when exposed to the environment (Johnson et

al., 2004). There are many preservatives that can be used to preserve *D.fragilis* such as the Sodium acetate-acetic acid formalin (SAF), Poviyl alcohol (PVA), and Schauddins preservative. PVA and Schauddins preservatives both contain mercuric chloride which is hazardous to humans (Johnson et al., 2004) and difficult to be prepared in the laboratory. Mercuric chloride was substituted with zinc sulphate in a previous study but the result was not as good when compared to using the mercuric chloride (Johnson et al., 2004). Till today, preservative using mercury substitutes does not give an optimum result.

The present study suggest potassium dichromate as a preservative as the results showed that the parasite remained intact and could easily be stained even after preservation for more than 12 months. This preservative can now prevent the disintegration of the parasite and thereby increases the possibility of detection. Potassium dichromate have been used previously to preserve other parasites and have commonly been used in routine surveys (Lee et al., 2014). A prevalence study on soil transmitted helminths using potassium dichromate to preserve the parasites such as the *Ascaris lumbricoide, Tricuris trichuria* and hookworm have been shown (Lee et al., 2014). The preservative therefore can be used for *D.fragilis,* and for other parasites. The fact that *D.fragilis* could be kept as long as 12 months at 4^{o}C in potassium dichromate provides a good alternative to the current recommended SAF preservative.

This finding has important implications. Often prevalence studies carried out on faecal samples from different countries employ direct microscopy without using any preservatives. Others use preservatives which has mercuric chloride. Parasitological findings with such differing practices cannot enable the effective comparison of prevalence data obtained from different countries or regions. The study recommends

that potassium dichromate be used. It is safer and easier to prepare and the preservative is more wholesome as it can also preserve the eggs of helminthes.

5.4 *In vitro* cultivation of *D.fragilis*

Studies have previously shown that *D.fragilis* grows only in xenic conditions as it appears to require the presence of bacteria for survival purposes (Johnson et al., 2004). In order to sustain the growth of bacteria in *in vitro* cultures, rice starch, used for carbohydrates, can ensure the growth of the bacteria (Johnson et al., 2004). Therefore in order to maintain *in vitro* cultures of *D.fragilis*, it is imperative that rice starch be included for this purpose.

Four culture media were used in the present study to maintain *D.fragilis* in *in vitro* and amongst them were Loeffler's and Jones' medium which was supplemented with 10 % horse serum and rice starch were used. Loeffler's medium showed a higher parasite count compared to Jones' medium, however Jones' medium had the advantage of a simple preparation protocol and the capability of long term storage before use. Furthermore Jones' medium could easily be used for rapid detection for *D.fragilis* in stool field surveys. These parasites can then be subsequently transferred to Loeffler's medium for long term maintenance.

The preparation of Loeffler's medium is time consuming and more tedious compared to Jones' medium. This is because, Loeffler's medium need to be placed at 80^{o}C for the inspissation of the medium. When placed in the oven, the medium requires constant checking from time to time so as to not cause overheating of the co-factors found in the serum which would then compromise the quality of the culture medium

(Barratt et al., 2010). In addition, the preparation of Loeffler's medium is costly as 700 ml of horse serum is needed for every 1000l of preparation whereas only 10 % of horse serum is usually needed for Jones' medium.

The present study has important implications. Due to the expensive preparation of Loeffler's medium, it is recommended that modified Jones' medium with rice starch should be used as the initial detection method to isolate the parasite from infected stools. The parasites then could then be transferred to Loeffler's medium for long term maintenance in *in vitro* culture. This two tier *in vitro* culture method not only enhances the sensitivity of the detection but also can be carried out much cheaper when compared to merely detecting the parasite using Loeffler's medium.

5.5 Staining to differentiate *D.fragilis* and *Blastocystis* sp.

Stools with mixed infections when cultured will have more than one parasite growing in the culture medium (Johnson et al., 2004). In the present study the growth of *D.fragilis* and *Blastocystis* sp. growing together in Loeffler's and Jones' medium supplemented with horse serum and rice starch posed a challenge when it came to identifying both these parasites. A simple and effective stain was needed to differentiate both of these parasites in a mixed culture as both were found to ingest rice starch making the identification a greater challenge.

Although in the past other stains have been used such as Iron hematoxylin, Trichrome and Giemsa stains to stain *D.fragilis* (Johnson et al., 2004), the present study provides evidence that Modified Fields' stain can be used as the duration of the procedure takes only 3 minutes compared to 20 mins with Giemsa stain . Moreover, Modified Fields' stain gives a better contrast when visualized for both parasites. Giemsa

stains the slide darker which causes a difficulty when it comes to differentiating the nucleus and the peripheral cytoplasm of the *Blastocystis* sp. It has been reported that *D.fragilis* can have up to five nuclei which can cause confusion with the *Blastocystis* sp. which is also known to have many nuclei. Although Iron haematoxylin clearly differentiates the nucleus in both *D.fragilis* and *Blastocystis* sp., the stain however takes an hour to stain when compared to Modified Field's stain which takes 3 minutes to stain.

This study is vital as most of the previous studies and the present one undertaken showed mixed infection of *D.fragilis* and *Blastocystis* sp. When cultured, *Blastocystis* sp. which mostly exist in vacoular forms looks almost similar to *D.fragilis* which is why a good staining method that provides clarity and contrast which can differentiate both will be ideal to identify the parasite in cultures, In Loeffler's medium, the presence of rice starch could be seen in both parasites. There has been only sporadic and inconclusive evidence that *Blastocystis* sp. does ingest starch but in the current study due to the staining the prominent granules of starch was made obvious. This finding further complicates the identification of the parasite when grown in mixed culture with *D.fragilis.* Modified Fields' stain is a better stain than Giemsa and Iron haematoxylin when it comes to differentiating the two organisms in cultures and therefore provides a better, faster and more reliable diagnostic method to precisely identify one from the other.

This study makes a strong recommendation that every stool culture positive for *Blastocystis* sp. should also be stained using Modified Fields' stain to differentiate *D.fragilis.* Modified Fields' stain should be used also to confirm the presence of *D.fragilis* as the staining characteristic is obvious and confers clarity for the purposes of identification.

5.6 Cytochemical staining to differentiate *D. fragilis* and *Blastocystis* sp.

Blastocytsis sp. has been shown to grow freely in all culture media found to be suitable for the growth of *D. fragilis* (Johnson et al., 2004). It was also reported that identifying ingested rice starch can differentiate *Blastocystis* sp. from a mixed growth of both parasites as *Blastocystis* sp. would show prominent rounded granules as evidence of ingested rice starch (Johnson et al., 2004). However, in the present study *Blastocystis* sp. was seen to ingest rice starch thereby causing confusion when it came to distinguish a mixed culture of *Blastocystis* sp. and *D.fragilis*.

In the present study, using DAPI and acridine orange stains, *D.fragilis* and *Blastocystis* sp. could be differentiated by identifying the number and location of nucleus within the respective parasite . The nuclei of *D.fragilis* were seen to be closer to each other and located in the middle of the parasite whereas the nuclei of *Blastocystis* sp. were seen far apart and located at the periphery of the cell body . This clearly provides a sharp contrast and the study has provided an easy to use method for differentiating *D.fragilis* from a mixed infection seen in *in vitro* cultures. This has been shown to be time saving.

It must be remembered that *Blastocystis* sp. is the most common parasite seen in any stool survey and often the gold standard is the *in vitro* culture method using Jones' medium. Jones' medium allows *Blastocystis* sp. to grow and rarely researchers assess the presence of *D.fragilis* which also could be growing in *Blastocystis* sp. infected stools. Hence to ascribe pathogenicity to *Blastocystis* sp. when correlating with symptoms seen in infected patients without verifying for the presence of *D.fragilis* in the same cultures would provide inaccurate results.

5.7 Evidence of another additional reproductive process

Sub-culturing on day 2 in *in vitro* cultures of *D.fragilis* have been suggested for the purposes of long term maintenance in cultures (Barratt, 2010) however the growth of the trophozoites was shown to be low and did not sustain parasite numbers when subjected to continuous culture. The present study showed 18th hour of culture is the best time to harvest the parasites as the parasite yield have been shown to be the highest and when sub-cultured , parasites remained viable and grew for at least the next eight days in culture. The parasite growth at the 18th hour, showed a peak parasite count of 6.93X10^{4} /ml. Earle's Balanced Salt Solution (EBSS) was reported earlier (Munasinghe et al .,2012) to have been used as an alternative to phosphate buffered saline (PBS) which resulted in an increase in the number of trophozoites. This has opened to new opportunities for more research to be carried out on parasites harvested at the 18th hour sub-culture.

This is the first study that reported on the generation time for *D.fragilis.* The generation time was calculated to be 7.00 hour and this doesn't justify the high parasite count if binary fission was the only reproductive process that this parasite was supposed to have (Baratt et al., 2011b). Staining with Modified Fields' stain revealed another reproductive process taking place where the progeny was shown to be released from the mother organism. The present study showed a typical bi-nucleated *D.fragilis* which elongated itself to show prominently the two nucleus. Then at one end, the nucleated progeny like organism is released leaving an empty space in the original mother cell. (refer to the figure 4.16) Parasites harvested at the 18th hour when stained showed two forms of trophozoites, the typical rounded form of *D.fragilis* as well as an elongated form showing the empty space, evidence of the release of the progeny. Thus we

conclude that *D.fragilis* undergoes two method of reproduction which is the reported binary fission and also another process which releases the progeny from the mother cell.

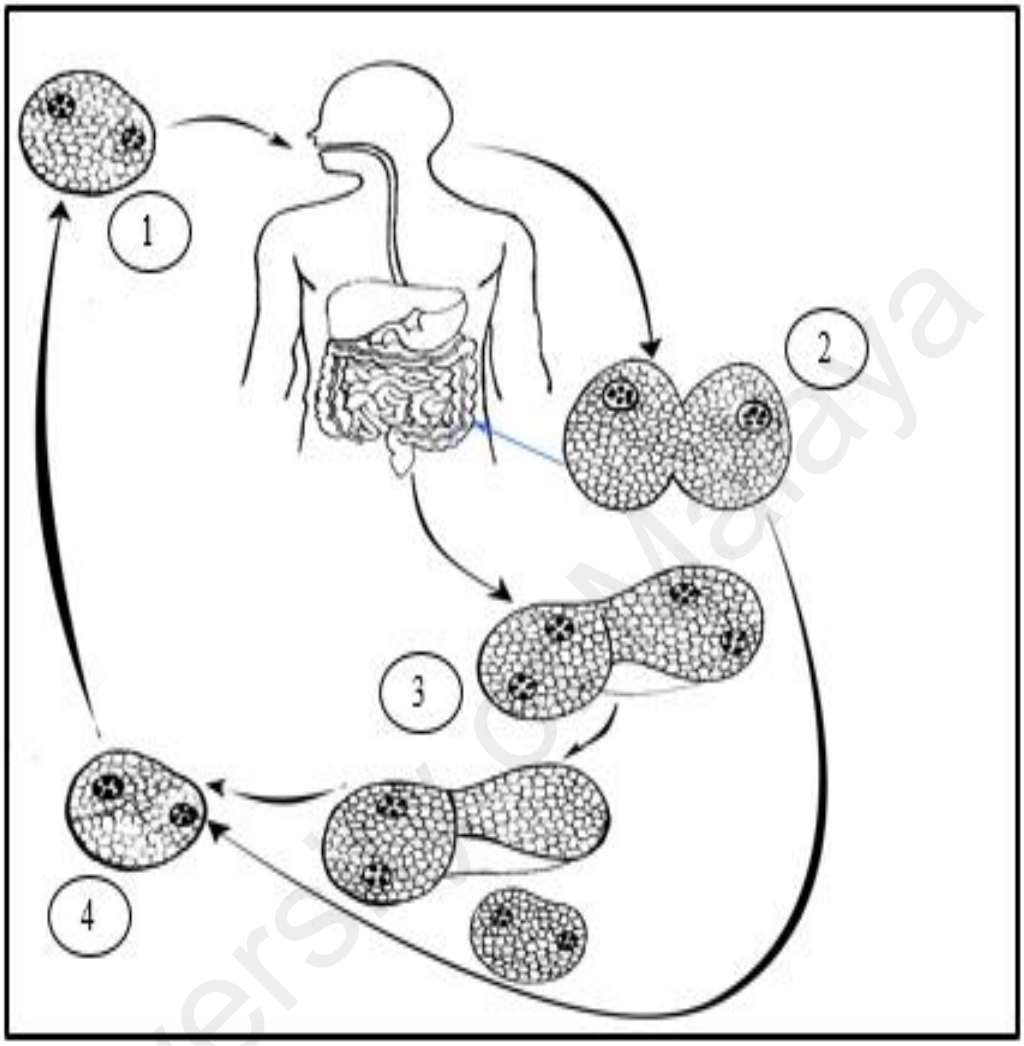

Figure 5.1 Proposal of a new life cycle for *D.fragilis* (1) cyst-like structures of *D.fragilis* can be transmitted through humans via the oral fecal route. Once the parasite ingested, the parasite undergo two modes of reproduction (2) binary fission and (3) the elongation of the mother cell with the subsequent release of the progeny with one or two nuclei whilst the original mother cell retains its two nuclei. (4) *D.fragilis* expelled into the surroundings where it can contaminate source of food and water.

5.8 The existence of cyst-like structure

The complete life cycle of *D. fragilis* is still not defined (Clark et al.,2014). Studies on the host distribution and zoonotic potential of *D.fragilis* have been investigated (Johnson et al., 2004) and humans have been reported to be the main host of this parasite .

In the present study, an attempt was made to identify cyst- like structures. Culture samples obtained were washed repeatedly with distilled water. Distilled water has been used previously to lyse mixed cultures of trophozoites and cysts of *Blastocystis* sp. (Zaman et al 1997). Distilled water has been known to lyse trophozoites leaving only sediment containing the cysts. This same approach was used in the present study. Rats inoculated with cyst-like structures of concentration of 1x 10^4/ml were not infected. However inoculation 1 x 10^6 cyst-like structures/ml at 24^{th} hour post-inoculation, the number of trophozoites in the stools of infected rats were seen to be the highest implying that 24^{th} hour post-inoculation the infection would have been at the peak. The study shows that infection involving *D.fragilis* is dose dependent and there is a threshold concentration which is needed to cause the infection in rats. The failure to grow the trophozoites when the stool samples from infected rats were cultured cannot be explained. This is probably due to the fact that the parasite may have gone through some changes during the course of the infection including the gaining of the thickening of the membrane which was obviously seen in stained preparation using Modified Fields stain failed to excyst when grown in cultures. This requires further studies to explore the reasons as to why this has happened despite the PCR results showing that stools were confirmed positive for *D.fraglis* . In any case cyst-like structures with a thickened membrane have been shown to cause experimental infection in Spraque

dawley rats. This is the first study to provide evidence that cyst-like structures do exist in the life cycle and can cause experimental infection in Sprague dawley rats. A previous study using trophozoites from *D. fragilis* culture could not infect Sprague dawley rats (Munasinghe et al., 2013). The thick membrane provided a resistance lyse in distilled water and these forms for the first time have been shown to cause experimental infections in Spraque dawley rats. Staining of the trophozoites taken from the stools of infected rats showed the thickening of the trophozoite membrane as evidenced by Modified Fields' stain and transmission electron microscopy studies which implies that this could be a survival mechanism of the trophozoites.

The study conclusively provides evidence that a simple technique to detect *D. fragilis* in the stool should adhere to the following procedure:-

a) Culture stool sample in Jones' medium supplemented with rice starch.
b) Make smears on glass slides from the culture sediment.
c) Stain with Modified Fields's stain
d) Some portion of the sediment should be subjected to cytochemical staining using DAPI and acridine orange staining

   The above method will clearly identify *D.fragilis* even if it is mixed with *Blastocystis* sp.

e) The remaining sediment should be sub-cultured and on the 18th hour of culture be transferred to Loeffler's medium for long term maintenance.

The study provides evidence for the existence of another life cycle stage which is the elongated form with an empty space at one end. It substantiates that binary fission alone cannot be the only mode of reproduction and another asexual mode of reproduction involving in the elongation of the cell with a subsequent fragmentation-like to release the cell. A cyst-like structure does exist for the parasite and this has been shown to cause experimental infection in rats.

5.9 Limitation of the study

There were two limitations that were encountered during the study which were:

1) Difficulty in encouraging the Orang Asli and school children to participate in this study. Many of them were reluctant to participate but eventually through persuasion joined in the study. The head of the village was included and he was of help to make the study feasible. Also, with the help of headmistress and teachers, school children participated in this study. Many school children were embarrassed to bring their respective stool samples and therefore found reluctance in volunteering their fecal samples. The sample size should be have been larger and more cohort groups could have been included.

2) The trophozoite of *D.fragilis* was shown to be very fragile. It is very important to extract immediately the DNA of the trophozoites. After extraction, PCR was carried out on the positive samples.

CHAPTER 6

CONCLUSION

6.1 Conclusion

The study for the first time have shown the prevalence of *D.fragilis* in two main groups namely the Orang Asli and school children. This parasite has been shown to be found mainly mixed with *Blastocystis* sp. The study therefore has provided some easy techniques to differentiate both these parasites. The study also reports for the first time a new life cycle stage demonstrating mode of reproduction other than binary fission. The study also confirms the existence of a cyst- like structure. In general the findings of the study can be e summarized as follows:-

1. The study clearly provided evidence that *D.fragilis* do exist in the Malaysian population especially in the Orang Asli population. This is the first study to document this finding. The fact that rural school children have been found positive shows that active transmission of this parasite still goes on.

2. The study for the first time has shown that participants whose ages were less than 18 years ,showed association with live stock , did not wash hands after handling animals , showed gastrointestinal symptoms and showed greater frequency of visits to the toilet per day were identified as significant ($p<0.05$) risk factors for acquiring *D.fragilis* in these communities.

3. This is the first finding to show that Potassium dichromate can be used as a preservative. The study recommends that future stool surveys should be carried out with this preservative substituting preservatives that have mercuric chloride. .

4. Jones' medium which is easier to prepare and have been successfully used previously for the detection of *Blastocystis* sp. should be used to isolate *D.fragilis* from fecal samples. The study showed that the growth of the parasite cannot exceed for more than two days. The study recommends that this culture medium be used for the initial detection especially in stool surveys and then positive cultures be transferred to Loeffler's medium for longer maintenance.

5. The study for the first time identifies the 18^{th} hour of culture as the best time point to harvest time the parasite as the parasite count appears to be at the optimum. When harvested parasites are sub-cultured the parasite could be kept *in vitro* as long as 8 days is break-through finding enables the right time point to sub culture in order to propagate continuous culture for longer *in-vitro* maintenance.

6. The study demonstrates another mode of reproduction other than binary fission. The organism elongates to release a nucleated progeny leaving an empty space at the far end in the original mother cell. This proves that binary fission is not the only mode of reproduction that *D. fragilis* has.

7. The study shows that cyst-like structures do exist which show a thickened membrane that provides a robustness to resist distil water. These forms for the first time have been shown to cause experimental infections in Spraque dawley rats.

1.2 Future studies

1. The present study reported prevalence study carried out on *D.fragilis* in only two vulnerable group which are the orang asli and school children. There is a need to carry out more prevalence studies in other groups such as patients suffering from Irritable bowel syndrome colorectal cancer, HIV, people who work in the zoo and farms, rural communities, day care centers housing children. There is a need to correlate the presence of the parasite and gastrointestinal symptoms to ascertain further its pathogenic role.

2. All hospital laboratories should include screening of *D.fragilis* as this parasite could go unidentified in mixed cultures with *Blastocystis* sp. It is important to differentiate both these parasites.

3. The cyst- like structures obtained from the fecal samples of infected rats could not excyst in cultures and therefore more excystation studies need to be carried out .

4. More experimental animal infection studies should be carried out with better molecular approaches to identify the parasites in stool samples of infected rats.

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