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

<table>
<thead>
<tr>
<th>Location</th>
<th>Subgroup</th>
<th>Approximate distances from Kuala Lumpur (km)(GPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kampung Orang Asli</td>
<td>Suku Temuan</td>
<td>39.7 (3.210037,101.882715)</td>
</tr>
<tr>
<td>Pangsun</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kampung Orang Asli Bukit</td>
<td>Suku Temuan</td>
<td>62.2 (2.898936,101.574000)</td>
</tr>
<tr>
<td>Cheeding, Jenjarom</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kampung Orang Asli</td>
<td>Suku Temuan</td>
<td>42.7 (3.052892,101.870861)</td>
</tr>
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<td>Sungai Lalang Baru</td>
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<td></td>
</tr>
<tr>
<td>Kampung Orang Asli</td>
<td>Suku Temuan</td>
<td>50.4 (3.367189,101.6114859)</td>
</tr>
<tr>
<td>Serendah Rawang</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kampung Orang Asli</td>
<td>Suku Temuan</td>
<td>41.9 (3.018254,101.904570)</td>
</tr>
<tr>
<td>Broga</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kampung Orang Asli</td>
<td>Suku Temuan</td>
<td>23.2 (3.219237,101.779538)</td>
</tr>
<tr>
<td>Kemensah</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kampung Orang Asli Batu 12, Gombak</td>
<td>Suku Temuan</td>
<td>29.5 (3.267447,101.572125)</td>
</tr>
<tr>
<td>Tanjung Sepat</td>
<td>Suku Mahmeri</td>
<td>87.3 (2.676831,101.550540)</td>
</tr>
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</table>
### Table 3.2: List of rural and urban school

<table>
<thead>
<tr>
<th>Rural school</th>
<th>Approximate distances from Kuala Lumpur (km)(GPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sekolah Rendah Pangsun, Hulu Langat</td>
<td>44 (3.109210,101.856023)</td>
</tr>
<tr>
<td>Sekolah Rendah Lebuk Kelubi, Hulu Langat</td>
<td>20 (3.086167,101.790012)</td>
</tr>
<tr>
<td>Sekolah Jenis Kebangsaan (T) Highland, Klang</td>
<td>42 (2.992425,101.441479)</td>
</tr>
<tr>
<td>Sekolah Kebangsaan Sg Serai, Hulu Langat</td>
<td>45 (3.085770,101.790466)</td>
</tr>
<tr>
<td>Sekolah Kebangsaan Dato Abu Bakar Baginda,</td>
<td>38 (2.959104,101.729125)</td>
</tr>
<tr>
<td>Sekolah Kebangsaan Bukit Tandom,</td>
<td>60 (2.805363,101.622279)</td>
</tr>
<tr>
<td>Sekolah Kebangsaan Bukit Changgang, Banting</td>
<td>55 (2.830133,101.626976)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Urban school</th>
<th>Approximate distances from Kuala Lumpur (km)(GPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sekolah Rendah Jenis Kebangsaan Vivekananda</td>
<td>8  (3.131096,101.6886728)</td>
</tr>
<tr>
<td>Sekolah Rendah Jenis Kebangsaan Batu Caves</td>
<td>25 (3.235584,101.682904)</td>
</tr>
<tr>
<td>Sekolah Kebangsaan Methodist, Petaling Jaya.</td>
<td>42 (3.110412,101.656201)</td>
</tr>
<tr>
<td>Sekolah Kebangsaan Bandar Rahman Putra</td>
<td>31 (3.207915,101.568366)</td>
</tr>
<tr>
<td>Sekolah Kebangsaan Sinaran Budi Rawang</td>
<td>42 (3.316476,101.568366)</td>
</tr>
</tbody>
</table>
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).

\[
\frac{n}{\frac{z}{m}} \geq \left(\frac{z}{m}\right)^2 \times p \left(1 - p\right)
\]

n= sample size
z=standard score (1.96)
m=rate of sampling (5%)
p=estimated rate or case which happened in the population
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 ($\chi^2$) 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) (2.5 g/L), nutrient broth (Merck, Germany) (6.25 g/L) and heat inactivated horse serum (Gibco, New Zealand) (700 ml/L) in distilled water. In a 15 ml falcon tube, 5 ml of this mixture are added, sloped and insipissated at 80°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 5 ml 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 25 µl contained 1.5 unit of *Taq* Polymerase, 10 mM Tris-Hcl at pH 9, 50 mM KCl, 1.5 mM MgCl₂, 200 mM dNTP, BSA and 1.0 µl genomic DNA extract. The thermocycling profile was carried out as follows:- 3 minutes of denaturation, hold at 94°C followed by 30 cycle of 1 minute at 94°C, 1 to 5 minute at 57°C and finally 2 minute at 72°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™ PCR purification kit (QIAGEN) and sent for sequencing.
3.8 \textit{In vitro} cultivation of \textit{Blastocystis} sp.

\textit{Blastocystis} sp. was isolated from the stool samples of school children and local communities by \textit{in vitro} cultivation using Jones’ medium (Jones, 1946) supplemented with 10\% horse serum and incubated at 37\degree C (Zaman et al. 1997). \textit{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 \textit{Blastocystis} sp.

\textit{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 \textit{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 MgCl₂, 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 95⁰C for 5min; followed by 30 cycles of denaturation at 95⁰C for 1min, annealing at 56.3⁰C for 1min 30sec and extension at 72⁰C for 1min; one cycle of final extension at 72⁰C for 10min and final infinite holding at 10⁰C (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.

<table>
<thead>
<tr>
<th>Subtypes</th>
<th>STS primer sets</th>
<th>Product size (bp)</th>
<th>Sequence of forward (F) and reverse (R) primers (5’-3’)</th>
<th>Source of primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SB83</td>
<td>351</td>
<td>F:GAAGGACTCTCTGACGATGA R:GTCCAAATGAAAAGGCAGC</td>
<td>Nand II</td>
</tr>
<tr>
<td>2</td>
<td>SB155</td>
<td>650</td>
<td>F:ATCAGCCTACAATCTCCTC R:ATCGCCACCTCTCAAT</td>
<td>B*</td>
</tr>
<tr>
<td>3</td>
<td>SB227</td>
<td>526</td>
<td>F:TAGGATTTGGGTGTTGGAGA R:TTAGAAGTGAGGAGATGGAAG</td>
<td>HV93-13</td>
</tr>
<tr>
<td>4</td>
<td>SB332</td>
<td>338</td>
<td>F:GCATCCAGACTACTATCACCATT R:CCATTTCAGACAAACCCTTA</td>
<td>HJ96A S-1</td>
</tr>
<tr>
<td>5</td>
<td>SB340</td>
<td>704</td>
<td>F:TGGTCTTGTGTCTTCTCAGCTC R:TTCTTTCAGACACTCCGTCAT</td>
<td>HJ96-1</td>
</tr>
<tr>
<td>6</td>
<td>SB336</td>
<td>317</td>
<td>F:GTGGGTAGAGGAAGGAAAACA R:AGAAACAGTGATGAGATGAGA</td>
<td>SY94-3</td>
</tr>
<tr>
<td>7</td>
<td>SB337</td>
<td>487</td>
<td>F:GTCTTTCCCTGTCTATTCTGCA R:AATCGGTCTGCTTTCTCTG</td>
<td>RN94-9</td>
</tr>
</tbody>
</table>
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*. 

Chapter 3: Methodology
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 37ºC 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℃, 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. $1 \times 10^4$ cysts/ml, $1 \times 10^5$ cysts/ml and $1 \times 10^6$ 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 $1 \times 10^4$ and $1 \times 10^6$ 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™ 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 stain 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 ultrastructural studies following the procedure as stated in 3.15.

The experiment were repeated using Balb/c mice