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 \textit{D.fragilis} is higher in children than in adults (Girginkardesler et al., 2008).

The transmission of \textit{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 \textit{D. fragilis} infected person (Barratt, et al., 2011) which implicates this association to be an indicator of pathogenicity for \textit{D.fragilis} (Adnan et al., 2013).

In the present study a strong association of \textit{D.fragilis} have been shown with \textit{Blastocystis} sp although studies have shown other parasites namely helminthes to have an association with \textit{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°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°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.
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(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 vacuolar 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.

*Blastocystis* 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 18\textsuperscript{th} 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 18\textsuperscript{th} hour, showed a peak parasite count of $6.93 \times 10^4$ /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 18\textsuperscript{th} 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 18\textsuperscript{th} 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.

![Diagram of life cycle of D. fragilis](image)

**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 $1 \times 10^4$/ml were not infected. However, inoculation $1 \times 10^6$ cyst-like structures/ml at 24th hour post-inoculation, the number of trophozoites in the stools of infected rats were seen to be the highest implying that 24th 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. fragilis*. 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.