MOLECULAR EPIDMIOLOGY OF *GIARDIA DUODENALIS* INFECTIONS AMONG INDIGENOUS COMMUNITIES IN RURAL MALAYSIA

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ABSTRACT

Giardia duodenalis is a protozoan parasite that can cause significant diarrhoeal diseases and is the most common intestinal protozoan parasite worldwide. It affects especially children from the rural areas, who are also the most vulnerable group that suffers from nutritional disorders that have been linked to this parasite. Being part of the complex group of parasitic, bacterial and viral diseases that debilitate the susceptible communities in developing regions from achieving full development potential, *Giardia* was included in the 'Neglected Diseases Initiative' in 2004. In Malaysia, information on the epidemiology of *Giardia* infection among different indigenous communities is limited. *Orang Asli*, the indigenous people that live in West Malaysia are the communities most at risk of acquiring parasitic infections. Meanwhile, the prevalence of the infection among indigenous people in East Malaysia has not been well explored. In addition, there is a scarcity of information on the genetic diversity and the dynamics of transmission of *G. duodenalis.* This cross-sectional study was carried out to investigate the prevalence and risk factors of *Giardia* infection among indigenous people in rural Malaysia. It also aims to identify *G. duodenalis* assemblages and sub-assemblages present in these communities based on multilocus genotyping approach. Moreover, the genetic data obtained by the present study were combined with a larger global sequence data for genetic diversity analyses. Faecal samples were collected between April 2011 and February 2013 from 1,330 participants from seven states of Malaysia. The samples were examined by wet mount and formalin-ether sedimentation methods while demographic, socioeconomic and environmental information was collected using a pre-tested questionnaire. Samples positive for *Giardia* were genotyped by using markers targeting the *glutamate dehydrogenase* (*gdh*), *beta-giardin* (*bg*) and *triose phosphate isomerase* (*tpi*) genes. The *tpi* sequences obtained by the present study as well as sequences from the global data obtained from the NCBI GenBank were used to analyse the population structure of *G. duodenalis.* The overall prevalence of *Giardia* infection was 11.6%. The prevalence was found to be significantly higher among the aboriginal population in West Malaysia (13.6%) when compared to the indigenous people in East Malaysia (5.8%). Multivariate logistic regression identified age of ≤ 12 years, lacking of toilet at household, not washing hands before eating, not washing hands after playing with animals, not boiling water before consumption, bathing in the river, and not wearing shoes when outside as the significant risk factors of *Giardia* infection among these communities. A significant association between *Giardia* infection and diarrhoea

among the studied population was reported. The frequency of diarrhoeal cases was significantly higher among *Giardia*-infected participants from West Malaysia when compared to their counterparts from East Malaysia. Of the 154 positive samples, 138 successfully yielded amplification by at least one of the markers (*gdh*, *bg* and *tpi*). Genotyping result showed that 69 of the isolates were classified as assemblage A and 69 were classified as assemblage B. Mixed infections were detected in 49 samples using a *tpi*-based assemblage specific protocol. At the sub-assemblages level, isolates belonged to assemblage A were AII. High nucleotide variation found in isolates of assemblage B made subtyping difficult to achieve. Infection with *Giardia* assemblage A was significantly associated with the age of ≤ 12 years, not boiling water before drinking and had close contact with domestic animals. With regard to assemblage B, large family size, bathing in river, practicing indiscriminate/open defecation, not washing hands before eating, and playing with soil were the associated factors. No association between the assemblages and the presence of symptoms was found. Analysis of the Malaysian and global data showed that assemblages A, B, and E (the most prevalent assemblages in humans and animals), have different level of genetic diversity. Assemblage B had the highest level of both haplotype diversity and nucleotide diversity, followed by assemblage E. The analysis also revealed population expansion and high gene flow in all assemblages. In conclusion, the present study shows that the prevalence of *Giardia* infection is still high and of public health concern among indigenous populations in rural Malaysia. The findings of assemblage B and the anthroponotic genotype AII implicate human-to-human transmission as the most possible mode of transmission among Malaysian indigenous people. Meanwhile, the population genetic study provides new insight into the genetic diversity of *Giardia* assemblages in different geographical regions and should have brought enlightenment to the dynamics and distribution of *Giardia* infection. In view of the significant difference in the prevalence of *Giardia* infection among the different indigenous communities, implemented policies that may help in controlling the infection should be identified. Providing proper sanitation, as well as provision of clean drinking water and proper health education regarding good personal hygiene practices will help significantly in reducing the prevalence and burden of *Giardia* infection in these communities.

ABSTRAK

Giardia duodenalis merupakan parasit protozoa usus yang boleh menyebabkan cirit-birit. Ia merupakan parasit protozoa yang paling umum dijumpai di seluruh dunia terutamanya di kalangan kanak-kanak di kawasan luar bandar, di mana golongan inilah juga yang paling kerap mengalami ganguan nutrisi yang disebabkan oleh parasit ini. Sebagai sebahagian daripada kumpulan jangkitan parasite, bakteria, dan virus yang boleh melemahkan komuniti-komuniti terdedah dari kawasan negara sedang membangun ke arah mencapai pembangunan yang menjayakan, *Giardia* telah disenaraikan dalam 'Inisiatif Penyakit Terabai" ('Neglected Diseases Initiative'). Di Malaysia, maklumat mengenai epidemiologi jangkitan *Giardia* di kalangan masyarakat penduduk asal yang berlainan adalah terhad. Orang Asli, iaitu penduduk asal di Semenanjung Malaysia merupakan golongan yang paling terdedah kepada jangkitan parasit. Manakala, kajian prevalens yang melibatkan pribumi di Sabah dan Sarawak jarang dilakukan. Di samping itu, terdapat kekurangan maklumat mengenai kepelbagaian genetik dan dinamik penyebaran *G. duodenalis* juga. Kajian melintang adalah dijalankan untuk menyiasat prevalens dan factor-faktor yang berkait rapat dengan jangkitan *Giardia* kalangan penduduk asal di luar bandar Malaysia dan juga untuk menentu genotip *G. duodenalis*. Tambahan pula, tidak banyak penyelidikan dijalankan bagi memahami evolusi and genetik populasi parasit ini. Oleh itu, data genetik yang diperoleh dari kajian ini disertakan dengan data dari seluruh dunia telah digunakan untuk analisis kepelbagain genetik. Sampel najis dikumpulkan daripada 1,330 peserta dan diperiksa dengan mikroskop manakala maklumat demografi, sosioekonomi dan alam sekitar telah dikumpulkan dengan menggunakan soal selidik pra-diuji. Genotip sampel *Giardia* yang positif telah ditentukan dengan menggunakan locus yang menyasarkan *glutamate dehydrogenase* (*gdh*), *beta-giardin* (*bg*) dan *triose phosphate isomerase* (*tpi*). Di samping itu, urutan *tpi* diperoleh dari kajian ini berserta dengan data global dari GenBank telah digunakan untuk mengkaji struktur popuplasi *Giardia*. Prevalens keseluruhan adalah 11.6%. Orang Asli didapati mempunyai jangkitan yang lebih tinggi berbanding dengan penduduk pribumi. Analisis multivariat mengesahkan usia $\leq$ 12 tahun, kekurangan tandas di rumah, tidak mencuci tangan sebelum makan, tidak mencuci tangan selepas bermain dengan haiwan, tidak mendidih air sebelum penggunaan, mandi di sungai, dan tidak memakai kasut ketika di luar sebagai factor-faktor utama yang berkaitan dengan jangkitan *Giardia.* Selain itu, kaitan yang rapat di antara jangkitan *Giardia* dengan cirit-birit telah dijumpai. Di kalangan

orang yang dijangkiti *Giardia*, kekerapan Orang Asli yang mengalami cirit-birit adalah lebih tinggi jika dibandingkan dengan penduduk pribumi. Sebanyak 138 daripada 154 sampel yang positif telah berjaya menghasilkan urutan dengan menggunakan sekurang-kurangnya salah satu penanda. Hasil analisis menunjukkan bahawa 69 sampel dicirikan sebagai assemblage A dan 69 sebagai assemblage B. Campuran jangkitan telah dikesan dalam 49 sampel dengan menggunakan protocol berasaskan tpi yang khas. Pada tahap sub-assemblages, semua assemblage A dicirikan sebagai AII. Manakala, disebabkan variasi nukleotida yang tinggi, pencirian di tahap sub-assemblage sukar untuk dicapai bagi sampel assemblage B. Jangkitan dengan *Giardia* assemblage A berkait rapat dengan umur ≤ 12 tahun, tidak mendidih air sebelum minum dan mempunyai kontak yang rapat dengan haiwan domestik. Sementara itu, saiz keluarga yang besar, mandi di sungai, mengamalkan pembuangan air besar di tempat tidak tentu/ terbuka, tidak mencuci tangan sebelum makan, dan bermain dengan tanah adalah faktor-faktor yang berkaitan dengan assemblage B. Hubungan antara jangkitan assemblage yang berlainan dengan gejala-gejala jangkitan tidak dijumpai. Analisis data Malaysia dan global menunjukkan bahawa assemblage-assemblage A , B, dan E, mempunyai tahap kepelbagaian genetik yang berbeza. Assemblge B mempunyai tahap tertinggi dalam kedua-dua kepelbagaian haplotaip dan kepelbagaian nukleotida, diikuti oleh assemblage E. Kajian juga mendapati perkembangan penduduk dan aliran gen tinggi dalam semua assemblage. Sebagain kesimpulan, hasil kajian ini menunjukan bahawa prevalens jangkitan *Giardia* masih tinggi dan masih merupakan kebimbangan kesihatan awam di kalangan penduduk asal di luar bandar Malaysia. Hasil daripada penjumpaan assemblage B dan AII iaitu genotip yang berkaitan dengan jangkitan anthoponotik mencadangkan bahawa penyebaran dari manusia ke manusia sebagai mod penyebaran utama di kalangan penduduk asal Malaysia. Selain itu, maklumat genetik populasi memberikan wawasan baru terhadap kepelbagaian genetik *Giardia* di rantau geografi yang berbeza dan membawa penelitian kepada dinamik dan penyebaran jangkitan *Giardia*. Memandangkan terdapat perbezaan yang ketara dalam prevalens jangkitan *Giardia* di kalangan penduduk asal yang berlainan, polisi yang berjaya dalam usaha mengawal jankitan telah dikenalpasti. Penyediaan sanitasi, air minuman yang bersih serta pendidikan kesihatan mengenai amalan penjagaan kebersihan diri adalah usaha-usaha yang dapat mengurangkan prevalens dan beban jangkitan *Giardia* dalam komuniti ini.

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LIST OF SYMBOLS AND ABBREVIATIONS

≈	:	Almost equal to
&	:	And
asb	:	Assemblage
bp	:	Base pair
BI	:	Bayesian Inference
BLAST	:	Basic Local Alignment Search Tool
cm	:	Centimeter
CDC	:	Centre for Disease Control and Prevention
X^2	:	Chi-square test
CI	:	Confidence Interval
°C	:	Degree centigrade
DALYs	:	Disability-Adjusted Life Years
dNTPs	:	Deoxynucleotide triphosphates
DNA	:	Deoxynucleotide acid
=	:	equal
et al,	:	*et alia* (others)
e.g.,	:	*exempli gratia* (example)
FERG	:	Foodborne Disease Burden Epidemiology Reference Group
g	:	gram
g/L	:	Gram per liter
i.e.,	:	*id est* (that is)
≥	:	Greater than or equal to
JAKOA	:	*Jabatan Kemajuan Orang Asli*
km		Kilometer

p	:	Level of significance
$MgCl_2$	:	Magnesium chloride
NCBI	:	National Centre for Biotechnology Information
µg	:	Microgram
µL	:	Microliter
µM	:	Micromolar
ml	:	Mililiter
mm	:	Micrometer
MOH	:	Ministry of Health
OD	:	Odd ratio
%	:	Percent
PCR	:	Polymerase Chain Reaction
RAPD	:	Random Amplification of Polymorphic DNA
RPM	:	Revolution Per Minutes
RM	:	Ringgit Malaysia
RFLP	:	Restriction Fragment Length Polymorphism
SSCP	:	Single-Stranded Conformation Polymorphism
~	:	Similar to (tilde)
STH	:	Soil-Transmitted Helminth
spp	:	Species
SD	:	Standard Deviation
SPSS	:	Statistical Package for the Social Sciences
≤	:	Smaller or equal to
U	:	Unit
UV	:	Ultraviolet
WHO	:	World Health Organization

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CHAPTER I: INTRODUCTION

1.1 GENERAL INTRODUCTION

Giardia duodenalis (syn. *G. intestinalis*; *G. lamblia*), a flagellate enteric protozoan, is the most frequently reported intestinal parasite in the world, with about 280 million people suffering from symptomatic *Giardia* infection every year (World Health Organisation, 1996; Robertson, 2013). It is a major cause of acute and chronic diarrhoea, particularly among children in underprivileged communities, with a prevalence range of between 10% and 50% in developing countries (Savioli et al., 2006; Daly et al., 2010). Moreover, it is the most common human intestinal parasite in many developed countries, with a prevalence range of between 2% and 5% (Nygard et al., 2006; Yoder et al., 2012). A review by Baldursson and Karanis (2011) disclosed that 199 protozoan-related outbreaks were documented during the period 2004 to 2010 and *Giardia* was the culprit in 35.1% (70) of the cases. *Giardia* had long been disregarded as a public health threat despite its substantial occurrence and causing illnesses until it was finally included in the 'Neglected Diseases Initiative' in 2004 (Savioli et al., 2006; Alum et al., 2012).

The ingestion of *Giardia* cysts through contaminated food or water is the most common mode of transmission. In addition to which, person-to-person transmissions may occur through direct faecal-oral contact among family members (Balcioglu et al., 2003), children in day care centres (Duffy et al., 2013), and by sexual practices (oral-anal contact) (Pakianathan & McMillan, 1999; Escobedo et al., 2014). The cysts are instantly infectious once they are passed out through faeces, with the potential to remain infectious for several months as they can withstand unfavourable environmental conditions (Duffy et al., 2013).

The clinical presentation of *Giardia* infection varies from an asymptomatic carrier state to a severe disease which is associated with fat malabsorption and lactose intolerance due to disaccharidase deficiency (Gardner & Hill, 2001; Buret, 2008). Furthermore, *Giardia* infection contributes substantially to the 2.5 million annual deaths from diarrhoeal disease (Adam, 2001). Several studies have revealed that a chronic infection of *Giardia* during childhood contributes to protein-energy malnutrition, vitamin A deficiency, iron deficiency anaemia, zinc deficiency and poor cognitive and educational performance (Berkman et al., 2002; Gendrel et al., 2003; Al-Mekhlafi et al., 2010; Quihui et al., 2010; Al-Mekhlafi et al., 2013). Socioeconomic factors such as poverty, lack of adequate sanitation and water treatment systems, illiteracy, and poor hygienic practices have been identified as significant risk factors associated with *Giardia* infection in different communities (Faustini et al., 2006; Anuar et al., 2012a).

Giardia is well known as a ubiquitous parasite of a wide range of vertebrates from mammals to birds and amphibians. Despite its ubiquitous presence, the genus of *Giardia* which used to have over 50 described species (Thompson & Monis, 2004) now comprises six highly host specific members with only *G. duodenalis* being isolated from humans and other mammalian hosts (Robertson, 2013). Molecular analyses revealed that *G. duodenalis* is species complex with at least 8 distinct assemblages designated as A to H, which demonstrate similarity in morphologic characteristics but are phenotypically and genotypically heterogeneous (Plutzer et al., 2010). Of the host restricted assemblages, assemblages C and D infect dogs, assemblage F infects cats, assemblage E infects hoofed livestock, assemblage G infects rats, and assemblage H infects marine animals. Only assemblages A and B can cause infection in humans with the exception of a small fraction of cases where animal host specific assemblages C–F were reported in human (Sprong et al., 2009). Besides, both assemblages A and B are also capable of infecting animals. The subdivision into AI–AIII has provided greater

insights on the dynamic of transmission of these assemblages. Sub-assemblage AII has been regarded as anthroponotic whereas AI and AIII are predominant in livestock and wildlife, respectively (Thompson et al., 2000; Sprong et al., 2009; Nolan et al., 2010; Feng & Xiao, 2011). With regards to clinical manifestation, studies on the correlation between the assemblages and clinical symptoms have reported controversial results. Some studies have pointed out that symptoms are more associated with assemblage A (Haque et al., 2005), while others have found that assemblage B infections are more likely to be symptomatic (Pelayo et al., 2008).

Identification of cyst/trophozoite through microscopy observation or detection of antibodies or antigens by immunodiagnostic tests are common means of diagnosis for *Giardia* infection. However, the use of these techniques has its limitation in discriminating the different genotypes of *G. duodenalis* and the ensuing study of genetic diversity. Knowing the genetic diversity of *Giardi*a is an essential component in enhancing our understanding on the taxonomy, epidemiology and population genetics of this parasite. Molecular characterization and in the recent years, multilocus genotyping (MLG) have become the prevailing tool used for genotyping and subtyping of *G. duodenalis*. This has in turn facilitated in outbreak surveillance, contamination source-tracking, and unveiling zoonotic potential, dynamic transmission, and relationship of genotypes and hosts (Feng & Xiao, 2011).

1.2 PROBLEM STATEMENT

In Malaysia, data on the risk factors of *Giardia* infection is limited. However, several studies on various parasitic infections concerning the indigenous people living in Peninsular Malaysia (West Malaysia) have been enthusiastically carried out since the 1970s, these revealed that the prevalence of *Giardia* infection among *Orang Asli* (Aboriginal) communities could be as high as 29.2% (Bisseru & Abdul Aziz, 1970; Norhayati et al., 1998; Al-Mekhlafi et al., 2005; Mahdy et al., 2008; Anuar et al., 2012a). In addition to which, *Giardia* infection has been associated with protein-energy malnutrition and vitamin A deficiency among the children in these communities (Al-Mekhlafi et al., 2010; Al-Mekhlafi et al., 2013). Unfortunately, the majority of existing studies were conducted across a small sample size and limited geographic areas, most of which were exclusively limited to the *Orang Asli* population. This makes it difficult to apply the results to a larger population, or to make strong conclusions about risk factors or control intervention. To make matters more difficult, data on *Giardia* infections in East Malaysia is not available.

On the other hand, most of the previous studies in Malaysia did not molecularly characterize assemblages of *Giardia* or only enrolled participants from selected population and restricted geographic localities. Data on the prevalence of assemblages from human are scarcely available with only 144 isolates (46.5% assemblage A vs. 53.5% assemblage B) being identified using a single locus (i.e. *tpi* gene) in one immunocompromised study and two community based studies (Mahdy et al., 2009a; Lim et al., 2011; Anuar et al., 2014a). Besides, assemblage A was isolated from environmental samples including recreational lake water and water bodies in a zoo (Lim et al., 2009a; Lim et al., 2009b). Genotyping study was also conducted on animals and assemblages A and E were detected among goats (Lim et al., 2013). However,

subtyping of assemblages A and B in these studies was not conducted and this has limited our understanding on the transmission dynamics and the source of infection of giardiasis in this country. Apart from that, there is very little population genetic studies on this parasite, most of which gave emphasis in looking for evidence of sexual reproduction or recombination in *G. duodenalis* (Cooper et al., 2007; Lasek-Nesselquist et al., 2009; Takumi et al., 2012).

Within this context, this community-based study was conducted to provide a comprehensive dataset regarding the prevalence and potential factors associated with *Giardia* infection among different indigenous groups in Malaysia, with a preference towards evaluating the connection of *Giardia* infection in indigenous groups with socioeconomic status in rural Malaysia. Furthermore, it has been thought that risk factors of giardiasis are associated with different occurrence of genotype (Laishram et al., 2012a). Hence, corresponding to the lack of information about *G. duodenalis* assemblages in Malaysia and its potential association with risk factors, a multi-locus genotyping approach was used to investigate the epidemiology of *Giardia* infection among indigenous people from East and West Malaysia. Moreover, a population genetic analysis was made to explore the genetic diversity of different assemblages distributed in different regions and to measure the genetic structure in order to draw inferences regarding driving forces that control the evolutionary process of *G. duodenalis* and the potential factors that affect the dynamics and distribution of *Giardia* infection.

1.3 GENERAL OBJECTIVE

To study the epidemiology and genetically characterize *Giardia duodenalis* among indigenous communities living in rural areas of Malaysia.

1.4 SPECIFIC OBJECTIVES

1) To determine the prevalence and distribution of *Giardia* infection among the indigenous population of Malaysia.
2) To determine the risk factors of *Giardia* infection among the indigenous population of Malaysia.
3) To determine the genotype and subtype of *G.duodenalis* in the indigenous population of Malaysia using multilocus genotyping approach.
4) To investigate the population genetic structure of *G. duodenalis* and to understand the transcontinental distribution of the parasite genotypes by using isolates from Malaysian population as well as global sequence data based on *tpi* gene.

1.5 SIGNIFICANCE OF STUDY

1) The outcome of the study provides beneficial information on the prevalence and distribution of *Giardia* infection in Malaysian indigenous populations from different areas and background. Such data are crucial for designing and implementing innovative and effective control measures among these populations.
2) The findings of prevalence, genetic diversity and the associated risk factors provide basis to assess the burden of disease and the need for health services to the groups at risk.
3) The better understanding of the transmission dynamics provides evidence for designing of effective measures and intervention programs to control, prevent and eliminate the disease.

CHAPTER II: LITERATURE REVIEW

2.1 INTESTINAL PARASITES

2.1.1 Intestinal parasitic infections and their global burden

Human gastrointestinal infections especially those that lead to diarrhoea can be caused by a variety of enteric pathogens such as bacteria, viruses and parasites (O'Ryan et al., 2005). Children with moderate-to-severe diarrhoea were reported to have close association with nutritional insult (Kotloff, 2013). Among the infectious agents, intestinal parasitic infections (IPIs) are globally endemic and responsible for substantial morbidity and mortality particularly in least developed countries. As it is less likely for the parasites to kill the hosts, measurement of health impacts using disability-adjusted life year (DALY) instead of mortality rate alone is adopted. This measurement which combines both years of life lost from premature death (YLLs) and years lost due to disability (YLDs) has been used since it was developed for the Global Burden of Disease (GBD) study in 1990 to access the burden of diseases and injuries. Each DALY is regarded as one year of healthy life lost (Lopez et al., 2006). Of all the human parasitism caused by helminths (phyla Nematoda and Platyhelminthes), infections in the intestine by *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms (*Necator americanus* and *Ancylostoma duodenale*), collectively known as soil-transmitted helminthiasis (STHs) are the most common and persistent parasitic infections worldwide, followed by schistosomiasis and lymphatic filariasis (Lustigman et al., 2012).

The global burden due to STHs infections was estimated at 5.2 million DALYs with 3.23 million caused by hookworm, 1.31 million by *A. lumbricoides* and 0.64 million by *T. trichiura* (Pullan et al., 2014). These figures were much lower than the estimation made by Chan (1997) i.e. 39 million DALYs probably due to the adoption of

different thresholds and emphasis such as burden thresholds and long-term consequences related to morbidity (Brooker, 2010). Another member of the STHs, *Strongyloides* is believed to infect an estimated 30-100 million people and its health consequences in immune-suppressed individuals has been continuously reported in medical literature (Lustigman et al., 2012). Besides STHs, intestinal parasitic infections caused by other helminth groups have also been reported to cause significant health concern. The global burden of food-borne trematodiases including the liver flukes (*Opisthorchis viverrini*, *Op. felineu*s, and *Clonorchis sinensis*), lung flukes (*Paragonimus* spp.) and intestinal fluke (*Fasciolopsis buski*) was estimated at 665,000 DALYs (Furst et al., 2012) whereas burden linked to schistosomiasis (*Schistosoma mansoni* and *S. japonicum*) resulted in approximately 4.5 million DALYs (Utzinger & Keiser, 2004). Cysticercosis caused by the cestode *Taenia solium* led to global burden estimated at 503,000 DALYs (Murray et al., 2013). Other helminthic infection associated with the intestinal tract but were less prevalent include the nematode infections such as Angiostrongyliasis (*Angiostrogylus cantonesis*) (Deng et al., 2011), Anisakiasis (*Anisakis marina*) (Baird et al., 2014), Capillariasis (*Capillaria philippinensis*) (Limsrivilai et al., 2014), Enterobiasis (*Enterobius vermicularis*) (Pohl, 2013), Trichinellosis (*Trichenella spiralis*) (Murrell & Pozio, 2011); cestode infections such as Hymenolepiasis (*Hymenolepis nana*) (Soares Magalhães et al., 2013); trematode infections such as heterophyiasis (*Heterophyes heterophyes*), metagonimiasis (*Metagonimus yokogawai*), echinostomiasis (*Echinostoma revolutum* and *E. echinatum*) (Wu et al., 2009).

In 2006, the Foodborne Disease Burden Epidemiology Reference Group (FERG), an advisory body established by the WHO identified parasites that were transmissible from food to humans and produced substantial disease burden. The parasites which include *Giardia*, *Entamoeba*, *Cryptosporidium* spp., *Fasciola* spp.,

Trichinella spp., *Toxoplasma gondii*, *Echinococcus* spp., *Opisthorchis* spp., *Clonorchis* spp., *T. solium*, and *A. lumbricoides* would be prioritised for systematic reviews conducted by a group of experts (Torgerson et al., 2014). Amoebiasis, giardiasis and cryptosporidiosis caused by the infectious agents *Entamoeba histolytica, G. duodenalis* and *Cryptosporidium* spp. respectively are widely acknowledged as the most common pathogenic intestinal protozoan infections. A preliminary finding from the systematic literature review involving 10,450 studies by FERG showed that among the three parasites, *Giardia* had the highest global prevalence (median= 10.8%), followed by *Entamoeba* (median= 4.3%) and *Cryptosporidium* (median= 4.0%). The calculation of DALY was still ongoing and was expected to be published within the next 2 years (Torgerson et al., 2014). However, it was reported that infection with *E. histolytica* could result in 40,000 to 100,000 annual deaths and was deemed one of the deadliest parasitic infection (Stanley, 2003). With regard to incidence that associated with global waterborne outbreaks of protozoan parasites, *Cryptosporidium* spp. caused the highest number of incidence i.e. 120 (60.3%) in 199 outbreaks documented during 2004 to 2011. *Giardia* on the other hand contributed to 35.2% of the outbreaks followed by etiological agents (4.5%) caused by other protozoa such as *T. gondii*, *Cyclospora cayetanensis*, *Acanthamoeba* spp., *E. histolytica* and *Blastocystis hominis* (Baldursson & Karanis, 2011).

2.1.2 Intestinal parasitic infections and the indigenous people in Malaysia

Infection rates can often reflect standards of hygiene and levels of sanitation because STH and intestinal protozoa are infected through direct or indirect contact with faecal-derived materials contaminated with infective stage of parasites such as larva, ova and (oo)cysts. In line with this, urban dwellers in Kuala Lumpur, the capital city of Malaysia who stayed in good living condition with sufficient sanitation and water supply was reported to have low parasitic infection rate (6.9%) (Jamaiah & Rohela, 2005). In

contrast, several studies revealed that high infection rate still persist in communities living in rural setting, especially the aboriginal group in West Malaysia, the *Orang Asli.* Huat et al. (2012) has reported intestinal helminths infection rate of 37.0% among 79 children from a rural Malay community while an overall parasitic infection as high as 76.8% was observed among 716 villagers living in rural and remote parts of West Malaysia (Ngui et al., 2011). Studies sampled only from *Orang Asli* after 2000s showed a parasitic prevalence range from 50.6-100%. The intestinal parasites that commonly reported among the *Orang Asli* were STH i.e. *T. trichura* (31.1-98.2%), *A. lumbricoides* (23.8-67.8%), hookworms (3.9-37%) while the protozoa were *G. duodenalis* (5.2-29.2%), *E. histolytica/dispar* (3.6-32.4%), *B. hominis* (3.9-52.3%), *Cryptosporidium* sp. (1.3-5.2%) (Al-Mekhlafi et al., 2005; Noor Azian et al., 2007; Mahdy et al., 2008; Ahmed et al., 2011a; Ahmed et al., 2011b; Abdulsalam et al., 2012; Anuar et al., 2012a; Sinniah et al., 2012; Al-Harazi et al., 2013; Anuar et al., 2013a; Anuar et al., 2013b; Hartini et al., 2013; Nasr et al., 2013; Al-Delaimy et al., 2014; Anuar et al., 2014b).

Orang Asli remained the vulnerable communities in the country. They are generally perceived as communities with high prevalence and intensity of intestinal parasite. Apart from that, non-acute parasitic infections seem to be regarded as a normal condition of life by these communities itself. Socioeconomic development programmes aiming at improving the quality of life of the *Orang Asli* has been implemented since 1978. However, poor planning and implementation rather than inherent defect of the programme appeared to be the major concern that this vulnerable minority is still associated with poverty and continuously plagued with parasitic infections (Lim et al., 2009c). In a case study that involved an educational concept, known as Clusters of Excellence Policy (CoEP), it was explicitly shown how lack of coordination among government agencies and failure in recognizing the nature and culture of the *Orang Asli* had exposed various shortcomings during implementation. For example, the concept of

CoEP is to identify the niche areas and further enhance student's performance through allocation of special funds. When English language was selected as one of the niche areas, the teacher found it a formidable task as English is the third language of the *Orang Asli* and is rarely used in the community. The concept might sound ideal but the outcome might not be as expected due to programme design that was irrelevant to the *Orang Asli.* This had led to lack of interest and commitment from both the parents and students. Meanwhile, no in-depth deliberation for student's future development had seen high dropout rate in secondary education (Mohd Noor, 2012b). This report also highlighted other barriers in education related to beliefs and traditions of the *Orang Asli*. Parents tend to bare the perception that education is not important for female children. In addition, *Orang Asli* students are hyperactive in nature due to their traditional hunting and gathering way of life, therefore making it difficult for them to stay focus in class. Similarly, *Orang Asli* children often get certain habits that predispose them to parasitic infection such as eating/ playing with the soil and going around without proper footwear (Al-Delaimy et al., 2014). When a health education programme is designed to instil proper healthcare practice and hygiene habit, it needs to be relevant to their lifestyle. It is also important to make sure that they understand and will practice what had been learnt in their daily activities. It has been advocated that health awareness programme accompanied by sanitation improvement and periodic deworming to be conducted in school to effectively prevent the spread of disease in the community (Ahmed et al., 2011b), but more importantly is the regular evaluation and monitoring of the programmes (Lim et al., 2009c).

Besides, it should also be emphasized that raising school attainment of *Orang Asli* is imperative in integrating their participation with mainstream economy and minimizing social marginalization. These are in line with the effort of the government to advance their socioeconomic status and to eliminate poverty from their communities.

Infections with STH and intestinal protozoa are not only the effects of poverty but are also the causes of poverty. Though it is uncommon for IPIs leading to fatality, these infections are major health problems among the poor that cause illnesses, reduce physical fitness, impair growth and cognitive development and contribute to school absenteeism (Ahmed et al., 2011b; Ngui et al., 2011; Huat et al., 2012). Poverty on the other hand is linked with overcrowding, poor housing and environmental sanitation, under nutrition, low level of education and lack of access to healthcare. These are the interconnected factors that trap the *Orang Asli* in a vicious cycle of repeated infections and a number of health consequences (Lim et al., 2009c; Ngui et al., 2011; Hotez, 2014).

2.2 Historical background of *Giardia*

2.2.1 Discovery and description

In 1681, Antony van Leeuwenhoek made observation of his own diarrhoeic stool and found many tiny creatures which he described as animalcules moving prettily yet slowly with quick motion of its sundry little paws. It was later confirmed by Clifford Dobell (1932), the famous protozoologist who examined Leeuvenhoek's work that the microorganism in which the vivid description was made was trophozoite of *G. duodenalis*. The observation of *Giardia* was also one of the first finding of single-celled eukaryotes amongst many other great discoveries made by the 'Father of Microbiology' through the single-lensed microscope that he designed (Ford, 2005).

The first name given to this organism was *Cercomonas intestinalis* by Vilém D. Lambl in 1859 (Ford, 2005). The Czech physician who rediscovered *Giardia* in the stool of pediatric patient with diarrhoea presented the first formal description of the parasite including the size and shape of the trophozoite and the presence of ventral disc. Subsequently, the morphology of cyst and structures in trophozoite such as flagella and nuclei as well as the orientation of the ventral disc were described by Grassi in 1881. In

the next few decades, various detailed description and illustration of the parasites based on light microscope were actively published.

2.2.2 Nomenclature

Lambl was the first person that contributed the detailed description in 1859. However, *Cercomonas intestinalis* was found to be in homonymy with another flagellate initially known as *Bodo intestinalis* and was transferred to the genus *Cercomonas* nine years earlier before the use of the name by Lambl. On top of that, based on the taxonomic description of the genus *Cercomonas,* the placement of the parasite in that genus classification was found to be incorrect. In 1875, Davaine observed the species in another mammal i.e. rabbit and named it *Hexamita duodenalis*. It was noted later by researcher that the parasite did not match the description of genus *Hexamita.* Seven years later, *Giardia* was introduced as generic name by Kunstler (1882) for the flagellate that he found in tadpoles in commemoration of the French zoologist Alfred M. Giard. This name became widely accepted. At about the same period, Blanchard (1888) who did not realize the work done by Kunstler had suggested the name *Lamblia intestinalis* to commemorate contribution made by Lambl. It was only until 1914 that Alexeieff noted the species *Lamblia intestinalis* was in fact a member of the genus described by Kunstler and hence was synonymized with *Giardia* Kunstler, 1882 and the genus *Lamblia* became invalid (Thompson & Monis, 2011). Ever since then, several specific names were proposed for species that cause giardiasis in human and over 50 species had been described for *Giardia* occurred in various hosts.

Filice (1952) conducted a thorough evaluation of the described species and the existing differentiation criteria. His proposal of a discriminatory scheme to replace the host-specific taxonomy garnered recognition. Three distinct morphological groups consist of *G. duodenalis*, *G .muris* and *G. agilis* were established based principally on

morphology and morphometry of trophozoite (Figure 2.1). In addition, Filice put forward Stile's observation that the species described by Davaine (1875) in rabbit is identical to the form of *Giardia* found in human and considered that the specific name '*duodenalis*' should remain as a valid name. He adopted this name for the species infecting most mammals including humans (Smith & Paget, 2007; Thompson & Monis, 2011). Due to different interpretations of the International Code of Zoological Nomenclature and personal preference, *G. intestinalis* is another name used in parallel with *G. duodenalis* while *G. lamblia* is widely used in the medical literature referred to the parasite that infects human (Feng & Xiao, 2011; Thompson & Monis, 2011). The usage of *G. lamblia* has been described by Thompson et al. (2000) as an unfortunate case as it overshadows the epidemiological importance and recent findings which showed the presence of the same *Giardia* genotypes in both humans and animals. With the advent of ultrastructure screening, three additional species i.e *G. psittaci, G. ardeae* and *G. microti* were described and formed the current well recognized six members of the genus *Giardia* (Table 2.1).

2.2.3 Evolution of Eukaryotes

Giardia possesses several uncommon features such as the presence of two identical transcriptionally active nuclei and a unique ventral sucking disc. In addition, *Giardia* trophozoites produce energy by bacterial anaerobic pathways, lack of subcellular organelles such as mitochondria, peroxisomes and have only rudimentary Golgi apparatus (Ortega-Pierres, 2009; Rivero et al., 2010). The exhibition of these prokaryotic features had led many to believe that *Giardia* represents the transitional form between prokaryotes and eukaryotes which developed eukaryotic features (i.e. a complex cytoskeleton and subcellular membranous system including nuclear envelope, endoplasmic reticulum, lysosome-like peripheral vacuoles and transport vesicles) before mitochondrial symbiosis (Cavalier-Smith, 1983, 1987).

Alongside with phylogenetic analysis of ribosomal RNA, *Giardia* had been described in the literature as the `early branching eukaryote' and was commonly used as a model system to study the development of basic cellular processes and evolution of eukaryotes (Rivero et al., 2010). However, over the past decade, the advancement in phylogenetic studies has shown that the placement of *Giardia* and other anaerobic unicellular organisms near the base of ribosomal RNA tree was attributed to long-branch attraction caused by rapid evolution of the organisms (Brinkmann & Philippe, 2007; Koonin, 2010).

Emerging evidences from genomic and ultrastructural studies indicate that all extant groups of amitochondrial organisms have genetic marker of mitochondria and mitochondria-derived organelles such as mitosome in *Giardia.* Thus, it is suggested that instead of the supposedly primitive peculiarity, the microbial organisms are probably more advanced eukaryotes that underwent loss or modification of complex structures by reductive evolution. This could be a consequence of their adaptation to parasitic life style where the host has taken over the role of carrying out fundamental cell activities (Hjort et al., 2010; Koonin, 2010).

2.3 *Giardia* and parasitology

2.3.1 Classification

Giardia belongs to the phylum Metamonada, order Diplomonadida and family Hexamitidae (Smith & Paget, 2007). Diplomonads are a group of flagellates that occupy low-oxygen environment characterized by the absence of mitochondria and Golgi apparatus. The diplozoic group of diplomonads typified by the paired organelles (two karyomastigonts) is divided into two major subgroups i.e. Giardiinae and Hexamitinae with respect to the presence of cystostome. Cystostome, the feeding structure which is lacking in Giardiinae enables the Hexamitines (e.g. *Spironucleus, Trepomonas*) to perform phagocytosis (Schaechter, 2011).

At species level, host of origin of the parasite has been an orienting feature used in the taxonomy of *Giardia* in conjunction with morphological characteristics until Filice revised and proposed the three-species discriminatory scheme in 1952. He expounded that differentiation based on morphological description might be referred as a provisional solution before emergence of other discriminatory tools and evidences (Thompson & Monis, 2011).

Subsequently, six valid *Giardia* species of distinct morphology and structure identified from several classes of vertebrate hosts (e.g. mammals, amphibians and birds) are established: *G. duodenalis, G. agilis, G. muris, G. ardeae, G. microti* and *G. psittaci* (Table 2.1). An additional species to be been verified by molecular data was found in water monitor (*Varanus salvator*). This species had binucleated cysts and possessed features similar to *G. duodenalis* but lacked median bodies (Upton & Zien, 1997).

2.3.2 Assemblages and sub-assemblage of *G. duodenalis*

The development of cultivation method has facilitated substantial production of materials for the application of allozyme electrophoresis to genotype *Giardia* (Ivanov, 2011). In recent years, genotyping studies were further improved with the advancement of molecular techniques that allowed direct identification of the parasites in environmental samples including faecal matters from human and animal source, soil and water.

Analysis at the protein and genomic levels had shown extensive genetic variation in that one species, *G. duodenalis*, and not only that, the analyses had demonstrated several defined evolutionary lineages among isolates of *G. duodenalis* recovered from various host groups. Many had referred *G. duodenalis* as a species complex that exhibits identical morphology of cyst and trophozoite but shows genetic and phenotypic variations including host specificity and biochemical characteristics (e.g. metabolism, drug sensibility, infectivity and susceptibility of animal host models and preference of varied *in vitro* conditions) (Plutzer et al., 2010; Feng & Xiao, 2011; Robertson, 2013). The above mentioned lineages supported clusters now known as assemblages encompassing at least eight distinct genetic groups (A to H).

In light of the variations found within these assemblages particularly the host specificity, it has been proposed that each assemblage be regarded as distinct species and the names formerly used to represent host distribution to be adopted (Table 2.2) (Thompson & Monis, 2012). The proposed scheme is not unfounded, however, before it can be established as valid classification, description of each species has to be reviewed and supported by complementary biological and molecular evidences in accordance with the International Code of Zoological Nomenclature (ICZN) (Feng & Xiao, 2011).

Despite considerable high host specificity shown by assemblages A to H, assemblages A and B have been reported to infect a diversity of hosts from domestic animals, terrestrial wildlife to marine animals. They are also the only assemblages associated with human infection although there were a few exceptions where humans were infected by other assemblages such as C, D, E and F (Gelanew et al., 2007; Foronda et al., 2008; Soliman et al., 2011; Helmy et al., 2014).

As cited by Monis et al. (1999) Assemblages A and B collectively with its subgroups (AI, AII and BIII, BIV) correspond to original systems used by previous authors: Nash groups 1,2 and 3 (Nash & Keister, 1985), Andrews groups I, II, III and IV(Andrews et al., 1989) and Homan groups Polish and Belgian (Homan et al., 1992).

2.3.3 Morphology and life cycle

Giardia is monogenetic parasite with a life cycle alternating between proliferative trophozoite and the resistant cyst harbours in the duodenum, jejunum and upper ileum region of the intestine (Roberts et al., 2013). When ingested by a host, each cyst emerges into excyzoite, an intermediate form that undergoes two rounds of cytoplasmic divisions and one round of nuclear division to produce four trophozoites containing two diploid nuclei (Figure 2.2). The process known as excystation is activated by the acidic environment in the stomach followed by alkaline stimulation in the upper small intestine to establish infection and clinical symptoms (Svard et al., 2003).

The bilaterally symmetrical trophozoite has four pairs of flagella (anterior, posterior, ventral and caudal) and shaped like a pear bisected lengthwise appearing dorsoventrally flattened and convex on the dorsal surface. The trophozoites of *G. duodenalis* are 12-15 μm long and 6-8 μm wide. It can be easily identified under the light microscope because of its appearance resembling a face of an old man wearing glasses. The two distinct nuclei on the ventral adhesive disc at the anterior part liken the

eyes on the face while the present of a pair of medium bodies in the mid part of the body give a smiley face to the old man. The ventral adhesive disc and the medium bodies are structures made of microtubules unique to the diplomonad with the former working in conjunction with ventral flagella to attach to the host epithelial cells while the function of the latter remain undefined in spite its differences in shape and position among the species has been used as a taxonomic criterion (Andersson et al., 2010; Roberts et al., 2013). Despite its unattractive feature in causing illness, *Giardia* is described as among the most beautiful organisms and the tumbling movement of the motile trophozoite likened to a falling leaf as the flagellate swims and rotates around its long axis (Ghosh et al., 2001; Smith & Paget, 2007).

Only active and feeding trophic stage present in the small intestine and watery stools. They multiply rapidly by asexual binary fission and colonize the mucosal surface. The flagellates transform to a spherical form and lose the ability to attach during the early phase of encystation when dehydration takes place as the intestinal contents migrate to the lower part of the small intestine. Cholesterol deprivation or high concentrations of primary bile salts coupled with changes of pH (7-8) are among factors that trigger the process (Svard et al., 2003). The differentiation of trophozoite that involves nuclear division and DNA replication without cytoplasmic division results in the forming of a mature cyst with four tetraploid nuclei (Figure 2.2). However, the transit time through the intestinal tract will also determine the presence of either binucleate or quadrinucleate cysts in feces (Smith & Paget, 2007). The cysts of *G.duodenalis* are 8-12 μm long and 7-10 μm wide. Most of the organelles and structures present in the trophozoite are dissembled or reorganized leaving only internalized flagella and disc fragments (Carpenter et al., 2012). Thick wall enveloping cyst is form to protect the parasite from adverse conditions when excreted in faeces and serve as a mode of transmission to infect a new host.

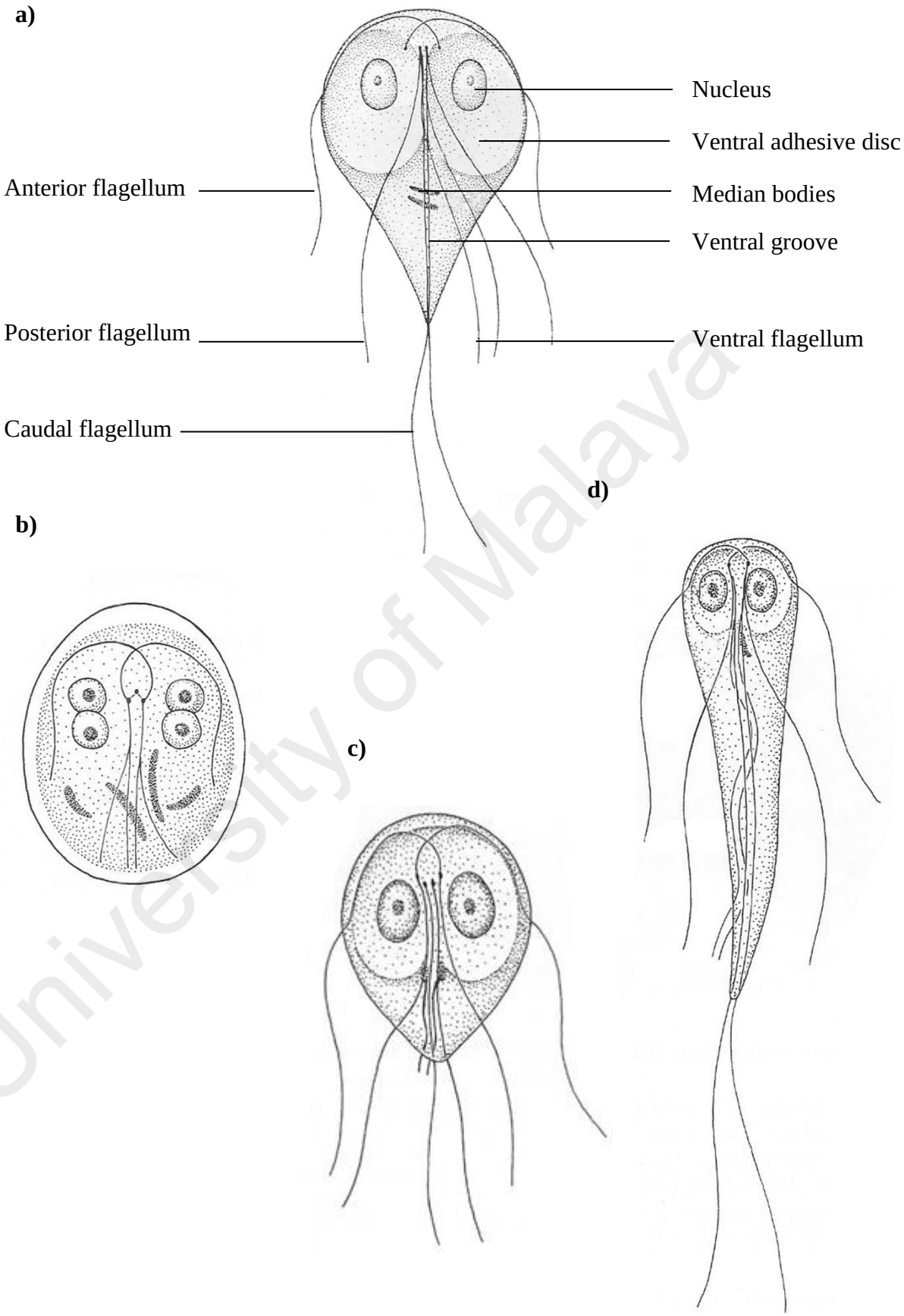

Figure 2.1: Trophozoites and cyst of *Giardia* (Roberts et al., 2013)
a) Trophozoite *G. duodenalis* b) Cyst of *G. duodenalis* c) *G. muris* d) *G. agilis*

Table 2.1: Members and their characteristics of the genus *Giardia*

Species	Host	Morphological characteristics by light microscopy	Additional characteristics by electron microscopy	Dimension of trophozoite length/width (µm)
G. duodenalis	Wide range of mammals including humans	Pear-shaped with one or two transverse, claw-shaped median bodies	Cysts contain single trophozoite without ventral disc	12–15/6–8
G. agilis	Amphibians	Long and narrow with club-shaped median bodies	N/A	20–30/4–5
G. muris	Rodents	Broad and rounded with small round median bodies	N/A	9–12/5–7
G. ardeae	Birds	Rounded with round-oval to claw shaped median bodies	Prominent notch in ventral disc; single caudal flagellum similar to *G. muris;* long, slender and tear-drop nuclei	~10/~6.5
G. psittaci	Birds	Similar to *G. duodenalis*	No ventro-lateral flange and marginal groove	~14/~6
G. microti	Rodents	Similar to *G. duodenalis*	Cysts contain two differentiated trophozoites with mature ventral discs	12–15/6–8
G. varani	Reptiles	Morphometry similar to *G. duodenalis*. Lacks median bodies	N/A	

Source: Adapted from Adam, 2001; Thompson & Monis, 2011

Table 2.2: Host distribution of *Giardia duodenalis* assemblages and their formerly used names

Assemblage	Host distribution	Proposed names based on original taxonomy
A	Wide range of mammalian hosts including humans, primates, livestock, dogs, cats and wild animals.	*Giardia duodenalis*
B	Wide range of mammalian hosts including human, dogs, cats and wild animals.	*Giardia enterica*
C	Dogs and other canids	*Giardia canis*
D	Dogs and other canids	*Giardia canis*
E	Hoofed livestock	*Giardia bovis*
F	Cats	*Giardia cati/ Giardia felis*
G	Rats	*Giardia simondi*
H	Marine mammals	?

Source: Thompson & Monis, 2012

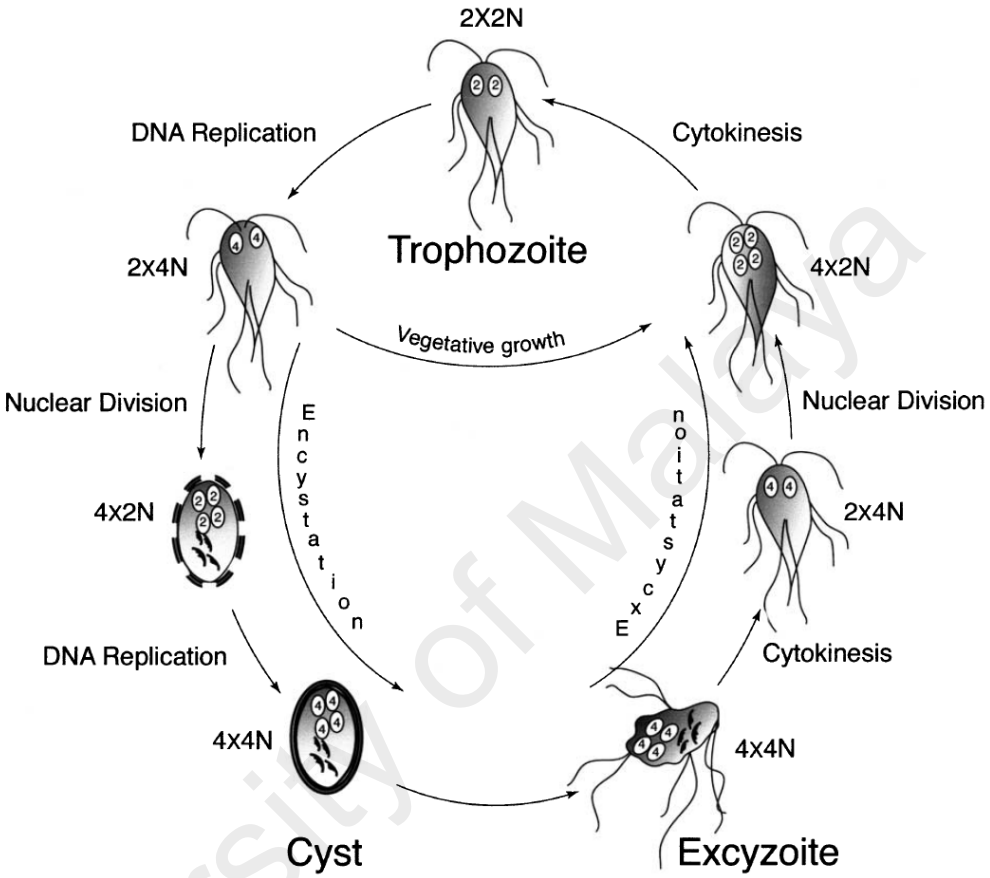

Figure 2.2: Life cycle of *Giardia* Svard et al., 2003

2.3.4 Epidemiology

Giardia is spread by faecal-oral route in which water is a major vehicle of transmission. Contamination of foodstuffs such as fruits and vegetables with cysts during production, collection, transport, and preparation is also a potential route for human infection (Smith & Paget, 2007; Dorny et al., 2009). Contaminant could be originated from faecal or faecal derived materials such as soil or carried over by vectors such as birds and insects (Robertson, 2013; Zhao et al., 2014). Being recognised as one of the major waterborne pathogens, *Giardia*-related outbreaks have been linked to exposure to contaminated swimming pools and recreational waters as well as consumption of cyst-containing water or food. The latter can be attributed to contamination of reservoirs, untreated or inadequate treatment of water supply, and post treatment contamination (Baldursson & Karanis, 2011). Person-to-person transmission has been reported in child care centre (Duffy et al., 2013) and individuals that conduct oral-anal sexual activity (Escobedo et al., 2014). Transmission of *Giardia* due to occupational exposure was also reported. *Giardia* cysts were detected in vegetables cultivated using untreated wastewater and this poses considerable risk of infection to the farmers, handlers, and residents working and staying around the irrigation water (Srikanth & Naik, 2004; Vuong et al., 2007).

Giardia has a global distribution but dwellers from developed countries generally have lower infection rate that ranges between 0.4-7.6% (Feng & Xiao, 2011). High prevalence rate did occasionally occur in poor regions while travellers returning from endemic areas are at high risk of contracting symptomatic giardiasis (Ortega & Adam, 1997; Feng & Xiao, 2011). Coupled with frequent report of outbreak related to drinking water and in child care centres, giardiasis is viewed as a re-emerging infectious disease in developed countries (Ortega-Pierres, 2009; Escobedo et al., 2010). It was

speculated that travellers contracted the infection may raise the risk of water source contamination with *Giardia* and change in the incidence of infections in non-endemic areas (Nygard et al., 2006).

In developing countries, prevalence is higher with most studies reported a range between 8% and 30% (Feng & Xiao, 2011). Although *Giardia* infection is more prevalent in developing countries, the reported prevalence rate varied from country to country depending on group or background of the recruited participants (e.g. hospital patients, rural or urban residents, children or adult). Hospital-based studies reported prevalence rate varied from 0.4% among residents of Bangkok, Thailand (Tungtrongchitr et al., 2010), 1.9% among children under five with diarrhoea in Tanzania (Moyo et al., 2011), and reached up to 73.4% among pre-school and school-going children with gastrointestinal illness in western Nepal (Easow et al., 2005).

Transmission of *G. duodenalis* is high in areas with substandard level of personal hygiene and poor sanitary infrastructure and causes substantial morbidity especially in socioeconomically disadvantaged populations (Escobedo et al., 2010; Lal et al., 2013). Aboriginal communities have been identified as one the high-risk populations vulnerable to *Giardia* infection and infectious diseases due to certain cultures and habits (Escobar-Pardo et al., 2010) and their unfavourable living condition such as overcrowding, inadequate access to safe water and sanitation facilities. The key underlying causes for these disadvantages are likely to be social inequality and powerlessness (McDonald et al., 2008). Prevalence reported in recent studies among aboriginal communities remained high. In the Northern Territory of Australia where approximately 80% of the indigenous population live in remote or very remote areas, a prevalence of 66.7% was reported (Asher et al., 2014). The prevalence rate was 30.7-38.2% among native Brazilian children aged 2 to 9 years (Escobar-Pardo et al., 2010), 27.7% among aboriginal people aged 1 to 49 years in Argentina (Menghi et al., 2007).

The first report of *Giardia* infection in Malaysia comprising *Orang Asli* children in hospital and school children of major races was made by (Bisseru & Abdul Aziz, 1970). This report together with subsequent studies of *Giardia* infection involved particularly *Orang Asli* communities undertaken in the 1970s-1990s recorded a prevalence rate of between 4.8% and 25.0% (Lim et al., 2008). Studies in the 2000s and early 2010s demonstrated that the prevalence of *Giardia* infection among the *Orang Asli* remained high at a range of 4.0% - 29.2% (Table 2.4). In East Malaysia, parasitic studies were relatively few and the infection rate ranged between 3.0-8.6% (Sagin et al., 2002; Nor Aza et al., 2003).

The general population of Malaysia had lower infection rate with most studies reported infection rate in the range between 3-9%. Four studies with infection rate exceeded 10% were conducted during 1970s-1990s. Two previous studies from 2000s forward showed prevalence rates of 5.7% and 10.4%. The former enrolled immunocompromised patients while the latter were subjects from rural residents (Table 2.3).

From the review by Baldursson and Karanis (2011), it was shown that majority of the giardiasis outbreak were recorded from developed countries. In this review, *Giardia* and *Cryptosporidium* were reported as the cause of an outbreak of diarrhoeal disease that occurred in Malaysia. However, it has been clarified that rotavirus was actually the main culprit of this outbreak (Hakim et al., 2007). In Malaysia, giardiasis is not notifiable under Prevention and Control of infectious Diseases Act 1988. Most diarrhoeal cases are generally given treatment based on diagnosis and stool examination is not routinely carried out. Only severe or suspected cases would be referred for further parasitological analyses. In Malaysia, giardiasis is not notifiable under Prevention and Control of infectious Diseases Act 1988. Most diarrhoeal cases are generally given treatment based on diagnosis with only severe or suspected cases would be referred for

further parasitological analyses. The lower incidence of outbreak reported in Malaysia and perhaps other developing countries could be due to different monitoring and reporting system and better investigation of diarrhoeal stool and in developed countries. Furthermore, due to self-limiting nature of the infection and the common occurrence of mild acute gastroenteritis in Malaysia, infected individuals tend not to seek further treatment unless symptoms are not resolved. Information about the prevalence of *Giardia* infections are mostly obtained from published cross-sectional studies or investigations of diarrhoeal samples in hospital that were not related to outbreak.

Table 2.3: Prevalence of human *Giardia* infection from 1970s- 2010s in different populations of Malaysia

Study population	Sample size	Infected (%)	Assemblage	References
Orang Asli				
Children (hospital)	100	25	N/A	Bisseru and Abdul Aziz (1970)
Children	162	23.1	N/A	Rahmah et al. (1997)
Children (2-15 years old)	281	24.9	N/A	Al-Mekhlafi et al. (2005)
Children (school)	307	21.8	N/A	Al-Harazi et al. (2013)
Children (school)	374	22.2	N/A	Al-Mekhlafi et al. (2013)
Children (4-12 years old)	111	12.6	N/A	Hartini et al. (2013)
Children (school)	498	28.3	N/A	Al-Delaimy et al. (2014)
All ages	1273	10.8	N/A	Dunn (1972)
All ages	126	4.8	N/A	Dissanaike et al. (1977)
All ages (urban)	159	6.9	N/A	Kamel et al. (1994)
All ages	59	11.9	N/A	Karim et al. (1995)
All ages (urban)	127	18.9	N/A	Lim et al. (1997)
All ages (diarrhoeal disease outbreak)	74	17.6	N/A	Hakim et al. (2007)
All ages	130	29.2	N/A	Noor Azian et al. (2007)
All ages	321	23.7	Assemblage A: 1; Assemblage. B: 41	Mahdy et al. (2009a)
All ages	75	4	N/A	Sinniah et al. (2010)
All ages	77	5.2	N/A	Sinniah et al. (2012)
All ages	500	20	Assemblage A: 62; Assemblage B:36	Anuar et al. (2014a)
All ages	269	3.7	N/A	Lee et al. (2014a)
Indigenous in Sabah and Sarawak				
All ages (interior communities in Sarawak)	330	3	N/A	Sagin et al. (2002)
All ages	150	8.6	N/A	Nor Aza et al. (2003)

Table 2.3: (Continued)

Study population	Sample size	Infected (%)	Assemblage	References
Others				
Children (school, major races)	678	5.6	N/A	Bisseru and Abdul Aziz (1970)
Children (<7 years, major races)	305	2.6	N/A	Hamimah et al. (1982)
Children (school, major races)	271	8.5	N/A	Sinniah (1984)
Children (school, Island community)	297	9.3	N/A	Sinniah (1988b)
Children (school, various communities including OA)	456	14.7	N/A	Rajeswari et al. (1994)
Children (school)	7557	0.2	N/A	Shekhar et al. (1996)
Children (cancer patients)	237	6	N/A	Menon et al. (1999)
Adults (Indonesian immigrants)	198	7.1	N/A	Sinniah (1988a)
All ages (Indian palm estate workers)	150	11.3	N/A	Sinniah et al. (1978)
All ages (island community)	83	6	N/A	Nawalinski and Roundy (1978)
All ages (major races)	529	9.5	N/A	Che Ghani et al. (1987)
All ages (various communities)	7995	8.4	N/A	Lai (1992)
All ages (various communities)	1220	15.5	N/A	Che Ghani (1993)
All ages (rural communities)	917	19.2	N/A	Norhayati et al. (1998)
All ages (rural and remotes areas)	716	10.4	N/A	Ngui et al. (2011)
Adults (immunocompromised patients)	122	5.7	Assemblage A: 4	Lim et al. (2011)

N/A: Data not available

2.3.5 Clinical manifestation and pathogenesis

Individuals infected with *Giardia* have varied clinical manifestations but most often the infection is asymptomatic and self-limiting. In symptomatic cases, the degree of symptoms and severity varies among different individuals and present in two disease phases: acute and chronic (Smith & Paget, 2007; Buret, 2008). Acute giardiasis develops symptoms including fatty diarrhoea, yellowish foul-smelling stools, bloating, nausea, vomiting, abdominal pains and fatigue with an incubation period of one to two weeks (median period of 7 days) (Hopkins & Juranek, 1991; Smith & Paget, 2007). Blood and mucus are seldom present in stool specimens which rarely contain pus cells when observed using microscope. Sometimes, symptoms develop before excretion of cysts or trophozoites can be detected in the stool when prepatent period (average of 9.1 days) exceeds incubation period (Smith & Paget, 2007). *Giardia* infection is slower to manifest symptoms, thus it can often be distinguished from bacterial and viral infections (Gardner & Hill, 2001). The symptoms of acute stage are usually short-lived (3-4 days) but pass into chronic stage if the duration prolongs to months or years characterized by persisting or recurrent signs leading to malabsorption, weight loss and debilitation (Robertson, 2013).

Although giardiasis has a wide clinical spectrum, the pathogenesis is not fully understood. The infection is not invasive in nature but it involves enterocyte and microvillus alterations. Studies done in epithelial cell lines and *in vivo* showed both parasite and host immune system have their roles in the alterations that lead to diarrhoea and malabsorption. Adherence and colonization of trophozoites to the epithelial cells not only creating physical obstruction that diminish absorptive surface area but also causing enzyme deficiencies and producing suction that damages the microvilli (Robertson, 2013). Subsequent histopathological alterations are associated with the increase of intestinal permeability (Scott et al., 2002), enterocyte apoptosis (Chin et al.,

2002; Troeger et al., 2007), shortening of microvilli and inflammation targeted at the enterocytes (Hodges & Gill, 2010). The combined effect of the physical obstruction and histopathological alterations causes reduction in digestive enzymatic activity such as lipase and intake of water, electrolytes and nutrients as well as triggers fluid accumulation in intestinal lumen leading to diarrhoea and steatorrhoea (Smith & Paget, 2007; Buret, 2008; Halliez & Buret, 2013).

In young children, chronic or repeated infection especially in endemic areas with poor sanitation and where asymptomatic cases often pass unnoticed may cause retarded growth and pose negative impacts on their cognitive development possibly due to malabsorption of carbohydrate, vitamins such as vitamin A and B 12 and trace elements such as zinc and iron (Ignatius et al., 2012; Al-Mekhlafi et al., 2013; Halliez & Buret, 2013). In additional to the typical clinical features that often observed in symptomatic infections, the symptomatology of the disease can be more intricate as some atypical manifestations including allergies, pruritis, urticarial, uveitis, ocular complications, arthritis as well as post-infectious irritable bowel syndrome have been reported in patients infected with *Giardia* (Halliez & Buret, 2013; Robertson, 2013).

The different clinical onset may lie in host factors such as nutrition mode, age, immune response (Robertson et al., 2010). Other contributing factors could be co-infection with other agent (e.g. virus, bacteria and other parasites), infective dose and route of infection. There was evidence showed that the interaction with the environment such as intestinal microbiota may be responsible for the parasite's virulence and pathogenicity (Torres et al., 2000; Shant et al., 2002). Acquired immunity after initial infection may emerge as an important protection toward the parasite (Hanevik et al., 2011).

Besides, it is thought that *Giardia* can evade host immune detection by exhibiting antigenic variation on its surface although the role of variant-specific surface protein (VSP) can be more complex (Nash, 2002). This, together with other parasites factor such as multiplication rate, and different response to treatment and host immune system might be associated with genotype and contribute to the differences in clinical presentation (Robertson et al., 2010). Attempts have been made to relate type of assemblage with pathogenesis, symptom and severity of disease. However, reports that indicated this association are not conclusive and appeared to be more complex that was initially thought (Table 2.4). The differences could be related to the type of samples with some studies restricted to clinical and/or symptomatic samples while some studies obtained from communities where asymptomatic infections were high and symptomatic infection might be co-infected with other intestinal pathogens. Hanevik et al. (2007) found that genotype that was less prevalent in a community appeared to be causing most symptoms. In two separate studies from Norway and Ethiopia where assemblage A was the most commonly found genotype, it was assemblage B that was found to be associated with diarrhoea and other digestive symptoms (Gelanew et al., 2007; Hanevik et al., 2007). In another study, Haque et al. (2009) reported similar finding where assemblage B was the more prevalent genotype, however more patients with assemblage A developed diarrhoeal symptom compared to assemblage B. Consistent with these findings, Robertson et al. (2010) speculated that the introduction of a new genotype to communities that have established an endemic genotype might induce particularly severe symptoms while mixed infections of two different genotypes might have synergistic pathology effects. On the other hand, it was suggested that host factors may affect the presence of symptoms more than *Giardia* genotypes (Sahagun et al., 2007).

Table 2.4: Correlation between assemblages A and B with symptoms

Location	Type of study	Symptoms	Asb. A	Asb. B	Methodology	Conclusions and other relevant findings	Reference
Australia	Children from day-care centres, under age of 5	No symptoms	1	13	Sequencing of *18S rDNA* gene	Children infected with asb. A were 26-fold greater risk to have diarrhoea than assem. B; No mixed infection.	Read et al. (2002)
		Diarrhoea	6	3			
Turkey	Stool and duodenal aspirate samples from individuals referred by outpatient specialists	No symptoms	2	22	PCR-RFLP at the *tpi* gene	Asb. A was associated with diarrhoeal symptoms while asb. B was typically seen in asymptomatic infections.	Aydin et al. (2004)
		Diarrhoea	17	3			
Spain	Outpatients clinics, asymptomatic patients from endemic areas, and a study of the patient's knowledge about parasitic infection	No symptoms	29	26	PCR-RFLP at the *tpi* gene	Asb. A was correlated with symptomatic infections and asb. B was correlated with asymptomatic infections.	Sahagun et al. (2007)
		Diarrhoea, abdominal pain, rapid weirght loss, abdominal cramps, flatulence, and nausea	14	35			

Table 2.4: (Continued)

Location	Type of study	Symptoms	Asb. A	Asb. B	Methodology	Conclusions and other relevant findings	Reference
Bangladesh	Patients admitted to Dhaka Hospital (all ages)	No symptoms (control group)	10	174	Real-time PCR on *18S rDNA* gene	Asb. A was associated with acute diarrhoea; Inverse correlation between parasite load and diarrhoea; Mixed infection in 4.2% of symptomatic cases and 7.5% in control cases.	Haque et al. (2009)
		Acute diarrhoea	29	109			
India	Children from urban slums in South India	No symptoms	2	48	PCR-RFLP at the *tpi* gene	Asb. A and mixed infection were correlated with symptomatic infection.	Ajjampur et al. (2009)
		Diarrhoea, vomiting, and fever	5	40			
Netherlands	Dutch patients aged 8- 60, who consulted their GP	Intermittent diarrhoea and moderate symptoms	9	0	PCR-RFLP at the *gdh* gene	Asb. A was correlated with mild, intermittent type of diarrhoea while asb. B was correlated with severe, actual/persistent diarrhoea.	Homan and Mank (2001)
		Persistent diarrhoea and severe symptoms	0	9			
Egypt	Patients with giardiasis	No symptoms	2	6	PCR-RFLP at the *gdh* gene	Asb. A was correlated with symptomatic infections.	Sadek et al. (2013)
		Diarrhoea, abdominal cramp, and flatulence.	25	2			

Table 2.4: (Continued)

Location	Type of study	Symptoms	Asb. A	Asb. B	Methodology	Conclusions and other relevant findings	Reference
Iran	Samples from clinical laboratories	No symptoms	4	19	PCR-RFLP at the *tpi* gene	Asb. A was correlated with symptomatic infections and asb. B was correlated with asymptomatic infections.	Pestechian et al. (2014)
		Diarrhoea	36	6			
Ethiopia	From two hospitals, a day-care centre, a primary school, and two rural communities	No symptoms	12	0	PCR-RFLP /sequencing of the *bg* gene	Asb. B was correlated with symptomatic infection; Higher number of mixed infection (25%), mostly associated with symptoms (83%).	Gelanew et al. (2007)
		Diarrhoea, abdominal pain, and nausea	19	12			
Cuba	Primary school children, mean age of 9 years	No symptoms	4	1	Sequencing of the *gdh* and *bg* genes	Asb. B was correlated with symptomatic infections; Correlation between load of cysts and symptoms	Pelayo et al. (2008)
		Diarrhoea and at least two other symptoms (nausea, vomiting, loss of appetite, weight loss, and abdominal pain)	4	10			

Table 2.4: (Continued)

Location	Type of study	Symptoms	Asb. A	Asb. B	Methodology	Conclusions and other relevant findings	Reference
Argentina	Community samples from rural and shanty towns from Argentina	No symptoms	0	2	PCR-RFLP at the *tpi* gene	Asb. A (AII) was correlated with oligosymptomatic infection (none had diarrhoea) while asb. B with asymptomatic/ polysymptomatic infections (many presented diarrhoea).	Minvielle et al. (2008)
		Diarrhoea, abdominal pain, appetite loss, and vomiting.	3	38			
Malaysia	Aboriginal community, mean age 10 years	No symptoms		14	Sequencing of *18S rDNA* gene	Asb. B was correlated with symptomatic infection.	Mahdy et al. (2009b)
		Diarrhoea, abdominal discomfort, vomiting, and nausea	1	18			
Saudi Arabia	Primary school children aged 6 to 12 years	No symptoms	16	0	PCR-HRM at the *IGS* region of rDNA gene	Asb. B was correlated with symptomatic infection.	Al-Mohammed (2011)
		Diarrhoea	7	15			
Albania	Children with acute gastroenteritis	All symptomatic	10	12	Sequencing of *18S rDNA* gene	No mixed infection was found.	Berrilli et al. (2006)

Table 2.4: (Continued)

Location	Type of study	Symptoms	Asb. A	Asb. B	Methodology	Conclusions and other relevant findings	Reference
Argentina	School children from suburban community	No symptoms	7	5	PCR-RFLP at the *tpi* gene	Only asb. B was associated with diarrhoea, vomiting, and weakness; One mixed infection case had diarrhoea and abdominal pain	Molina et al. (2011b)
		Diarrhoea, abdominal pain, vomiting, and weakness	3	8			
Cuba	Mixture of clinical cases and asymptomatic children enrolled in a school-based surveillance program	No digestive symptoms	8	0	Assemblage-specific PCR at the *tpi* gene	Asb. B was correlated with characteristic symptoms of *Giardia* such as diarrhoea, flatulence, and abdominal pain	Puebla et al. (2014)
		Diarrhoea, flatulence, and abdominal pain	26	36			
Brazil	Children from an urban shanty town	No symptoms	2	14	Real-time PCR on *18S rDNA* gene	No significant differences between symptoms and genotypes; Mixed infection in 10% of the samples; Higher load of cysts in mixed infection or asb. B infection.	Kohli et al. (2008)
		Diarrhoea	7	29			

Table 2.4: (Continued)

Location	Type of study	Symptoms	Asb. A	Asb. B	Methodology	Conclusions and other relevant findings	Reference
United Kingdom	Symptomatic cases of giardiasis in south-west London (all ages)	Diarrhoea, vomiting, nausea, headache, weight loss, fever, and abdominal pain	14	31	Sequencing of *tpi* and *18S rDNA* genes	Both genotypes caused similar illnesses, however, asb. A was associated with fever; mixed infection in 3% of samples.	Breathnach et al. (2010)
Rwanda	Children from communities and health facilities under age of 5	Diarrhoea Vomiting Abdominal pain Severe malnutrition Underweight	1 1 5 28 47	12 2 3 2 4	Sequencing of *tpi* gene	Infection with asb. A isolates showed increased proportions of abdominal pain and vomiting; asb. B infections associated with underweight and clinically assessed severe malnutrition.	Ignatius et al. (2012)
Sweden	Patients with giardiasis	Diarrhoea Flatulence Vomiting	48 33 17	86 73 29	Sequencing of *tpi*, *gdh*, and *bg* genes	Asb. B was correlated with flatulence but no significant difference between both asb. with diarrhoea, abdominal pain, and vomiting.	Lebbad et al. (2011)
Australia	*Giardia* samples from pathology laboratory	N/A	N/A	N/A	Sequencing of *18S rDNA* and *gdh* genes	Diarrhoea was the most common symptoms for both asb. A and B.	Yang et al. (2010a)
Iran		N/A	N/A	N/A	PCR-RFLP at the *gdh* gene	Similar illness in both asb. But asb. A more frequently associated with abdominal pain, nausea and vomiting	Sarkari et al. (2012)

N/A: Data not available.

Source: Adapted from Caccio and Sprong (2011)

2.3.6 Diagnosis

Diagnosis of *Giardia* infection can be done through the examination of faecal specimens for the presence of trophozoites/cysts or through the detection of antigen or antibody. The appearance of the faeces can sometimes provide clues for the infection. Diarrhoeic stool from infected patient are usually fatty, pale in colour and may carry an offensive smell (Cheesbrough, 2005). *Giardia* can be demonstrated in direct wet preparations or concentrated faecal materials with or without staining. The unstained specimens can be prepared using saline suspension while the stained specimens can be prepared either using temporary stain such as iodine or permanent stain such as Trichrome (Smith & Mank, 2011). Direct examination has the benefit of detecting motile trophozoites that may present especially in unformed specimens. Conventional approaches for faecal concentration includes sedimentation technique in which centrifugal force is used to sediment the parasites and floatation technique in which solution with high specific gravity is used to float the parasites to the surface (Cheesbrough, 2005). The former is usually performed using biophasic technique where two immiscible solutions are used e.g. the formol ether concentration whereas the latter is usually performed using monophasic technique where only one solution is used e.g. zinc sulphate method (Smith & Mank, 2011). For convenience purpose, faecal parasite concentrator kits such as Mini Parasep® (APACOR, United Kingdom) are available commercially to reduce filter process and to eliminate the use of tubes.

In laboratories where fluorescence microscopy service is available, immunofluorecent assay (IFA) can be used to identify *Giardia* cysts. This method utilizes the binding of *Giardia* cyst with specific fluorescent monoclonal antibodies. Due to its high specificity and sensitivity in detection, it was recommended for clinically suspected specimens following negative microscopy result (El-Nahas et al., 2013). Occasionally, in cases where clinical suspicion is high but no parasites are

detected after repeated faecal examinations, diagnosis of *Giardia* from duodenal contents is considered (Cheesbrough, 2005). Several enzyme-linked immunosorbent assay (ELISA) tests are available for the detection of *Giardia* antigen in faeces. This technique can detect infection even the parasite cannot be traced in faeces. This rapid test can be performed on large batch of samples. However, due to the low specificity in detection, it was suggested that the ELISA test to be performed in combination with microscopy examination to improve the result (Elsafi et al., 2013; Singhal et al., 2015).

2.3.7 Treatment

There are at least six classes of drugs can be used as antigiardial agents in humans (Robertson et al., 2010). Nitroimidazoles includes metronidazole, tinidazole, ornidazole, and secnidazole was found to be effective against giardiasis and several other protozoan infections such as trichomoniasis and amoebiasis (Gardner & Hill, 2001; Khan, 2008). Metronidazole is the most prescribed drug for the treatment of giardiasis. Not only the mechanism of metronidazole in killing *Giardia* which involves activation of the drug by the enzyme pyruvate:ferredoxin oxidoreductase (PFOR) and ferredoxin was well documented but the mechanism in metronidazole-resistant *Giardia* has also been well studied (Upcroft & Upcroft, 2001).

Moreover, albendazole and mebendazole, two members of benzimidazoles, have been used to treat giardiasis. Albendazole has the advantage to treat helminth infections including *T. trichiura, A. lumbricoides*, and hookworms with comparatively benign side effects. It was investigated as an alternative and/or a replacement for metronidazole and was found to show promising result in the treatment of giardiasis (Solaymani-Mohammadi et al., 2010). Nitazoxanide belongs to the thiazole family is also a drug of choice for the treatment of giardiasis besides cryptosporidiosis. It is also found to be effective against helminths and some anaerobic microorganisms and viruses (Hemphill

et al., 2006). Pregnant women infected with *Giardia* can be treated with paromomysin which is a minimally absorbed aminoglycoside. Other alternative antigiardial agents include quinacrine, furazolidone and bacitracin zinc (Gardner & Hill, 2001).

Besides metronidazole, treatment failures and drug resistance from *in vitro* setting have been reported for most of the common antigiardial agents such as tinidazole, furazolidone and albendazole (Upcroft & Upcroft, 2001). However, when investigating drug resistance, it is important for the clinicians or researchers to distinguish whether the treatment failure is due to actual resistance to the drug, poor compliance with the course of treatment by the patients or reinfection (Gardner & Hill, 2001; Khan, 2008). Reinfection is common in areas where *Giardia* infection is endemic (Gilman et al., 1988; Saffar et al., 2005; Fallah, 2011; Al-Mekhlafi et al., 2013). It is also an issue to consider whether to treat asymptomatic patient as high rate of reinfection and treatment failure often observed in children living in endemic areas (Gardner & Hill, 2001; Saffar et al., 2005; Blackwell et al., 2013). Veenemans et al., 2011 reported protective association against diarrhoea and seemingly did not support treatment of *Giardia* infections in symptomatic children. However, if left untreated, it might lead to long-term consequences such as retarded growth and *Giardia* infection might spread because of cysts excretion by the asymptomatic carriers. Albendazole has been used for the treatment of *Giardia* and STH infections in rural communities of Malaysia (Penggabean et al., 1998) and significant improvement in weight gain was observed among the *Giardia*-infected participants following treatment (Al-Mekhlafi et al., 2013). Therefore, treatment should be provided regardless of presence or absence of symptoms for the benefit of the communities, however, risk factors should be identified and additional healthcare education is required in order to avoid re-infection (Gardner & Hill, 2001).

2.4 Molecular characterization

2.4.1 Molecular tools for discrimination of genetic variation within *Giardia duodenalis*

In September 2004, *Giardia* together with *Cryptosporidium* were included in the World Health Organisation's 'Neglected Diseases Initiative' with the main prospect being the incorporation of molecular approaches into to the study of these parasites will significantly enhance our understanding about their biology, epidemiology and the interaction between host and parasite. This initiative refers to a group of parasitic, bacterial and viral diseases that putatively linked with poverty and contribute considerable and increasing global burden which limit the potential of those affected to development and socioeconomic achievement (Savioli et al., 2006).

Studies of many infections have benefited from molecular genotyping to obtain better clarified epidemiological information (Anderson, 2001; Miles et al., 2009; Campino et al., 2011). For *Giardia*, the continuous development of molecular approach and its application in the investigation of various scenarios such as human population, domestic and wild life animals, environmental medium, and production have significantly enhanced our understanding especially in the aspects related to the host range of the parasite species and genotypes, the possibility of cross-species transmission, and the environmental factors that could lead to the exposure to the parasite (Carmena & Cardona, 2012). It has been the subject of several studies in both developed and developing countries to understand how genetic variability of the parasites and host factors contribute to the clinical establishment of this disease (Caccio & Sprong, 2011). Summary about correlation of symptoms with genotypes has been shown in Table 2.3. It is apparent that not all *Giardia* infections have the similar clinical presentations. There is no strict distinction of pathogenic and non-pathogenic genotypes in *Giardia* as

those exhibited by *Entamoeba* spp. in which the morphologically similar but non-pathogenic *E. dispar* can be genetically differentiated from the pathogenic *E. histolytica* (Homan & Mank, 2001). The presence of morphologically indistinguishable subtypes and the frequent occurrence of mixed infection are complicating factors that might have masked the genetic, biological, and epidemiological differences of *G. duodenalis*, and hence, care should be taken in the selection genotyping approaches (Thompson et al., 2000; Caccio & Sprong, 2011).

Detection of *Giardia* can be done through examination of duodenal, faecal, tissue, and environmental samples by light microscopy (stained or unstained preparations), immunofluorescence microscopy using fluorescein isothocyanate (FITC) and immunological methods such as ELISA (Smith & Mank, 2011). Some of these methods are convenient for routine and mass diagnosis for *Giardia* infection however the disadvantage is that genotype of *Giardia* cannot be identified and hence are not likely to access source or cycle of transmission (Lee et al., 2006). Molecular tools have been widely used over other methods for its high specificity to characterize *Giardia* at species, assemblage, and sub-assemblages and genotype. Various nucleic acid-based methods have been developed for this purpose (Table 2.5). The selection of the methods is based on the objective of each study, however the usefulness, strengths and weaknesses of the methods must be carefully weighed (Wielinga & Thompson, 2007; Smith & Mank, 2011). The variations in the methods are also influenced by the gene targeted and the specificity of the assay such as those listed in Table 2.6, as well as the region and the number of loci examined (Wielinga & Thompson, 2007; Feng & Xiao, 2011).

Substitution rates among the most commonly used markers, *18S r DNA*, *bg*, *gdh*, and *tpi* genes were 0.01, 0.03, 0.06, and 0.12 substitutions per nucleotide respectively (Wielinga & Thompson, 2007). The difference in substitution rates gives rise to

different application of these markers. The more conserved gene, *18S rDNA* is usable to detect and compare older groups (more distant) (Wielinga & Thompson, 2007) but its low resolution is of little use to study genetic variation within assemblages (Ryan & Caccio, 2013). Though having high sensitivity for PCR due to its multi copy number, GC richness of this gene has made it difficult for amplification while primers targeted this gene which usually amplify very small fragments and largely rely on a small number of polymorphic sites for differentiation have partially led to discordant findings (Feng & Xiao, 2011; Ryan & Caccio, 2013). On the other hand, the quicker evolving which are also the more variable genes such as *tpi* and *gdh* can identify new and emerging groups (Wielinga & Thompson, 2007). Given that most primers amplify 40% to 60% of these genes, they are good candidates for genotyping and subtyping and sometimes with the incorporation of conserved gene such as *18S rDNA* can provide fundamental data for current systematic classification of members within *Giardia* (Feng & Xiao, 2011; Wielinga et al., 2011; Koehler et al., 2014). Other repeated DNA sequences and molecular markers which is high in genetic variation such as microsatellites will be beneficial to identify sub-structuring but these sequences are not commonly represented in genome (Ryan & Caccio, 2013).

Multilocus genotyping (MLG) has become the trend in recent epidemiological studies (Mukherjee et al.; Caccio et al., 2008; Yang et al., 2010b; Lebbad et al., 2011; Soliman et al., 2011; Helmy et al., 2014; Zhao et al., 2014). The transition for single locus to multilocus was prompted by the recognition that larger datasets would contain more informative sites (Brito & Edwards, 2009). In addition, there appear to be insufficiency or possible misinterpretation of data based on single marker to gain an informative picture on epidemiology of *Giardia* amidst the growing concerns about the inconsistent genotype assignment using different makers (Caccio et al., 2008; Broglia et al., 2013).

Genetic variations within assemblage especially those of assemblages A and B are important to elucidate the relationship between subtypes and hosts including the investigation of zoonotic potential, to track contamination source in case of outbreak as well as to characterize the transmission dynamics in endemic areas (Feng & Xiao, 2011). However, the terminology used by different researchers to describe isolates that are closely related or the genetic variants within each assemblage has led to some extent of confusion (Ryan & Caccio, 2013). The inter- and intra-genotypic substitutions that used in the classification scheme by Wielinga and Thompson (2007) and Caccio et al. (2008) have been widely used to determine the grouping/sub-groupings of *G. duodenalis* isolates. Roman numeral was used as suffix for the established sub-assemblages such as AI and AII while other genotype variation found within assemblage was referred as subtype. According to analysis of pulsed-gel electrophoresis of chromosomes hybridization, the *tpi* gene was mapped to chromosome 5 while the *gdh* and *bg* genes were mapped to chromosome 4 (Caccio et al., 2008).

Table 2.5: Nucleic acid-based methods

Nucleic acid technique	Principle	Technique features
Fluorescent in situ hybridisation (FISH)	Hybridization of fluorescently-labelled probes or oligonucleotides to target sequences within RNA or DNA.	Reliant on RNA rather than DNA, thus not suitable for dead cysts due to rapid degradation of RNA. Less reliable in discerning species or assemblages.
Random amplification of polymorphic DNA analysis (RAPD)	Random use of primers without requiring specific knowledge of genome or genes.	Rapid screening of genome for variation within and among populations but low in specificity and reproducibility.
PCR-based restriction fragment length polymorphism (PCR-RFLP)	DNA amplification using specific primers followed by enzymatic cleavage and gel electrophoresis.	Not all sequence and length variations can be detected because only a few variable sites are recognizable by the endonuclease. Incomplete digestion can lead to misinterpretations of RFLP profiles.
Terminal restriction fragment length polymorphism (T-RFLP)	DNA amplification that incorporate a fluorescent primer that generate fluorescent PCR product followed by enzymatic cleavage and capillary electrophoresis.[a]	Discern assemblage and sub-assemblage and fragments of similar sizes more effectively than RFLP.
Direct DNA sequencing	Sequence data of amplified DNA.	The 'gold-standard' for detection of polymorphism. Direct approach for comparative analyses and phylogenetic investigations.

Table 2.5: (Continued)

Nucleic acid technique	Principle	Technique features
Single-strand conformation polymorphism (SSCP)	Detection of mutation by identifying conformational pattern of single-stranded DNA molecule through electrophoretic migration in a non-denaturing gel.[b]	Capable of detecting single point mutations in amplicons of <500 bp but detection rate decrease for amplicons >500 bp.
Restriction endonuclease fingerprinting (REF)	A modified SSCP approach.	Capable of accurately scanning long fragments (≤2 kb) for mutations.
High resolution melting curve (HRM)	Real-time PCR-based approach. Measure rate of dissociation of the dsDNA template in order to differentiate isolates based on melting profile.	For rapid and specific detection of *G. duodenalis* (sub-assemblages). Efficiency of sequence type distinction may decrease if melting temperatures are similar.
Multiplexed tandem-PCR (MT-PCR); Luminex technology; *Taq*Man array card (TAC)	Real-time PCR based amplification of multiple genetic loci either simultaneously or in tandem using micro-beads or probes.	Simultaneous detection and quantification of multiple pathogens.

Main content source from: Koehler et al. (2014)

[a] From Asher et al. (2012)

[b] From Leung and Yip (2008)

Table 2.6: Genotyping markers

Genetic marker	Function	Sequence availability/ Level of differentiation	Gene copy	Unique to *Giardia*
Mlh1 (*mlh*)	Involved in DNA repair	*G. duodenalis* assemblages A and B	Low	No
Glutamate dehydrogenase (*gdh*)	Housekeeping enzyme	*G. duodenalis* assemblages/ sub-assemblages, *G. muris*, and *G. ardeae*	Low	No
Triose phosphate isomerase (*tpi*)	Housekeeping enzyme	*G. duodenalis* assemblages/ sub-assemblages, *G. microti*, *G. muris*, and *G. ardeae*	Low	No
Beta-giardin (*bg*)	Structural protein	*G. duodenalis* assemblages/ sub-assemblages and *G. muris*	Low	Yes
Elongation factor 1-α (*ef1α)*	Involved in translation	*G. duodenalis* assemblages/ sub-assemblages, *G. muris*, and *G. ardeae*	Low	No
Ferredoxin (*fd*)	Mediates electron transfer	*G. duodenalis* assemblages A and B	Low	No
Open reading frame C4 (*orf-C4)*	Hypothetical heat shock protein	*G. duodenalis* assemblages A and B/ sub-assemblages	Low	Yes
18S ribosomal DNA (18S rDNA)	Small subunit of the ribosome	*G. duodenalis* assemblages, *G. microti*, *G. muris*, *G. ardeae, and G. agilis*	High	No
Intergenic spacer (*IGS*)	Non-coding DNA	*G. duodenalis* assemblages A and B	High	No
ITS 1, ITS 2, and 5.8S rDNA	Ribosomal	*G. duodenalis* assemblages/ sub-assemblages, *G. microti*, *G. muris*, and *G. ardeae*	High	No
Ribosomal protein L7a (*rp*)	Ribosomal	*G. duodenalis* assemblages A and B	Low	No
Variable surface protein (*vsp*)	Surface antigen	*G. duodenalis* sub-assemblages	Low	Yes

Source: Adapted from Koehler et al. (2014) and Ryan and Caccio (2013)

2.4.2 Distribution of genotypes and its epidemiological implications

The global data on molecular genotyping of human isolates in the review by Feng and Xiao in 2011, followed by an update by Ryan and Caccio in 2013, as well as additional reports mostly from recent data has been summarised in Table 2.7. A statement made by Feng and Xiao (2011) that only assemblages A and B are almost exclusively associated with human *Giardia* infections is still applicable in the present context. Genotyping data of 4474 samples revealed that assemblage B (53.7%) was the more commonly present assemblage worldwide compared to assemblage A (39.2%). The proportion of assemblage A vs. B was almost similar in developing countries (43.9% vs. 56.1%) but higher proportion of assemblage B (61.2%) was seen in developed countries than assemblage A (38.8%). The prevalence of assemblage B also predominated in most of the regions except Southern America. Different distribution of assemblages A and B was observed between different studies within the same region such as New Zealand and Australia and within the same country such as Brazil, Peru, Egypt, Bangladesh, Iran, and Thailand. Of all the 300 mixed infections detected, 249 infections occurred in developing countries.

The variation in the distribution of *Giardia* genotypes in different geographic regions could be due to different routes and sources of transmission, but the exact reasons behind it remained uncertain (Mahdy et al., 2009b; Feng & Xiao, 2011). Higher excretion in samples of patients infected by assemblage B as detected by microscopy or real time PCR (Haque et al., 2005; Kohli et al., 2008; Saksirisampant et al., 2012) was suggested as an explanation for the predominance of assemblage B in certain regions (Lebbad et al., 2011). On the other hand, several studies showed that assemblage B infections were found to be more prevalent than assemblage A in patients with persistent diarrhoea (Homan & Mank, 2001; Helmy et al., 2009; Al-Mohammed, 2011) and some studies have seen higher assemblage B infections in asymptomatic individuals

when compared with assemblage A (Aydin et al., 2004; Sahagun et al., 2007; Pestechian et al., 2014). These possible relationships between type of assemblage and type of diarrhoea or asymptomatic carriage have been hypothesized as reasons for discrepancy finding in genotyping data between infections of travellers who returned from a particular destination and local infection of that destination (Breathnach et al., 2010). For example, a study conducted in Portugal found all 25 symptomatic cases to be assemblage A infections (Sousa et al., 2006). However, infections of British travellers returned from Portugal were found to be predominant in assemblage B (3/4) (Breathnach et al., 2010). Besides, Lebbad et al. (2011) found that assemblage B dominated in infections of Swedish travellers returned from other European countries, Africa, Asia, and North America but not Latin America where equal distribution of both genotypes was seen. Of note, assemblage A was predominant in Southern America (Table 2.7).

In view of whether assemblage has possible association with gender and age, it was previously reported that children ≤ 12 years and females were at higher risk of being infected with assemblage B in Malaysia (Mahdy et al., 2009b). In a study from Scotland where assemblage A was the predominant genotype (21/30), it was found that majority of the infections (18/30) were from adult males (Alexander et al., 2013). On the contrary, Breathnach et al. (2010) found that both assemblages had a bimodal age distribution and proportion of almost similar in males and females where males accounted for 44% of assemblage A infections and 49% of assemblage B infections. Another study among Swedish patients showed that both genders had higher infection of assemblage B compared to assemblage A (Lebbad et al., 2011). Both of the latter studies showed that correlation between assemblage and gender is less likely. Nevertheless, high risk for females in contacting *Giardia* infection could be due to their role as caretakers with direct and frequent contact with faeces. Thus, it is possible that

the transmission route of *Giardia* infection is different between females and males from the same area (Feng & Xiao, 2011). Although correlation of assemblage with age is also unlikely and needs more evidence to support, it was suggested that age factor might play a role in susceptibility of the infected individuals to virulence assemblage (Al-Mohammed, 2011). A correlation between assemblage A and symptomatic infections, and between assemblage B and asymptomatic infections was found in the overall age group of a study conducted in Spain. However, the relationship was not statistically significant when only patients of age more than 5 years were involved (Sahagun et al., 2007). In addition, most of the studies summarized in Table 2.3 that reported correlation between assemblage B with symptomatic infections were predominantly cases of infected children (Gelanew et al., 2007; Pelayo et al., 2008; Al-Mohammed, 2011; Lebbad et al., 2011; Molina et al., 2011b; Puebla et al., 2014). Immaturity of immune system among the young patients is believed to be the reason for the susceptibility to symptomatic infection (Feng & Xiao, 2011).

In an *in-vivo* study, gerbils were infected with *G. duodenalis* trophozoites to explore the association between genotype and virulence. Among the genotypes being investigated, (sub)assemblage A1 and B were more virulent than (sub)assemblage AII and E where they were found to have higher infectivity, excrete of higher load of trophozoite for a longer period, and show relatively greater pathogenicity (Bénéré et al., 2012). Although the small number of isolates used was regarded as a limitation by the authors and additional isolates would be needed to corroborate the finding, this study provides preliminary evidence that shows variations in the level of virulence elicited by the different genotypes. In addition, variations found even within the same assemblage (i.e. AI and AII) demonstrated that the presence of subtypes in an assemblage is also an important issue to consider when investigate the association between genotypes and virulence or symptoms.

Subgroup classification within assemblages A and B was derived from isoenzyme analysis of laboratory-adapted strains and was subsequently confirmed by extensive evaluation of genetic data obtained from different loci (Wielinga & Thompson, 2007; Sprong et al., 2009). There are at least three subgroups present in assemblage A, namely AI, AII, and AIII. Subtyping results of human isolates showed that both sub-assemblages AI and AII were capable of infecting humans, in which AII was the predominant sub-assemblage. The approximate proportion between sub-assemblages AI and AII was 26% and 74% respectively (Feng & Xiao, 2011). Among the three established sub-assemblages, sub-assemblage AI was generally regarded as the zoonotic genotype as it is more prevalent in domestic animals. Sub-assemblage AII predominantly takes place between humans, and may also transmit from humans to animals while AIII is chiefly restricted to wild animals (Caccio & Sprong, 2011). Assemblage A has gathered the most focus in the study of zoonotic transmission because it is the most common non-host-specific assemblage occurred in animals. According to Feng and Xiao (2011), the proportion of sub-assemblage infection in animals for AI, AII, and AIII was 67%, 24%, and 10% respectively. The overall picture for sub-assemblages distribution in humans and various animals and in different regions was illustrated by Sprong et al. (2009) and was summarized in Table 2.8. Sub-assemblage AII dominated in human in most of the regions except Asia and Australia while the opposite was seen in animals where AI was the predominant sub-assemblage globally.

On the other hand, isoenzyme analysis of assemblage B had yielded protein polymorphisms consist of subgroups BIII and BIV, this clustering however, was not consistent with DNA polymorphisms analysis and was not as robust as those found in assemblage A where sequences of assemblage B containing ambiguous nucleotides and mixed infection of subgroups were frequently reported (Wielinga & Thompson, 2007;

Caccio & Sprong, 2011). According to Sprong et al. (2009), the relative distributions of assemblage B (n=1037) in different sources were 62% in humans, 2% in cats, 10% in dogs, 2% in cattle, 1% in goat/ sheep, 1% in pigs, and, 22% in wildlife. In 2011, Feng and Xiao had also reviewed the infection rates of different genotypes in three groups of animals. In farm animals, the commonly found genotypes were assemblages A, B, and E and their relative proportions were 45.3%, 8.3%, and, 46.4% respectively. The relative proportions of assemblages A, B, C, D, E, and F reported in companion animals were 26.2%, 4.6%, 25.2%, 33.5%, 1.6% and 8.9% respectively. Assemblages A (60.3%) and B (28.7%) were the most prevalent genotypes in wild animals. Other detected assemblages were assemblages H (5.2%) and E (3.2%). It appeared that assemblage B occurred predominantly in humans and was relatively rare in farm and companion animals. Based on these genotyping data, it has been suggested that humans are the major source of assemblage B and wildlife might play a role in maintaining the zoonotic transmission cycle of *Giardia* infection (Sprong et al., 2009; Feng & Xiao, 2011) .

Animals are commonly assumed to be reservoirs of *G. duodenalis* infections for human largely due to the early finding of this parasite in aquatic animals such as beavers and muskrats and later with evidences from molecular studies which showed that humans are infected by the common A and B assemblages found in other mammalian hosts including companion animals, livestock and wild terrestrial and marine animals (Baruch et al., 1996; Thompson & Lymbery, 1996; Traub et al., 2004; Traub et al., 2009; Johnston et al., 2010; Lasek-Nesselquist et al., 2010; Helmy et al., 2014). Giardiasis has been regarded as a zoonotic disease by WHO since 1979 (Lebbad et al., 2011). It is thought that humans can contract infection from animals or vice versa in endemic areas where humans and animals live closely within the same locality. However, direct evidence to prove this mode of transmission and to determine the primary source is limited owning to the shedding nature of *Giardia* cysts to the

environment (Takumi et al., 2012). The comparative analysis of genetic polymorphisms between human and animal isolates has been used and interpreted as indirect evidence for zoonotic potential (Caccio & Sprong, 2011).

Occurrence of animal-specific assemblages in humans has been reported occasionally in a few studies. In a study in Bangkok, Thailand, where samples were obtained from humans and dogs living in temple communities, Traub et al. (2009) detected the presence of assemblage C in humans. Other significant finding included dominance of assemblage A in both humans and dogs. It was suggested that human-to-dog and dog-to-human could have taken place in the transmission of *Giardia* and assemblage A may be the most significant genotype with regard to zoonotic potential. Nevertheless, the subtyping of the assemblage A to identify whether humans were infected with the same subtypes with dogs was not available as the marker used was 18S rDNA, thus strong conclusion cannot be drawn. In a more recent study, dominance of assemblage C was found in patients with giardiasis in Shanghai, China, suggesting dogs as the source of infections (Liu et al., 2014b). In contrast, a study conducted in a region with high *Giardia* endemicity in Peru showed that household transmission between humans and dogs was uncommon as the genotypes found in the two sources were different from each other (i.e. assemblages A and B in humans and assemblage C and D in dogs) (Cooper et al., 2010). This finding was also consistent with a study in Cambodia that showed only 2% of the dogs infected with assemblage B (n=94) and all human isolates were typed as assemblages AII and B (Inpankaew et al., 2014). In another study involving the companion animal, it was also found that humans and dogs harboured dissimilar genotypes, suggesting limited occurrence of zoonotic transmission. It was however, the consumption of water from mountain streams as the possible source of infection in which the water samples were found presence of *G. duodenalis* belonged to the same genotype with humans (Liang et al., 2012). Small sample sizes, few

reported cases of dogs and cats showing the same genotypes with humans living in close proximity as well as lacking in subtyping data are the limitations in providing definite answer to determine the zoonotic role of cats or dogs in *Giardia* infection of humans (Ballweber et al., 2010). The detection of assemblages C and D in humans could represent carriage instead of infection with these genotypes (Tysnes et al., 2014).

A MLG study of human and animal isolates conducted in Egypt showed that zoonotic transmission was infrequent, even in an area with high prevalence of *Giardia* infection in livestock. Although assemblage E was found in two human isolates, most of the humans and animals were predominantly infected with assemblages B and E respectively (Helmy et al., 2014). In Tanzania, zoonotic risk was also suggested to have a minor impact on human infections. Isolates from humans, goats, and, zebus were genotyped using *18S rDNA* and *gdh* genes, none of the human isolates was genotyped as assemblage E, while assemblages A and E were the major genotypes present in animals. Assemblage A in humans were typed as AII using *gdh* gene but animal isolates of assemblage A could not be typed until sub-assemblage level (Di Cristanziano et al., 2014). Similar with the data from companion animals, subtyping data are still not sufficient to support widespread occurrence of zoonotic transmission (Feng & Xiao, 2011). Conversely, based on the finding of (sub)assemblages AII and B in majority of the human isolates, anthroponotic transmission has been suggested as the main route of transmission in a number of studies (Ajjampur et al., 2009; Laishram et al., 2012b; Sarkari et al., 2012; Asher et al., 2014; Boontanom et al., 2014).

Molecular characterization has been used in recent years to identify the possible source of the parasite in environmental samples including surface and spring water, raw and treated wastewater from treatment plant. Predominance of assemblages A and/or B was found in most of these samples, indicating the risk of water as source of *Giardia* infection and higher possibility of anthroponotic transmission or human-originated

contamination in Italy (Di Cave et al., 2005), France (Bertrand & Schwartzbrod, 2007), Hungary (Plutzer et al., 2008), Brazil (Fernandes et al., 2011; Durigan et al., 2014), Malaysia (Lim et al., 2009a), China (Li et al., 2012), Taiwan (Liang et al., 2012), South Africa (Samie & Ntekele, 2014) and Poland (Adamska, 2014). Some of these studies revealed the low efficiency of treatment plant in eliminating the protozoa while some indicated the presence *Giardia* contamination in recreational water bodies thus constituting risk of infection by these genotypes either through direct consumption of the contaminated water or through recreational activities. Samie and Ntekele (2014) found that although assemblage A was the more prevalent genotype, it was more easily removed from the wastewater samples than assemblage B. A water-borne related giardiasis outbreak in Norway was presumed to be related to assemblage B and was resulted from sewage leakage into the water supply (Robertson et al., 2006).

DNA-based genotyping approaches have been widely applied in epidemiology and epizootiology of parasites. Most of the studies that investigate zoonotic transmission of *Giardia* in environmental samples relied only on single gene or gene that cannot discriminate subtype. The use of more sensitive tools and standardization on genotyping scheme to compare human and animal isolates as well as water samples, for example the use of MLG are needed to gain comprehensive understanding in population sub-structure, geographic, and host segregation (Lebbad et al., 2011; Ryan & Caccio, 2013). Better characterization of the population genetic of *G. duodenalis* is required for a thorough understanding of the dynamic transmission of the parasite in humans and animals (Feng & Xiao, 2011). From the perspective of population ecology, molecular approaches provide a means for parasitologist to understand processes such as transmission of parasites, patterns of speciation, evolution of host specificity and spread drug resistance (Charles et al., 2005). The evolutionary potential of parasite is driven by

the distribution, flow and degree of genetic diversity along with the magnitude of diversity recombination among genomes (Andras & Ebert, 2013).

Table 2.7: Prevalence and genotype of human *Giardia* infection in different regions of the world

Location	Total No. of samples	Positive samples (%)	No. of samples genotyped	Asb. A	Asb. B	Mixed/ Other infections	Reference
Europe							
Albania	125	17.6	22	10	12	-	Feng and Xiao (2011)
Belgium	373	4	72	18	54	-	Feng and Xiao (2011)
France	-	-	25	9	16	-	Feng and Xiao (2011)
Germany	202	1.5	3	3	-	-	Feng and Xiao (2011)
Italy	-	-	120	65	39	16 (A+B)	Giangaspero et al. (2007)
Netherlands	-	-	98	34	64	-	Feng and Xiao (2011)
Netherlands	892	2	18	9	9	-	Feng and Xiao (2011)
Norway	-	-	21	-	21	-	Feng and Xiao (2011)
Norway	-	-	63	3	60	-	Feng and Xiao (2011)
Poland	232	1.3	3	2	1	-	Feng and Xiao (2011)
Portugal	190	3.7	7	7	-	-	Feng and Xiao (2011)
Portugal	-	-	25	25	-	-	Feng and Xiao (2011)
Portugal	317	2.8	9	6	3	-	Ferreira et al. (2013)
Scotland	-	-	28	31	4	3 (A+B)	Alexander et al. (2013)
Spain	327	3	7	2	4	1 (A+B)	Cardona et al. (2011)
Spain	-	-	108	43	61	4 (A+B)	Feng and Xiao (2011)
Sweden	-	-	207	73	128	6 (A+B)	Lebbad et al. (2011)
United Kingdom	-	-	199	48	145	6 (A+B)	Feng and Xiao (2011)
United Kingdom	-	-	33	9	21	3 (A+B)	Feng and Xiao (2011)
Subtotal			1068	387	642	39	

Table 2.7: (Continued)

Location	Total No. of samples	Positive samples (%)	No. of samples genotyped	Asb. A	Asb. B	Mixed/ Other infections	Reference
North America							
Canada	658	0.5	3	3	-	-	Budu-Amoako et al. (2012)
Canada	52	28.9	15	3	9	3 (A+B)	Feng and Xiao (2011)
Canada	-	-	6	6	-	-	Feng and Xiao (2011)
United States	-	-	14	14	-	-	Feng and Xiao (2011)
United States	-	-	2	-	2	-	Feng and Xiao (2011)
Subtotal			40	26	11	3	
Oceania							
Australia	-	-	12	-	11	1 (A+B)	Hopkins et al. (1997)
Australia	353	7.6	23	7	16	-	Read et al., (2002)
Australia	-	-	124	31	93	-	Yang et al., (2009)
Australia	87	66.7	45	11	34	-	Asher et al. (2014)
New Zealand	66	7.6	5	1	4	-	Feng and Xiao (2011)
New Zealand	-	-	30	23	7	-	Feng and Xiao (2011)
Subtotal			239	73	165	1	

Table 2.7: (Continued)

Location	Total No. of samples	Positive samples (%)	No. of samples genotyped	Asb. A	Asb. B	Mixed/ Other infections	Reference
Central and Southern America							
Argentina			43	3	40	-	Minvielle et al. (2008)
Brazil	245	51.8	30	-	30	-	Santos et al. (2012)
Brazil	366	23.8	62	62	-	-	Feng and Xiao (2011)
Brazil	-	-	37	29	8	-	Feng and Xiao (2011)
Brazil	154	25.3	32	32	0	-	de Godoy et al. (2013)
Brazil	-	-	76	28	29	3 (A+B), 2 (A+C), 3 C[d]	Durigan et al. (2014)
Cuba	-	-	20	9	11	-	Feng and Xiao (2011)
Cuba	452	22.8	90	36	38	16 (A+B)	Puebla et al. (2014)
Ecuador	595	25.9	69	22	42	5 (A+B)	Atherton et al. (2013)
Guatemala	645	5.4	20	7	12	1 (A+B)	Velasquez et al. (2011)
Mexico	-	-	19	19	-	-	Feng and Xiao (2011)
Mexico	-	-	9	9	-	-	Feng and Xiao (2011)
Mexico	-	-	12	12	-	-	Feng and Xiao (2011)
Mexico	429	7.7	33	27	6	-	Torres-Romero et al. (2014)
Nicaragua	-	-	119	25	94	-	Feng and Xiao (2011)
Peru	1531	20.4	167	66	81	20 (A+B)	Feng and Xiao (2011)
Peru	845	23.8	16	10	6	-	Feng and Xiao (2011)
Peru	-	-	25	6	19	-	Feng and Xiao (2011)
Subtotal			879	402	416	47	

Table 2.7: (Continued)

Location	Total No. of samples	Positive samples (%)	No. of samples genotyped	Asb. A	Asb. B	Mixed/ Other infections	Reference
Africa							
Egypt	-	-	41	31	8	2 (A+B)	Helmy et al. (2009)
Egypt	-	-	15	1	13	1 (A+C)	Soliman et al. (2011)
Egypt	52	34.6	18	1	14	1 (B+E), 2 E	Feng and Xiao (2011)
Egypt	165	21.0	18 [a]	3	12	1 (A+B), 2 E	Helmy et al. (2014)
Egypt	161	29.2	35	27	-	-	Sadek et al. (2013)
Ethiopia	-	-	59	31	13	8 (A+B), 7 (A+F)	Feng and Xiao (2011)
Guinea Bissau	50	56	25	3	22	-	Ferreira et al. (2012)
Ivory Coast	307	19.8	61	25	36	-	Berrilli et al. (2012)
Nigeria	157	3.2	5	5	-	-	Maikai et al. (2012)
Morocco	673	19.8	11	2	9	-	El Fatni et al. (2014)
Rwanda	583	60.1	208	28	179	1 (A+B)	Ignatius et al. (2012)
Sahrawi	120	34.2	32	12	18	2 (A+B)	Feng and Xiao (2011)
Sao Tome and Principe	348	8.3	11	6	5	-	Lobo et al. (2014)
Tanzania	45	62.2	28	6	22	-	Di Cristanziano et al. (2014)
Uganda	427	20.1	34	5	25	4 (A+B)	Ankarklev et al. (2012a)
Uganda	62	5	3	3	-	-	Feng and Xiao (2011)
Subtotal			604	189	384	27	

Table 2.7: (Continued)

Location	Total No. of samples	Positive samples (%)	No. of samples genotyped	Asb. A	Asb. B	Mixed/ Other infections	Reference
Asia							
Bangladesh	-	-	35	3	32	-	Ng et al. (2005)
Bangladesh	2534	12.7	267	20	231	16 (A+B)	Feng and Xiao (2011)
China	-	-	8	4	4	-	Feng and Xiao (2011)
China	-	-	18	12	6	-	Feng and Xiao (2011)
China	4045	1.0	36	25	11	-	Wang et al. (2013)
China	252	6.8	17	0	1	16 C	Liu et al. (2014b)
India	51	27.4	14	6	8	-	Khan et al. (2011)
India	-	-	16	5	8	3 (A+B)	Feng and Xiao (2011)
India	-	-	19	6	9	4 (A+B)	Feng and Xiao (2011)
India	-	-	74 [a]	7	61	6 (A+B)	Laishram et al. (2012b)
Iran	-	-	38	33	3	2 (A+B)	Babaei et al. (2008)
Iran	-	-	31	17	13	1 (A+B)	Nahavandi et al. (2011)
Iran	-	-	172	128	36	8 (A+B)	Sarkari et al. (2012)
Iran	-	-	50	8	19	23 (A+B)	Roointan et al. (2013)
Iran	-	-	100	14	27	59 (A+B)	Rafiei et al. (2013)
Iran	-	-	67	40	25	2 (A+B)	Pestechian et al. (2014)

Table 2.7: (Continued)

Location	Total No. of samples	Positive samples (%)	No. of samples genotyped	Asb. A	Asb. B	Mixed/ Other infections	Reference
Iran	1000	10.7	40	32	8	-	Rayani et al. (2014)
Iran	720	4.7	30	2	28	-	Tappeh et al. (2014)
Iraq	-	-	30	9	21	-	Fadia et al. (2014)
Japan	-	-	3	2	1	-	Feng and Xiao (2011)
Japan	86	4.7	4	0	4	-	Abe and Teramoto (2012)
Laos	-	-	5	-	5	-	Feng and Xiao (2011)
Malaysia	321	23.7	42	1	41	-	Feng and Xiao (2011)
Malaysia	611	18.0	98	62	36	-	Anuar et al. (2014a)
Mongolia	138	3.6	5	5	0	-	Hong et al. (2014)
Nepal	1096	4.1	35	7	26	2 (A+B)	Feng and Xiao (2011)
Saudi Arabia	1500	6.5	40	23	15	2 (A+B)	Feng and Xiao (2011)
South Korea	-	-	5	5	-	-	Feng and Xiao (2011)
Taiwan	209	3.8	8	8	-	-	Liang et al. (2012)
Thailand	6967	0.9	61	5	31	25 (A+B)	Feng and Xiao (2011)
Thailand	204	20.3	35	25	1	2 (A+B), 6 (A/B+ C/D), 1 C	Feng and Xiao (2011)
Thailand	531	6.2	12	5	7	-	Feng and Xiao (2011)

Table 2.7: (Continued)

Location	Total No. of samples	Positive samples (%)	No. of samples genotyped	Asb. A	Asb. B	Mixed/ Other infections	Reference
Thailand	189	5.8	10	3	7	-	Boontanom et al. (2011)
Thailand	765	5.2	28 [a]	16	-	-	Saksirisampant et al. (2012)
Thailand	895[b]	6.1[c]	36	26	10	-	Boontanom et al. (2014)
Turkey	-	-	44	19	25	-	Feng and Xiao (2011)
United Arab Emirates	111	60.4	46	21	19	6 (A+B)	ElBakri et al. (2014)
Yemen	503	17.6	65	43	22	-	Alyousefi et al. (2013)
Subtotal			1644	676	784	183	
Total			4474	1753	2402	300	

Source: Adapted from Feng and Xiao (2011) and Ryan and Caccio (2013)

[a] MLG was used in these studies. As result obtained from each gene varied, the number reported here was according to the gene with the highest number of isolates amplified.

[b] Total samples from 481 individuals.

[c] Overall prevalence (54/892).

[d] Result of discordant genotype from MLG of *tpi*, *gdh*, and *bg*.

Table 2.8: Prevalence of subtypes of assemblage A in humans and animals and their distribution in different regions of the world

Sub-assemblage	**Source**							
	Cat	**Dog**	**Cattle**	**Goat, sheep**	**Pig**	**Wildlife**	**Human**	**Other**
AI	69%	73%	62%	78%	86%	44%	25%	55%
AII	25%	27%	35%	22%	14%	3%	75%	45%
AIII	5%	0%	4%	0%	0%	52%	0%	0%
Total (n)	59	120	113	36	14	86	594	80
	Region							
In humans	**Africa**	**Asia**	**Australia**	**Europe**	**Middle east**	**C/S -America**	**N-America**	
AI	12%	60%	69%	14%	13%	42%	44%	
AII	88%	40%	31%	86%	88%	58%	56%	
Total (n)	73	5	26	295	16	160	16	
In domestic animals[a]								
AI	67%	100%	92%	67%	0	77%	65%	
AII	33%	0%	8%	33%	0	23%	35%	
Total (n)	3	9	12	334	0	30	84	

Source: Adapted from (Sprong et al. (2009))

[a] Data from the source cats, cattle, dogs, goats and sheep, and pigs were grouped in 'domestic animals' by the authors.

CHAPTER III: MATERIALS AND METHODS

3.1 Malaysia profile

Malaysia occupies the central position within Southeast Asia and is comprised of two geographical regions which are separated by the South China Sea. It is a federation with principal administrative divisions consist of 13 states (Negeri) and three federal territories (Wilayah Persekutuan) divided between Peninsular Malaysia or West Malaysia (11 states and two federal territories) and East Malaysia (two states and one federal territory) (Saw & Institute of Southeast Asian Studies., 2007).

Malaysia is a multiracial and multicultural country with Bumiputra inclusive of Malay and Indigenous (67.4%), Chinese (24.6%) and Indian (7.3%) make up the three main groups with a total population of 28.3 million (Department of Statistics Malaysia [DSM], 2010). The indigenous communities in which the current study was particularly recruited are known as "*Orang Asal*" in Malay term, refers to the descendants of the earliest population in the country. It is also a term recognized and used by the Human Rights Commission of Malaysia (SUHAKAM). Based on the UN working definition, the aborigines in West Malaysia (specifically known as "*Orang Asli"*) and the native of Sabah and Sarawak in East Malaysia are regarded as indigenous peoples. The common characteristics that used to define indigenous people are: 1) the earliest inhabitants before pre-colonization 2) self-identification 3) distinct culture and language 4) non-dominance from other sectors of the mainstream society (Human Rights Commission of Malaysia [SUHAKAM], 2013).

Even though Malaysia has experienced rapid development since independence in 1957, the indigenous people still appeared to be the marginalized and disadvantaged group in the development process. *Orang Asli* communities remain one of the most impoverished groups in the country and have often been identified as socio-underclass.

On political and socio-economic concern, the indigenous peoples in East Malaysia attain a relatively better status compared to *Orang Asli*. They have fair participation in the decision making process whilst the *Orang Asli* has yet been represented by their own trait of political party in the government. In spite of this advantage, indigenous peoples in Sabah and Sarawak are still generally lagging behind in socio-economic perspective when compared with the dominant counterpart in Peninsular Malaysia.

3.2 Study areas

This community-based cross-sectional study was carried out with a particular focus on epidemiology and genetic classification of *Giardia* infection among indigenous communities in rural parts of Malaysia (both in West and East Malaysia) from April 2011 to February 2013. Overall, 28 villages from seven states of Malaysia, namely Pahang, Selangor, Negeri Sembilan, Kelantan, Kedah, Malacca and Sabah were involved (Figure 3.1). The populations in the areas under study lived with disparate lifestyles and environmental exposures, especially in terms of the indigenous groups in Peninsular Malaysia (West Malaysia) and Sabah (East Malaysia). In particular, the overall living standard was lower among the indigenous groups in West Malaysia compared to their counterparts in East Malaysia, though the housing conditions vary among the villages. The climate is equatorial with hot-humid conditions and rainfall throughout the year. The vegetation is the thick rain forest type and there are few water streams in the area.

In West Malaysia, most of the aboriginal communities lived in self-constructed wooden or bamboo house. There were also several villages e.g. Kg. Donglai Baru and Kg. Bukit Payung (Table 3.1) with mixed of wooden, bamboo and brick houses dispersed in the villages. In East Malaysia, there were villagers that lived in new settlement with standard wooden houses e.g. Kg. Penimbawan or new settlement with

standard brick houses (Kg. Salarom Taka). Other villages have mixed setting similar with those in East Malaysia. Pet animals such as dog and cat were seen wandering around the compound of the villages as well as in the houses. Livestock such cattle and goat were reared by some of the villagers and were let free in the compound. The cleanliness of the environment of the villages in East Malaysia was generally higher than those in West Malaysia.

Table 3.1: Villages and their main tribal group involved in the study

State	District	Village	Main tribal group
Sabah	Tuaran	Penimbawan	Bajau
	Tenom	Pangalat	Dusun
		Pamilaan	Dusun
	Nabawan	Naaturan	Murut
		Salarom Taka	Murut
Selangor	Hulu Langat	Donglai Baru	Temuan
Malacca	Alor Gajah	Bukit Payung	Temuan
		Bukit Sebang	Temuan
Negeri Sembilan	Jelebu	Dusun Kubur	Temuan
		Ulu Kelaka	Temuan
Kedah	Baling	Ulu Legong	Kensiu
Pahang	Temerloh	Paya Pelong	Jahut
		Paya Sendayan	Jahut
	Lipis	Kuala Koyan	Semai
		Sentoi	Semai
		Kuala Kennip	Semai
		Sarang	Semai
		Samut	Semai
		Kuala Milut	Semai
		Tual Baru	Semai
		Sat Baru	Semai
		Chekai	Semai
		Ulu Milot	Semai
		Tanjung Gahai	Semai
		Sungai Padi	Semai
		Semoi	Semai
Kelantan	Gua Musang	Sangwai	Temiar
		Jekjok	Temiar

3.3 Study population

The aboriginal communities in West Malaysia are recognized specifically as '*Orang Asli'*, a collective term used to address the heterogeneous minorities that are classified into three main groups namely Negrito (2.8%), Proto-Malay (42.3%) and Senoi (54.9%). Each of these tribal groups consists of six tribes, with an estimated population of 178,197 which make up 0.7% of the total Malaysian population according to population census 2010. The population of *Orang Asli* has increased to 76.8% since independence in 1957 which was then had only 41,360 people (DSM, 2010, Jabatan Kemajuan Orang Asli [JAKOA], 2011). The current 852 *Orang Asli* villages constitute of approximately 36,658 households are categorized into three location groups i.e. Interior (38%), Periphery (61%) and Town (1%) according to the area of inhabitation, level of economic development and availability of basic amenities and infrastructures. Incidence of poverty for *Orang Asli* is 31.2% of the total household, with 20.0% belongs to the hard-core poverty group (JAKOA, 2011). In contrast with West Malaysia, the indigenous people forms more than 50.0% of the total population of Sabah and Sarawak and represent the majority group in East Malaysia. In Sabah, there are 72 ethnic and sub-ethnic groups, with Kadazandusun (17.7%), Bajau (14.0%) and Murut (3.2%) making up the major composition of the Sabah population (DSM, 2010; SUHAKAM, 2013).

The lifestyles and means of pursuing a livelihood are diverse among the indigenous groups, though they tend to have a close connection with the various tribes traditional habitats and natural resources. Some of the aborigines in the West Malaysia still live in remote areas, however, due to implementation of development programmes that were initiated by the government, an increasing number of these minorities are moving to the periphery of urban areas where they are integrating with urban communities (Mohd Noor, 2012a). Their traditional subsistence orientated production

has seen a gradual adoption of a more modern mainstream economy as means of living. The common economic activities engaged in by the present *Orang Asli* are rubber tapping, small-scale cultivation of local crops (e.g. cassava and banana), wage-earning jobs in private sectors (e.g. oil palm/coco plantation, construction site and factory), as well as forest produce collection and selling (e.g. fruits and bamboo) to a lesser extent (Khor and Zalilah, 2008; Chung, 2010). Conversely, the indigenous communities in the rural areas of Sabah employ a more diversified subsistence economy. The coastal and riverine communities engage largely in the fishing industry, with a recent expedition in to seaweed cultivation. Whilst those living in the interior areas depend on farming, gardening and collecting forest resources, both for their own consumption and in order to sell the surplus for cash. Some of them are involved in cash crops plantation projects, such as oil palm, cocoa and rubber. The younger generations are leaving the agrarian way of life and move to urban areas. More and more are employed in service sectors such as tourism or work in civil service (Malaysian Rating Corporation Berhad [MARC], 2011; Oxford Business Group, 2011).

3.4 Sample size and sampling strategy

The minimum sample size required for this study was calculated according to the formula provided by Lwanga and Lemeshow (1991). At a 5% level of significance and a 95% confidence level, the minimum number of subjects required for this study was estimated as being 983, assuming that the prevalence of *Giardia* infection was 20%; as recently reported among three different Aboriginal tribes (Anuar et al., 2012). Overall, 1,330 individuals agreed to voluntarily participate in this study who met the inclusion criteria (signed written consent, completed questionnaire and delivered stool samples for examination).

The states and villages involved in this study were randomly selected from the available official administrative list in collaboration with the Department of Orang Asli Development (Jabatan Kemajuan Orang Asli, JAKOA) and Sabah Health Department (JKN SABAH), with consideration of the following criteria for villages: located in a rural area, accessible from the main roads and each village has more than 20 houses or ≥ 100 residents. The probability proportional to size sampling method was used to select the participants from each state, based on the total number of the indigenous population in the states and districts. This research strategy was used to facilitate inter-site comparisons with the emphasis on the epidemiology of the infection among the indigenous people. Before the commencement of the sampling, surveys were made by visiting the villages accompanied by officers from JAKOA and JKN SABAH, to meet the head of the village so as to explain and prepare the villagers for data and sample collections, as well as to obtain primary information on the existing conditions in the villages.

3.5 Questionnaire survey

A pretested questionnaire was used to collect information on the demographic (e.g. age, sex and number of household members), socioeconomic (e.g. household monthly income, occupation and educational status), environmental (e.g. availability and types of toilets in the household, types of water supply, garbage disposal and presence of domestic animals), personal hygiene (e.g. washing hands before eating, after defecation and after playing with animals, washing vegetables and fruits before consumption, boiling water before consumption and bathing place), and general health status of the participants (i.e. symptoms related to intestinal parasitic infections such as diarrhoea, nausea, vomiting, abdominal pain and a history of receiving anthelmintics treatment). The participants were regarded as symptomatic if they presented with any one of the signs and symptoms mentioned above in the past one week. The questionnaire was

designed in the English language and then translated into the Malay language. Two research assistants from the Department of Parasitology, University of Malaya were trained for the purpose of this study.

3.6 Faecal samples collection and examination

Fresh faecal samples were collected in 60 ml clearly labelled containers with wide mouths and screw-caps. The containers were distributed and the participants were informed on the proper method of sample collection e.g. not to mix their stool sample with urine or water, and to provide a sample amount of at least a thumb-size. Participants were invited to bring their early morning stool samples the next day. Samples were transported in suitable cool boxes to the Department of Parasitology, Faculty of Medicine, University of Malaya and stored in a cold room at 4°C. When immediate transfer of samples to the department was not possible, samples were kept in a refrigerator at local clinics or health offices.

The collected faecal samples were processed based on the formalin-ether sedimentation technique previously described by Cheesbrough (2005). Briefly, a small amount of stool samples (pea size) was added to a 15 ml conical test tube containing 7 ml 10% formol water. Applicator stick was used to mix and emulsify the faeces. The sample was then sieved through double-layered gauze and collected in a beaker. Three ml of diethyl ether was added after the sieved suspension was transferred to the washed test tube. The test tube with the suspension was capped and mixed by shaking before centrifuging for 5 minutes at 2500 rpm. The top three layers (ether, debris and formol water) were removed. Pellets of the sediment were used for examination by emulsifying them in 1-2 drops of iodine solution, then examining the results using a light microscope with the aim of finding the presence of *Giardia* cysts and/or trophozoites, as

well as other intestinal parasites. For quality control, duplicate slides were prepared from 20% of the samples and the slides were read by another microscopist.

3.7 DNA extraction

DNA was extracted directly from faecal samples either using PowerSoil®DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, California) or MACHEREY-NAGEL NucleoSpin® Soil (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) following the manufacturer's instructions. In brief, for PowerSoil®DNA Isolation Kit, approximate 0.25g of the stool samples was loaded to the PowerBead Tubes and gently vortexed. Solution C1 was added and subjected to vortexing for ten minutes for mechanical and chemical homogenization and cell lysis. The procedures were ensued by the addition of Solution C2 and C3 and a short incubation period aimed at removing inhibitors from the samples. The Solution C4 with high salt content was used to create a condition that enhance the binding of DNA to silica membrane when the sample was placed in Spin Filter and passing through the filter membrane. In the subsequent washing procedures, DNA was treated with Solution C5 in the filter membrane. Elution step was accomplished by adding reduced volume of Solution C6 (10 mM Tris) to obtain a final volume of 50 µl and the DNA was stored in freezer under -20°C. For MACHEREY-NAGEL NucleoSpin® Soil, during the sample lysis step where optional solutions were provided by the manufacturer, lysis buffer SL 2 (instead of SL1) was used as the lysis buffer with the additional of 150 µl of Enhancer SX. The principle of cell lysis was similar to PowerSoil®DNA i.e. attach the lysis tubes horizontally to a vortexer and vortex the samples to homogenize and disrupt the cells by ceramic beads. Proteins and PCR inhibitors were precipitated and removed in the incubation step with lysis buffer SL3 and filtering step with NucleoSpin® Inhibitor Removal (an additional step to PowerSoil®DNA). DNA was eluted in 50 µl of Buffer SE and stored at -20 ºC.

3.8 Genotyping of *Giardia duodenalis* using *gdh* gene

Genotyping of the 154 microscopy-positive samples were performed using three loci, *gdh*, *bg* and *tpi* genes. A 432-bp fragment of the *gdh* gene was amplified using semi-nested PCR primers described by Read et al. (2004) with some minor alterations adopted from Plutzer et al. (2008). Forward primer GDHeF (5'- TCA ACG TYA AYC GYG GYT TCC GT-3') was used in the primary PCR while GDHiF (5'- CAG TAC AAC TCY GCT CTC GG-3') was used in the secondary PCR. Reverse primer GDHiR (5'- GTT RTC CTT GCA CAT CTC C-3') was used in both set of the PCRs. The reactions were performed in a 50 µl PCR mix comprised 0.5 µM of each primer (Bioneer Q-Oligos, Korea), 2.5 U of HotStarTaq® Plus DNA Polymerase (Qiagen, Hilden, Germany),, 1 X PCR buffer (Qiagen, Hilden, Germany), 200 µM of each dNTP (Fermentas, Ontario, Canada), 1.5 mM MgCl2 (Qiagen, Hilden, Germany), 5% dimethyl sulfoxide (Sigma-Aldrich, USA) and 0.4 mg/ml BSA (New England Biolabs, Ipswich, USA). Two µl of DNA template were used in both amplifications that were run in the MyCycler thermal cycler (Bio-Rad, Hercules, USA) under the following conditions: Initial activation (95°C for 5 min), 1 cycle (94 °C for 2 min, 56 °C for 1 min, 72 °C for 2 min), followed by 55 cycles [33 cycles for secondary PCR](94 °C 30 s, 56 °C for 20 s, 72 °C for 45 s) and a final extension (72 °C for 7 min).

3.9 Genotyping of *Giardia duodenalis* using *tpi* gene

Nested-PCR amplification of 530-bp fragment of the *tpi* gene was performed using protocol described by Sulaiman et al. (2003) with slight modification. The modifications included adding BSA in the reaction, reducing final reaction volume to 50 µl instead of 100 µl, reducing the concentration of taq polymerase and increasing the annealing temperature of secondary PCR to 58 °C instead of 50 °C. Primary PCR was run using forward primer AL3543 (5'- AAA TIA TGC CTG CTC GTC G-3') and

reverse primer AL3546 (5'- CAA ACC TTI TCC GCA AAC C-3'). For secondary PCR, forward primer AL3544 (5'- CCC TTC ATC GGI GGT AAC TT-3') and reverse primer AL3545 (5'- GTG GCC ACC ACI CCC GTG CC-3') were used. Both PCR amplications were performed in a 50 µl PCR mix comprised 0.2 µM of each primer (Bioneer Q-Oligos, Korea), 1 U of HotStarTaq® Plus DNA Polymerase (Qiagen, Hilden, Germany), 1 X PCR buffer (Qiagen, Hilden, Germany), 200 µM of each dNTP (Fermentas, Ontario, Canada), 1.5 mM MgCl2 (Qiagen, Hilden, Germany), and 0.2 mg/ml BSA (New England Biolabs, Ipswich, USA). Two µl of DNA template were used and the prepared mix was incubated in the MyCycler thermal cycler (Bio-Rad, Hercules, USA) under the following conditions: Initial activation (95°C for 5 min), 35 cycles (94 °C 45 s, 50 °C for 45 s [58 °C for secondary PCR], 72 °C for 60 s) and a final extension (72 °C for 10 min).

3.10 Detection of mixed infection using *tpi* gene

The first PCR product of the reaction described by Sulaiman et al. (2003) was analysed using a set of separate A (Geurden et al., 2007) & B (Geurden et al., 2009) assemblage-specific primers. Present of mixed infection was detected by visualizing the occurrence of band in the agarose gel at 332 bp for assemblage A amplified using primers AssAF (5'-CGC CGT ACA CCT GTC-3') and AssAR (5'-AGC AAT GAC AAC CTC CTT CC-3') and at 400 bp for assemblage B amplified using primers AssBF (5'- GTT GTT GTT GCT CCC TCC TTT -3') and AssBR (5'- CCG GCT CAT AGG CAA TTA CA -3'). The PCR reaction mix consist of 0.2 µM (0.4 µM for assemblage B) of each primer (Bioneer Q-Oligos, Korea), 1.25 U of HotStarTaq® Plus DNA Polymerase (Qiagen, Hilden, Germany), 1 X PCR buffer (Qiagen, Hilden, Germany), 200 µM of each dNTP (Fermentas, Ontario, Canada), 1.5 mM MgCl2 (Qiagen, Hilden, Germany) and 0.1 mg/ml BSA (New England Biolabs, Ipswich, USA) to a final volume of 25µl. One µl of DNA template was added for assemblage A and 2 µl was added for assemblage B for

the PCR amplifications following the cycle parameter: Initial activation (95°C for 5 min), initial denaturation (94 °C for 10 min), and 35 cycles (94 °C 45 s, 64 °C for 45 s)[62°C for secondary PCR], 72 °C for 45 s).

3.11 Genotyping of *Giardia duodenalis* using *bg* gene

For amplification of 511-bp fragment of the *bg* gene, primers and conditions described by Caccio et al. (2002) and Lalle et al. (2005) were used. The primers for primary PCR were G7 (5'-AAG CCC GAC GAC CTC ACC CGC AGT GC -3') and G759 (5'- GAG GCC GCC CTG GAT CTT CGA GAC GAC -3'). For secondary PCR, BG511F (5'- GAA CGA ACG AGA TCG AGG TCC G -3') and BG511R (5'- CTC GAC GAG CT TCG TGT T -3') were used. The PCR master mix consisted of 0.4 µM of each primer (Bioneer Q-Oligos, Korea), 2.5 U of HotStarTaq® Plus DNA Polymerase (Qiagen, Hilden, Germany), 1 X PCR buffer (Qiagen, Hilden, Germany) and 200 µM of each dNTP (Fermentas, Ontario, Canada) in a total volume of 50 µl. Two µl of DNA template were added and the mixture was run in the MyCycler thermal cycler (Bio-Rad, Hercules, USA) under the following conditions: Initial activation (95°C for 5 min), initial denaturation (95 °C for 15 min), followed by 35 cycles (95 °C for 30 s, 65°C for 30 s (55°C for secondary PCR), 72 °C for 60 s) and a final extension at 72 °C for 7 min.

3.12 Determination of PCR results

In all the PCR reactions, a *Giardia*-positive DNA from previously sequenced and known sample and distilled water (nuclease free water Qiagen) were used as positive and negative control. Besides the use of negative control to detect occurrence contamination, separation of areas for sample extraction and PCR preparation were taken as preventive measure. Eight microliters of the PCR products were analysed using 2% agarose gel (Vivantis) electrophoresis stained with SYBR® Safe DNA (Invitrogen, Auckland, New Zealand). Positive and negative controls on the gel electrophoresis were carefully examined to confirm validity of the assays. Occurrence of ambiguous bands would be considered invalid and would be repeated. When samples were not amplified using the protocols mentioned above, several attempts were made by adjusting the amount DNA used or the concentration of reagent such as Taq Polymerase. The samples were considered positive if a 432 bp, 530 bp and 511 bp bands were amplified and visible for the nested-PCR of the *gdh*, *tpi* and *bg* assays respectively. Successfully amplified PCR products with the nested-PCR for each locus were sequenced in both directions in Applied Biosystems 3730 xl DNA Analyzer (Applied Biosystems, USA). For the result of tpi assemblage-specific PCR, amplification of at 332 bp and 400 bp were considered positive for assemblage A and B respectively. Amplification at both the assemblage-specific assays was regarded as mixed infection.

3.13 Phylogenetic analysis

The chromatograms and sequences generated from this study were viewed and assembled using BioEdit Sequence Alignment Editor Programme (http://www.mbio.ncsu.edu). Preliminary similarity comparison of the consensus sequence with the sequences in the Gen Bank database was made using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov). The isolate sequences were genotyped into assemblage and sub-assemblage using multiple alignments implemented by Clustal W (Thompson et al., 1994) with reference sequences retrieved from Gen Bank. Phylogenetic analysis was performed by Bayesian inference (BI) using Monte Carlo Markov Chain (MCMC) analysis in MrBayes 3.1.2 (Huelsenbeck et al., 2001; Holder & Lewis, 2003; Ronquist & Huelsenbeck, 2003) and neighbour-joining (NJ) algorithms with evolutionary distances calculated by Kimura-2 parameter method (Kimura, 1980) and 1000 bootstrap value in MEGA 5 (www.megasoftware.net).

3.14 Population genetic structure

Population genetic analysis was conducted to assess the genetic variation of *G. duodenalis* isolated from Malaysia as well as to understand the transcontinental distribution of the parasite genotypes by comparing with sequences recovered from other countries based on *tpi* gene. *Tpi* gene was chosen as the candidate as it produced the highest number of amplifications compared to the other two genes and had the relatively longer length of nucleotide sites for sufficient analysis. In addition, molecular characterization analysis using *tpi* was well established (Sulaiman et al., 2003) and the sequences were readily available in the NCBI GenBank. Thus, searches were carried out to retrieve as many *tpi* sequences as possible from the GenBank database. Of the 1465 *tpi* sequences available on the GenBank, only sequences with known sources were selected. A total of 932 sequences (119 sequences from Malaysia and 813 sequences from other countries) were enrolled in the analyses. The sequences were trimmed and

sorted into assemblages by visual inspection according to the positions of the single nucleotide polymorphisms (SNPs) using multiple alignments implemented by Clustal W (Thompson et al., 1994) in BioEdit Sequence Alignment Editor Programme (http://www.mbio.ncsu.edu). In order to obtain maximum length and maximum number of sequences with matching and continuous coverage for analyses, sequences that were too short and not in the common cover region were omitted. Besides, sequences with heterogeneous nucleotide were removed from the analyses.

A Median Joining Network analysis were performed with all individual *tpi* sequences obtained from the present study and the GenBank database to generate haplotype networks between closely related sequences using the program SplitsTree (Huson & Bryant, 2006) according to assemblages (i.e. A, B and E). Due to small number of sequences available for assemblage C, D, F, G and H, analyses for these isolates were not performed. The sequences were further classified into five populations according to continents (North and South America, Europe, Asia, Australia and Africa) to understand the distribution of *G. duodenalis* genotypes across different regions. Sequences of Malaysia generated from *tpi* gene comprised isolates of assemblages A and B obtained from different states in Malaysia were analysed within its own subset to access the genetic variation and the pattern of genotype distribution.

Genetic diversity analysis including identification of haplotypes, haplotype diversity (Hd), nucleotide diversity (Pi) were performed using DnaSP v.5.10.01 (Librado & Rozas, 2009). Using the same programme, the statistical tests of neutrality, Tajima's *D* (D) and Fu and Li's *D* (D*) as well as the mismatch distribution test were performed. The level of genetic differentiation measured using Wright's test indicated by fixation index (FST) with value ranging from 0 to 1 was rated as FST > 0.25 (huge differentiation), 0.15 to 0.25 (great differentiation), 0.05 to 0.15 (moderate differentiation) and FST < 0.05 (negligible differentiation) (Wright, 1978). Huge

differentiation would imply great separation between two populations while negligible differentiation would imply little divergence and free spreading between two populations. The point of reference for the level of gene flow was referred as high gene flow (Nm > 1), intermediate gene flow (0.25-0.99) and low gene flow (<0.25) (Govindajuru, 1989).

3.15 Statistical analysis

Data analysis was done by using SPSS for WINDOWS (version 18.0). Data was entered and reviewed by two different researchers. For descriptive analysis, the prevalence of infections and other categorical variables were expressed in percentages, while mean (standard deviation; SD) was used to present the quantitative data, with results being presented in tables and figures. Pearson's Chi Square test was used to examine the associations between *Giardia* infection as the dependent variable and demographic, socioeconomic, environmental and personal hygiene factors as the independent variables. All the variables used in the survey were coded in a binary manner as dummy variables. For example, *Giardia* infection (positive = 1, negative = 0); gender (male = 1, female = 0); availability of piped water supply of toilet in the house (no = 1, yes = 0) and washing hands before eating (no = 1, yes = 0). Family size was categorized into two groups (> 7 and ≤ 7 members), and age of participants was also categorized into two groups (≤ 12 and > 12 years) (Al-Mekhlafi et al., 2005; Mahdy et al., 2009a; Ngui et al., 2011). Furthermore, a monthly household income of < RM500 was considered as being low based on the poverty income threshold in Malaysia (Ngui et al., 2011). Odd ratios (OR) and 95% confidence intervals (CI) were computed. Multiple logistic regression analysis was used to identify the factors significantly associated with *Giardia* infection; OR and its corresponding 95% CI were calculated based on the final model. All tests were considered significant at $P < 0.05$.

3.16 Ethical consideration

The protocol of this study was approved by the Medical Ethics Committee of the University of Malaya Medical Centre (Ref. no: 788.74 and 878.19). Prior to the commencement of the survey questionnaire and sample collection, permissions were obtained from the heads of the villages. Then, the participants were informed about the objectives and methods of the study. They were informed that their participation was totally voluntary and that they could withdraw from the study at any time without citing any reason whatsoever. Written and signed or thumb-printed informed consent was obtained from those who agreed to participate, or from parents or guardians on behalf of their children, and these procedures were approved by the Medical Ethics Committee of the University of Malaya Medical Centre. The methods used in this research were carried out in accordance with the approved guidelines.

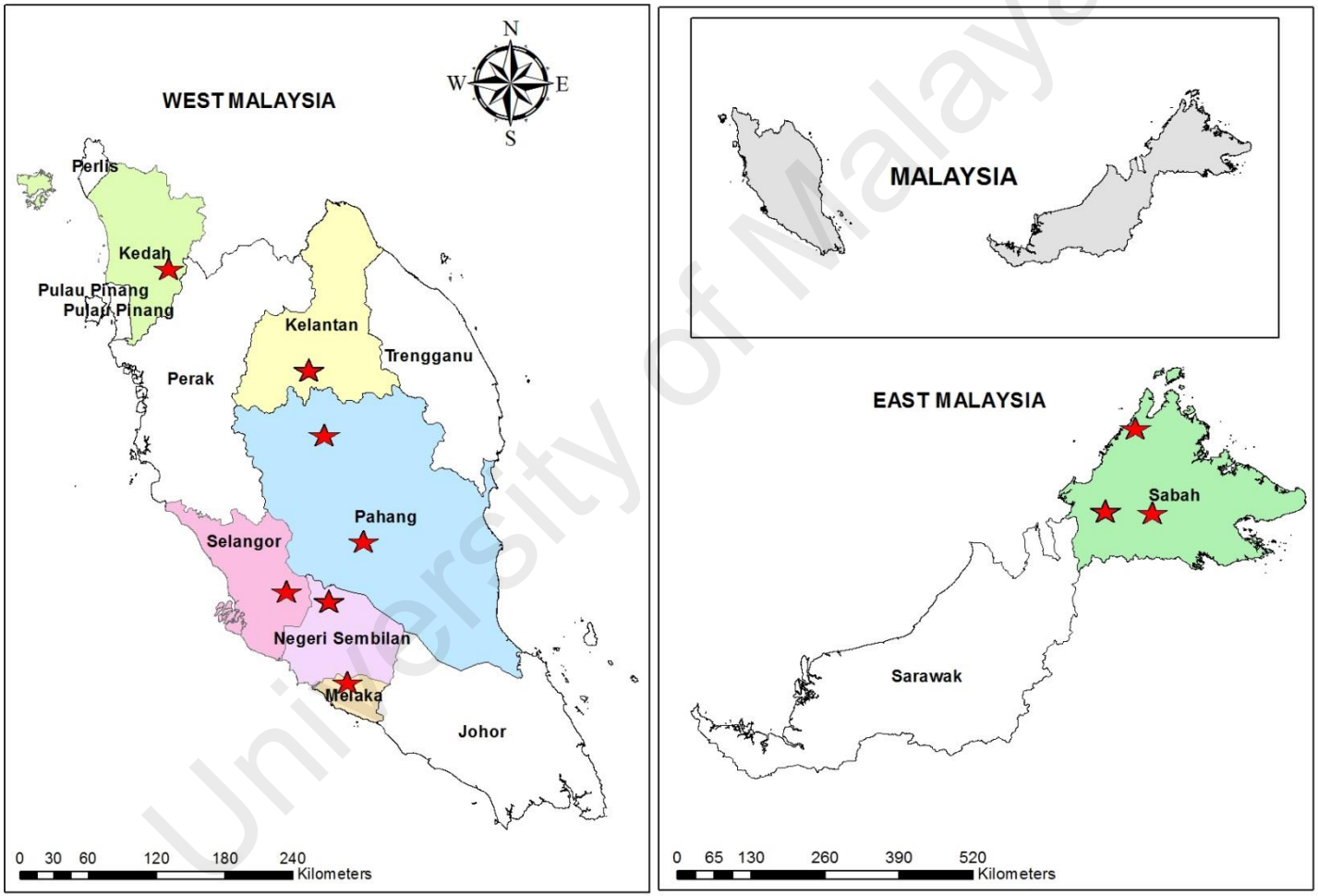

Figure 3.1: A map shows the study areas

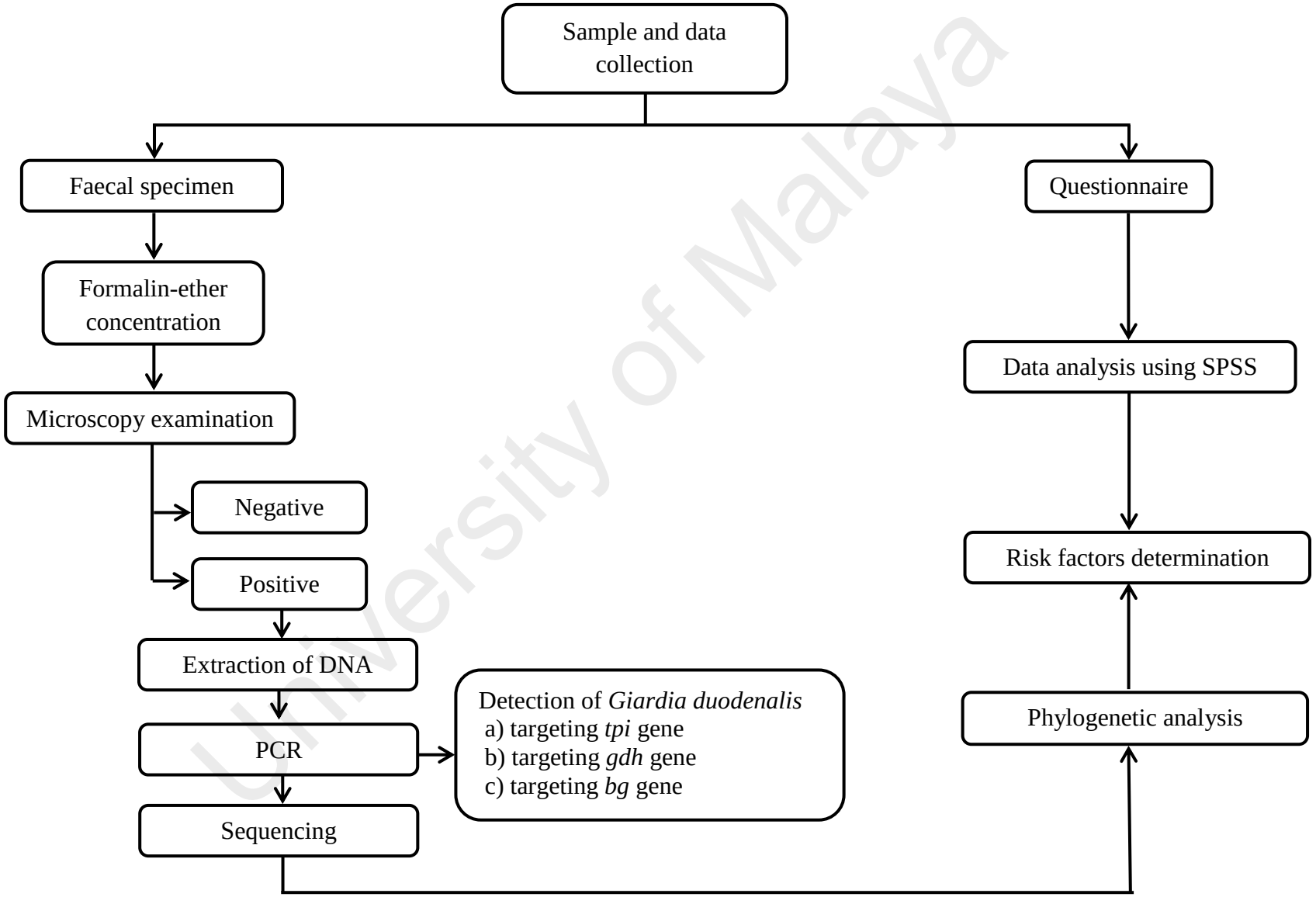

Figure 3.2: Workflow chart

CHAPTER IV: RESULTS

4.1 Prevalence and Associated Risk Factors of *Giardia* Infection among Indigenous Communities in Rural Malaysia

4.1.2 Study cohort and socioeconomic profile

A total of 1330 participants (50% males, 50% females) with a mean age of 15 years from seven states of Malaysia (986 from Peninsular Malaysia and 344 from Sabah, East Malaysia) were enrolled in the study. Among the cohort from Sabah, 175 (50.9%) were from the Dusun tribe, 97 (28.2%) from the Murut tribe, and 72 (20.9%) from the Bajau tribe. Among the cohort from Peninsular Malaysia, 484 (49.1%) were from the Semai tribe, 268 (27.2%) from the Temuan tribe, 99 (10.0%) from the Temiar tribe, 68 (6.9%) from the Jahut tribe, and 67 (6.8%) from the Kensiu tribe. Overall poverty prevails in these communities, with more than half (50.9%) of all families having a low monthly income (under RM500, the poverty income threshold in Malaysia). The greatest number of families with household incomes of less than RM500 is located in Peninsular Malaysia (56.6%), with Sabah also struggling with a spread low income households (34.0%). With regard to educational status, 62.9% of the participants had at least primary levels of education, with a higher percentage of subjects in Sabah being educated when compared to Peninsular Malaysia (75.7% vs 58.5%). Almost half of the adult participants are not working (48.6%), with almost similar frequencies of unemployment in both Peninsular Malaysia and Sabah (49.7% vs 46.8%). Those working were mainly farmers (39.5%) or otherwise engaged in agriculture (25.6%) (rubber and oil palm plantations), forestry, fishing and related occupations. General characteristics of the participants are presented in Table 4.1.

Table 4.1: General characteristics of the indigenous communities that participated in this study

Characteristics	Peninsular Malaysia (n = 986)	Sabah (n = 344)	Overall (n = 1330)
Age group			
≤ 12 years (1 month-12 years)	723 (73.3)	207 (60.2)	930 (69.9)
> 12 years (13-84 years)	263 (26.7)	137 (39.8)	400 (30.1)
Gender			
Male	500 (50.7)	164 (47.7)	664 (49.9)
Female	486 (49.3)	180 (52.3)	666 (50.1)
Socioeconomic status			
Low household income (< RM500)	558 (56.6)	119 (34.6)	677 (50.9)
> 7 members-large	353 (35.8)	79 (23.0)	432 (32.5)
Not working	100 (50.5)	60 (47.6)	160 (49.4)
Educational level			
Secondary education	66 (6.7)	63 (18.3)	129 (9.7)
Primary education	512 (51.9)	196 (57.0)	708 (53.2)
Non educated	408 (41.4)	85 (24.7)	493 (37.1)
Supplied with piped water	471 (47.8)	325 (94.5)	796 (59.8)
Presence of toilet at household	706 (71.6)	296 (86.0)	1002 (75.3)
Presence of animals at household	698 (70.8)	245 (71.8)	943 (71.1)

4.1.3 Prevalence of *Giardia* infection

The overall prevalence rate of *Giardia* infection was 11.6% (154/1330), with a significantly higher infection rate in Peninsular Malaysia when compared to Sabah (13.6%; 95% CI= 11.6, 15.9 vs 5.8%; 95% CI = 3.8, 8.8; *P*< 0.001). The prevalence of infection was also significantly higher among participants aged under 12 years of age (14.2%; 95% CI= 12.1, 16.6 vs 5.5%; 95% CI=3.7, 8.2; *P*< 0.001). There was a similar prevalence of infections reported among both males and females (12.1%; 95% CI= 9.8, 14.8 vs 11.1%; 95% CI= 8.9, 13.8; *P*= 0.593). With regards to the tribes in Peninsular Malaysia, the prevalence of *Giardia* infection was significantly higher among participants from the Semai tribe (17.8%), followed by the Kensiu (13.4%) and Temuan (10.8%) tribes. Similarly, the amount of infections were higher among the Dusun tribe of Sabah (8.6%) compared to the Murut and Bajau tribes. At the level of states, the highest prevalence of infections were in Pahang (15.9%) followed by Negeri Sembilan (14.9%) and Kedah (13.4%), while the lowest levels of infections were in Malacca (4.6%).

Faecal specimens were also screened for the presence of other intestinal parasitic infections, with participants being found to be infected with *Trichuris trichiura* (54.0%), *Ascaris lumbricoides* (28.7%), *Entamoeba histolytica/dispar/moshkovskii* (16.5%), and hookworms (10.5%). Overall, the prevalence of all detected infections was significantly higher among the participants from Peninsular Malaysia compared to those from Sabah (*P*< 0.001). About two-thirds of *Giardia* cases (104/154) were mixed infections with one or more parasite species, while one third were *Giardia* single infection (50/154). Regarding co-infections, *Giardia* and *Trichuris* was the most common co-infection, followed by *Giardia* with *Ascaris* and *Giardia* with *Entamoeba* species. The prevalence and distribution of *Giardia* and other parasitic infections according to location and tribes are shown in Table 4.2.

Table 4.2: Prevalence and distribution of intestinal parasitic infections among the indigenous communities that participated in this study

Parasite (%)	*Giardia*	*Trichuris*	*Ascaris*	*Entamoeba*	Hookworm
States					
Selangor	6.1	83.7	57.1	18.4	0.0
Malacca	4.6	53.8	27.7	9.2	0.0
Negeri Sembilan	14.9	61.7	34.4	5.2	11.0
Kedah	13.4	62.7	61.2	32.8	14.9
Pahang	15.9	69.9	40.6	27.0	17.6
Kelantan	8.1	9.1	3.0	15.2	1.0
Sabah	5.8	1.2	4.4	2.9	4.1
Tribes					
Semai	17.8	71.7	37.4	29.8	17.6
Kensiu	13.4	62.7	61.2	32.8	14.9
Jahut	2.9	57.4	63.2	7.4	17.6
Temiar	8.1	9.1	3.0	15.2	1.0
Temuan	10.8	63.8	36.9	8.6	6.3
Dusun	8.6	0.0	0.0	2.9	4.0
Murut	3.1	4.1	15.5	3.1	7.2
Bajau	2.8	0.0	0.0	2.8	0.0
Overall prevalence	11.6	46.0	28.7	16.5	10.5

4.1.4 Associated factors with *Giardia* infection

The associations of *Giardia* infection with demographic, socioeconomic and environmental factors are illustrated in Table 3. Besides location (Peninsular Malaysia) and age (≤ 12 years), participants from large households (with family sizes numbering more than 7 living together) experienced a significantly higher prevalence of *Giardia* infection than those from smaller families (14.8%; 95% CI = 12.1, 18.5 vs 10.0%; 95% CI = 8.2, 12.0).

There was also a significant association between *Giardia* infection and subjects educational level, with a higher prevalence among those who were either non educated or only had primary education when compared to those who had a secondary education (12.3%; 95% CI = 10.6, 14.3 vs 4.7%; 95% CI = 2.2, 9.8). However, prevalence of *Giardia* was not significantly different between those who had a primary education (12.7%; 95% CI= 10.5, 15.4) and those who were non educated at all (11.8%; 95% CI= 9.8, 15.0).

Furthermore, the prevalence of *Giardia* infection was significantly higher among those who live in houses without toilets when compared to those living in houses with functioning toilets (17.4%; 95% CI = 13.7, 21.9 vs 9.7%; 95% CI = 8.0, 11.7). With regards to hygienic practices, the results of univariate analyses revealed that the prevalence of *Giardia* infection is significantly associated with not washing hands before eating, not boiling water before consumption, bathing in the river, indiscriminate defecation, not washing vegetables/fruits before consumption, not wearing shoes when outside, not washing hands after playing with animals and indiscriminate garbage disposal.

Interestingly, when we stratified the univariate analyses according to location (Table 4.4), a significantly higher prevalence of *Giardia* infection was found among

Orang Asli from West Malaysia drinking piped water when compared to those who collect drinking water from unsafe sources (15.9% vs 11.5%; $\chi^2 = 4.181$; $P = 0.041$). It was found that participants from East Malaysia who still collect their drinking water from rivers, wells and rain have a higher prevalence of infection when compared to their counterparts; however the difference was not statistically significant(15.8% vs 5.2%; $\chi^2 = 3.655$; $P = 0.056$).

Overall, all the significant associations were retained by the Peninsular Malaysia group, while only not washing vegetables/fruits before consumption (OR = 3.4; 95% CI = 1.1, 11.1) was retained as a significant variable in regards to the Sabah group.

Table 4.3: Univariate analysis of factors associated with *Giardia* infection among the indigenous communities that participated in this study

Variables	No. Examined	No. Infected	% Infected	OR	95% CI	*P*-value
Location						
Peninsular Malaysia	986	134	13.6	2.5	1.6, 4.1	< 0.001
Sabah (East Malaysia)	344	20	5.8	1		
Age group						
<=12 Years	930	132	14.2	2.8	1.8, 4.5	< 0.001
> 12 Years	400	22	5.5	1		
Gender						
Male	664	80	12.0	1.1	0.8, 1.5	0.593
Female	666	74	11.1	1		
Size of household						
>7 members	432	64	14.8	1.6	1.1, 2.2	0.011
<=7 members	898	90	10.0	1		
Household income						
< RM500	677	88	13.0	1.3	0.9, 1.9	0.099
>= RM500	653	66	10.1	1		
Educational level (with three categories)						
Secondary education	129	6	4.7	1	1.3-7.0	
Primary education	708	90	12.7	3.0	1.2-6.5	0.008
Non educated	493	58	11.8	2.7	1.3-7.0	0.018
Educational level (with two categories)						
Secondary education	129	6	4.7	1		
Primary education/non educated	1201	148	12.3	2.9	1.2-6.7	0.010

Table 4.3: (continued)

Variables	No. Examined	No. Infected	% Infected	OR	95% CI	*P*-value
Employment status						
Not working	160	5	3.1	0.6	0.2, 2.0	0.421
Working	164	8	4.9	1		
Source of drinking water						
Unsafe water (river, well, rain)	534	62	11.6	1.0	0.7, 1.4	0.977
Piped water	796	92	11.6	1		
Use of water tank						
Not using	652	79	12.1	1.1	0.8, 1.5	0.568
Using	675	75	11.1	1		
Presence of toilet at household						
No	328	57	17.4	2.0	1.4, 2.8	< 0.001
Yes	1002	97	9.7	1		
Boiling water before consumption						
No	192	40	20.8	2.4	1.6, 3.5	< 0.001
Yes	1138	114	10.0	1		
Bathing place						
River	279	52	18.6	2.1	1.5, 3.1	< 0.001
Bathroom	1049	102	9.7	1		
Indiscriminate defecation						
Yes	437	77	17.6	2.3	1.6, 3.2	< 0.001
No	890	77	8.7	1		

Table 4.3: (continued)

Variables	No. Examined	No. Infected	% Infected	OR	95% CI	*P*-value
Eating using hands						
Yes	1158	145	12.5	2.5	1.3, 5.1	0.006
No	169	9	5.3	1		
Washing hands before eating						
No	327	49	15.0	1.5	1.0, 2.2	0.028
Yes	1003	105	10.5	1		
Washing hands after defecation						
No	269	35	13.0	1.2	0.8, 1.8	0.420
Yes	1058	119	11.2	1		
Consumption of raw vegetables						
Yes	453	45	9.9	0.8	0.5, 1.1	0.171
No	874	109	12.5	1		
Washing vegetables/fruits before consumption						
No	335	59	17.6	1.9	1.3, 2.9	< 0.001
Yes	993	95	9.6	1		
Wearing shoes when outside						
No	316	58	18.4	2.2	1.5, 3.1	< 0.001
Yes	1014	96	9.5	1		
Garbage disposal						
Indiscriminate	452	75	16.6	2.0	1.4, 2.8	< 0.001
Proper disposal	875	79	9.0	1		

Table 4.3: (continued)

Variables	No. Examined	No. Infected	% Infected	OR	95% CI	*P*-value
Playing in river						
Yes	391	56	14.3	1.5	1.0, 2.1	0.036
No	869	89	10.2	1		
Playing with soil						
Yes	748	97	13.0	1.4	0.9, 2.1	0.050
No	512	48	9.4	1		
Presence of domestic animals						
Yes	943	117	12.4	1.3	0.9, 2.0	0.153
No	384	37	9.6	1		
Washing hands after playing with animals						
No	266	50	18.8	2.1	1.5, 3.1	< 0.001
Yes	1061	104	9.8	1		

Table 4.4: Univariate analysis of risk factors associated with *Giardia* infection between Peninsular Malaysia and Sabah

	Peninsular Malaysia			Sabah (East Malaysia)		
Variables	Infected n (%)	OR (95% CI)	*P*-value	Infected n (%)	OR (95% CI)	*P*-value
Being aged <=12 years	117 (16.2)	2.8 (1.6, 4.7)	< 0.001	15 (7.2)	2.1 (0.7, 5.8)	0.163
Gender (Male)	70 (14.0)	1.1 (0.7, 1.5)	0.703	10 (6.1)	1.1 (0.4, 2.7)	0.830
Large Household size (>7 members)	62 (17.6)	1.7 (1.1, 2.4)	0.007	2 (2.5)	0.4 (0.1, 1.8)	0.155
Low household income (< RM500)	81 (14.5)	1.2 (0.8, 1.7)	0.333	7 (5.9)	1.0 (0.4, 2.6)	0.969
Not working	3 (3.0)	0.4 (0.1, 1.6)	0.183	2 (3.3)	2.2 (0.2, 20.0)	0.605
Primary education	75 (14.6)	2.7 (0.9, 7.5)	0.056	2 (3.2)	0.4 (0.1, 1.7)	0.258
Non educated	55 (13.5)	2.4 (0.8, 6.9)	0.090	3 (3.5)	1.1 (0.2, 6.8)	0.906
Using unsafe drinking water (river, hill & well)	75 (11.5)	0.7 (0.5, 0.9)	0.041	3 (15.8)	3.4 (0.9, 12.7)	0.056
Not using water tank	76 (12.8)	0.8 (0.6, 1.2)	0.384	3 (5.1)	0.8 (0.2, 2.9)	0.779
Not boiling water before consumption	39 (21.2)	2.0 (1.3, 3.0)	0.001	1 (12.5)	2.4 (0.3, 20.0)	0.384
Bathing in the river	49 (19.1)	1.8 (1.2, 2.6)	0.003	3 (13.6)	2.8 (0.7, 10.4)	0.129
No toilet at household	55 (19.6)	1.9 (1.3, 2.8)	< 0.001	2 (4.2)	0.7 (0.2, 3.0)	0.599
Indiscriminate defecation	77 (18.1)	2.0 (1.4, 2.8)	< 0.001	0	0	0

Table 4.4: (Continued)

	Peninsular Malaysia			Sabah (East Malaysia)		
Variables	Infected n (%)	OR (95% CI)	*P*-value	Infected n (%)	OR (95% CI)	*P*-value
Eating using hands	132 (13.8)	2.3 (0.5, 9.8)	0.239	13 (6.4)	1.3 (0.5, 3.3)	0.608
Not washing hands before eating	48 (15.0)	1.2 (0.8, 1.7)	0.371	1 (14.3)	2.7 (0.3,21.0)	0.348
Not washing hands after defecation	35 (13.2)	0.9 (0.6. 1.4)	0.810	0	0	0
Consumption of raw vegetables	41 (11.4)	0.7 (0.5, 1.1)	0.120	4 (4.3)	0.7 (0.2, 2.0)	0.469
Not washing vegetables/fruits before consumption	55 (17.8)	1.6 (1.1, 2.4)	0.009	4 (15.4)	3.4 (1.1, 11.1)	0.031
Not washing hands after playing with animals	46 (20.6)	2.0 (1.3, 3.0)	< 0.001	4 (9.3)	1.8 (0.6, 5.7)	0.305
Not wearing shoes when outside	57 (19.0)	1.9 (1.3, 2.7)	0.001	1 (6.2)	1.1 (0.1, 8.5)	0.946
Playing in river	47 (17.1)	1.5 (1.0, 2.2)	0.044	9 (7.8)	1.6 (0.7, 4.0)	0.285
Playing with soil	86 (14.5)	1.2 (0.8, 1.9)	0.295	11 (7.1)	1.5 (0.6, 3.8)	0.362
Indiscriminate garbage disposal	72 (16.9)	1.6 (1.1, 2.3)	0.009	3 (12.0)	2.4 (0.7, 8.8)	0.173
Presence of domestic animals at households	99 (14.2)	1.2 (0.8,1.8)	0.398	18 (7.3)	3.7 (0.8, 16.3)	0.063
Close contact with animals	86 (16.4)	1.7 (1.2, 2.5)	0.006	11 (8.7)	2.2 (0.9, 5.4)	0.090

4.1.5 Risk factors of *Giardia* infection

Table 4.5 shows that a multivariable logistic regression model retained 7 variables as being significant risk factors in terms of inducing *Giardia* infection among the studied indigenous people. The results confirmed that those aged ≤ 12 years (OR = 2.1) and living in a house without a functioning toilet (OR = 1.5) were at higher odds of having a *Giardia* infection when compared with their counterparts. Moreover, poor personal hygiene practices, including not washing hands after playing with animals, not boiling water before consumption, bathing in the river, not wearing shoes when outside and not washing hands before eating were also retained as significant risk factors of *Giardia* infection among these people.

4.1.6 Association of *Giardia* infection with diarrhoea

The majority of participants, 94.1% (1252/1330), had no complaints about gastrointestinal signs or symptoms. Of 78 symptomatic cases, 34.6% (27/78) had diarrhoea, 46.2% (36/78) had diarrhoea and abdominal pain, 6.4% (5/78) had abdominal pain, 10.3% (8/78) had vomiting, and 2.6% (2/78) had dysentery. As for *Giardia* positive cases, most of them were asymptomatic (129 out of 154 cases), and about one-third (25/78) of the symptomatic individuals were infected with *Giardia*. The prevalence of *Giardia* infection was significantly higher among the 63 participants who had diarrhoea when compared to their asymptomatic counterparts (36.5% vs 10.3%; χ^2 = 40.142; P< 0.001). Similarly, the prevalence of infection was higher among those who had abdominal pain compared to the asymptomatic individuals, however the difference was not statistically significant (19.5% vs 11.3%; $\chi 2$ = 2.601; P = 0.107). It was noted that 57 (90.5%) of the diarrhoea cases were from Peninsular Malaysia, compared to only 6 (9.5%) cases from Sabah (χ^2 = 9.209; P= 0.002).

Table 4.5: Multivariate analysis of risk factors associated with *Giardia* infection among the indigenous communities that participated in this study

Variables	Adjusted OR	95% CI	*P*-value
Location (Peninsular Malaysia)	1.5	0.9, 2.6	0.115
Being aged ≤ 12 years	2.1	1.3, 3.4	0.003
Gender (males)	1.1	0.7, 1.5	0.799
Large household members (>7 members)	1.4	0.9, 2.0	0.065
Low educational level	1.0	0.4, 2.7	0.968
No toilet at household	1.5	1.0, 2.2	0.049
Not boiling water before consumption	2.1	1.4, 3.3	0.001
Bathing in the river	1.7	1.2, 2.6	0.007
Indiscriminate defecation	1.2	0.7, 2.0	0.608
Not washing hands before eating	1.5	1.1, 2.2	0.029
Not washing hands after playing with animals	2.1	1.4, 3.1	< 0.001
Not washing vegetables/fruits before consumption	1.1	0.8, 1.7	0.561
Not wearing shoes when outside	1.6	1.1, 2.4	0.012
Indiscriminate garbage disposal	1.1	0.7, 1.7	0.673

4.2 Molecular genotyping of *Giardia duodenalis*

4.2.1 Preliminary assessment of nucleotide sequences and identification of assemblages

Microscopy examination of the 1330 samples revealed that 154 participants were infected with *Giardia*. Analysis using multilocus genotyping approach on the positive isolates had successfully amplified 138 samples using at least one of the markers (*tpi*, *gdh*, and *bg*) and seven additional isolates from assemblage-specific primers while nine isolates remained negative at all markers used. Forty eight isolates were amplified at three genes, 31 isolates were amplified at *tpi* and *gdh* genes, 18 isolates at *tpi* and *bg* genes, two at *gdh* and *bg* genes, while the rest were amplified at any one of the three loci. When sequences generated from the amplicons were analysed using multiple alignment with previously published reference sequences, 52/138 (37.7%) were genotyped as assemblage A and 59/138 (42.8%) were genotyped as assemblage B. Discordant genotyping of assemblage by different loci was found in 27 isolates (18.6%). For example, four isolates (DB2.1, BP14.2, JJ11.4, and PD20.1) were identified as assemblage B at *tpi* and *gdh* genes but were assigned to assemblage A at *bg* gene. Amplification using *tpi* gene produced the highest number of PCR amplicons i.e. 122 (79.2%). Sequence alignment patterns assigned 69 (56.6%) of the isolates to assemblage A and 53 (43.4%) to assemblage B. At *gdh* gene, the amplification rate was 91 (59.1%). Higher number of isolates i.e. 72 (79.1%) was identified as assemblage B compared to only 19 (20.9%) as assemblage A. Less than half of the isolates i.e. 72 (46.8%) were amplified by PCR based on *bg* gene. Among the isolates, 45 (62.5%) were identified as assemblage A and 27 (37.5%) as assemblage B (Table 4.6 and 4.7)

Table 4.6: The distribution of assemblages A and B based on the different loci and mixed infection

Genes	Assemblages n (%)			
	A	B	A+B	Total
tpi	69 (56.6)	53 (43.4)	-	122
gdh	19 (20.9)	72 (79.1)	-	91
b-giardin	45 (62.5)	27 (37.5)	-	72
MLGs[a]	15 (40.5)	22 (59.5)	-	37
tpi-Mixed[b]	15 (12.5)	56 (46.7)	49 (40.8)	120

[a]Multilocus genotypes (isolates with the same assemblage identified using *tpi* and *gdh* and *b*-giardin)

[b]*tpi*-based PCR used assemblage-specific primers to identify the mixed infection

Table 4.7: The distribution of assemblages A and B and mixed infection for all the isolates

Isolate	*tpi*	*gdh*	*bg*	*tpi*-Mixed	Isolate	*tpi*	*gdh*	*bg*	*tpi*-Mixed
PG 22.1	A(A2)	A(A2)	A(A2)	A	SE25.2	A(A2)			A+B
PG 3.1	A(A2)	A(A2)	A(A3)	A	KK1.6	A(A2)			A+B
PB 11.1	A(A2)	A(A2)	A(A2)		CK1.2	A(A2)			B
PM 13.1	A(A2)	A(A2)	A(A2)	A	CK10.2	A(A2)			A+B
PM 25.2	A(A2)	A(A2)	A(A3)	A	SB1.1	A(A2)			A+B
PM 31.2	A(A2)	A(A2)	A(A3)	A	SE25.4	A			A+B
PM 53.1	A(A2)	A(A2)	A(A3)	A+B	DK 28.3	A(A2)			B
SB 3.3	A(A2)	A(A2)	A(A2)	A	UK 6.7	A(A2)			B
DK 19.10	A(A2)	A(A2)	A(A2)	A	PM 54.1	A(A2)			A+B
UK 17.3	A(A2)	A(A2)	A(A2)		PB 14.5	A(A2)			
PG 26.2	A(A2)	A(A2)	A(A2)	A	ST10.3		A(A2)		B
SW16.3	A(A2)	A(A2)	A(A3)	A	TB12.3			A(A2)	
TB11.6	A(A2)	A(A2)	A(A2)	A+B	KK7.5			A(A3)	
TG2.5	A(A2)	A(A2)	A(A3)	B	SE29.4			A(A2)	
TG13.2	A(A2)	A(A2)	A(A3)	B	DK 10.4	B	B	B	B
TB13.3	A(A2)	A(A2)		A+B	DK 17.3	B	B	B	B
SB14.1	A(A2)	A(A2)			DK 17.6	B	B	B	B
ST15.1	A(A2)	A(A2)		A+B	DK 27.1	B	B	B	B
KK16.4	A(A2)		A(A2)		DK 28.2	B	B	B	
KK16.5	A(A2)		A(A2)	A+B	UK 6.2	B	B	B	B
TB7.3	A(A2)		A(A2)	A+B	UK 6.5	B	B	B	
SE10.1	A(A2)		A(A3)	B	UL 1.11	B	B	B	B
SE17.1	A(A2)		A(A2)	A	UL 1.3	B	B	B	B
TB15.3	A(A2)		A(A2)	A+B	UL 1.7	B	B	B	B
TG9.2	A(A2)		A(A2)	A+B	UL 4.4	B	B	B	B
PD1.1	A(A2)		A(A3)	A+B	NT 4.4	B	B	B	B
PD11.2	A(A2)		A(A2)	A+B	PG 2.7	B	B	B	B
SE29.3	A(A2)		A(A2)		SW14.3	B	B	B	B
DK 29.1	A(A2)		A(A3)	A	KM15.1	B	B	B	B
DK 3.3	A(A2)		A(A3)	A	TB2.3	B	B	B	B
UK 15.4	A(A2)		A(A2)	A	TB4.1	B	B	B	A+B
UK 15.5	A(A2)		A(A2)		TB5.1	B	B	B	A+B
DK 10.3	A(A2)		A(A2)	A	CK20.2	B	B	B	A+B
SP 11.1	A(A2)		A(A2)	A	SE7.1	B	B	B	A+B
KK11.3	A(A2)			A+B	SE12.1	B	B	B	B
KM4.1	A(A2)				SE24.2	B	B	B	B
TB3.3	A(A2)			A+B	DB 2.1	B	B	A(A3)	B
TB13.2	A(A2)			A+B	BP 14.2	B	B	A(A2)	B

Table 4.7: (Continued)

Isolate	*tpi*	*gdh*	*bg*	*tpi*-Mixed	Isolate	*tpi*	*gdh*	*bg*	*tpi*-Mixed
JJ 11.4	B	B	A(A2)	B	UL 3.3	A(A2)	B	B	B
PD20.1	B	B	A(A3)	A+B	DB 11.7	A(A2)	B	B	A+B
JJ 7.2	B	B			UK 12.2	A(A2)	B	A(A3)	
JJ 11.5	B	B		B	UK 15.2	A(A2)	B	A(A2)	
UL 13.2	B	B		B	UK 9.2	A(A2)	B	A(A2)	
UL 19.6	B	B		B	ST8.1	A(A2)	B	A(A2)	B
UL 3.4	B	B		B	BP 1.5	A(A2)	B	A(A2)	A+B
NT 6.2	B	B			DB 9.1	A(A2)	B		
SW10.1	B	B		B	PG 10.3	A(A2)	B		B
KN7.4	B	B		A+B	UK 15.3	A(A2)	B		B
SM1.2	B	B		A+B	UK 17.1	A(A2)	B		
KM13.2	B	B		A+B	UK 6.3	A(A2)	B		
TB9.2	B	B		A+B	PP 1.1	A(A2)	B		B
CK7.1	B	B		A+B	PS 7.3	A(A2)	B		B
SE13.1	B	B		B	KN7.2	A(A2)	B		B
TB15.1	B	B		A+B	SM6.2	A(A2)	B		A+B
SW11.2		B	B	B	KM3.2	A(A2)	B		B
SE9.3	B		B	A+B	KM12.3	A(A2)	B		A+B
PG 12.2	B			B	TB11.2	A(A2)	B		A+B
DK 11.2	B			B	SB15.1	A(A2)	B		A+B
PG 5.1	B			A+B	SE18.4	A	B		A+B
KN6.4	B			A+B	PM 53.2	B		A(A3)	A+B
TB9.1	B			A+B	NT 6.3		B	A(A2)	B
UM3.1	B			A+B	KK6.1				A+B
SE8.4	B			A+B	CK20.1				A+B
SE27.6	B			B	ST1.3				B
SE27.7	B			B	ST3.2				B
KK2.3	B			A+B	ST21.1				B
KK2.5	B			A+B	TB2.5				B
UL 19.5		B		B	SE13.2				B
SW5.3		B		B	SE 13.3				
KM7.3		B			PD 18.1				
KM8.1		B			ST 26.2				
KM15.2		B		B	KN 6.3				
ST13.2		B		B	ST 7.2				
ST20.3		B		B	TB 10.1				
KM8.4		B			PG 20.1				
CK1.4		B		A+B	PM 19.2				
SB4.1			B		SP 7.3				

4.2.2 Analysis of molecular data of respective markers and their distribution

4.2.2.1 Assessment of *tpi* sequences

Consensus sequence of 122 *tpi* isolates with the length of 504 base pairs were scrutinized and multiple aligned with published sequences identified as assemblage A (accession no. L02120 and U57897) and assemblage B (accession no. AF069560, L02116, AY368167, AY368165 and AY368163).

When the isolates were compared with the respective base reference sequence (Table 4.9), intragenotypic variations were detected within sequences of both assemblage A and B. Three different subtypes of assemblage A representing 66 isolates were defined based on the nucleotide substituted and the positions of nucleotide substitution. In contrast to assemblage A, greater number of subtypes that is as high as 32 representing 51 isolates was found in assemblage B (Table 4.8). Three isolates with moderate mixed templates as displayed by the multiple peaks in sequence chromatogram namely SE25.4, SE18.4 and PM54.1 could not be distinctly typed but were assigned to assemblage A by phylogenetic analysis and the nucleotide distribution pattern which predominantly resembled assemblage A. Similarly, two isolates (i.e. PD20.1 and KK2.5) of assemblages B were not grouped into the subtypes due to mixed templates. It was shown by the assemblage-specific primers that these isolates were mixed infection of assemblages A and B.

Further inspection of the sequences and the chromatograms revealed occurrences of heterogeneous nucleotide in eight of the 32 subtypes of assemblage B, characterized by the presence of double peaks at the sequence fragment. Nine positions with nucleotide heterogeneous were identified in the sequences i.e. 39 (A/G), 45 (C/T), 91 (C/T), 165 (C/T), 168 (C/T), 210 (A/G), 255 (A/G), 264 (A/G), 516 (A/G). The heterogeneous nucleotides were shown in Table 4.9 and were replaced with IUPAC

codes (R= A/G; Y= C/T). These heterogeneous nucleotides were examined at the multiple alignments to see about whether it occurred at positions that a) show polymorphism that define assemblages A and B (could imply inter-assemblage mixed infection; b) contain polymorphism restricted to assemblage B only (could imply intra-assemblage polymorphism/ mixed infection) and c) contain nucleotide common to both assemblages A and B but show polymorphism within assemblage B (could be due to both intra- and inter- assemblage mixed infections). It was found that none of the nucleotide heterogeneous present at positions that define assemblages A and B. Six were identified at positions common for both the assemblages while three at positions with polymorphism within assemblage B only. Conversely, no double peaks were observed in the sequences of the assemblage A isolates.

The most common subtype among the isolates of assemblage A was KC3132923. All the isolates with the exception of UK17.1, DK29.1 and SW16.3 were homologous to KC3132923 that is to say the isolates that under this subtype had the similar pattern of nucleotide substitution. It should be pointed out that this common subtype also had 100% similarity with the prototype isolate, JH (accession no. U57897). Besides, when the distribution of the subtypes in different localities (i.e. in different state) was studied, it was found that the subtype KC3132923 had a wide distribution and was obtained from all states (i.e. Sabah, Selangor, Pahang, Negeri Sembilan, Kedah and Melaka) except Kelantan which harboured the subtype KT124816 (Table 4.10).

As indicated earlier, assemblage B had higher number of subtypes compared to assemblage A. However, most of the subtypes were represented by only one representative sequence. Other subtypes were observed in two to twelve isolates. The most common subtype of assemblage B was KC313907 and was found in five states (i.e. Sabah, Pahang, Negeri Sembilan, Kedah and Kelantan). In proportion with the high

prevalence of *Giardia* infection in Pahang, the state had the highest number of subtype B (i.e. 16), while other states had one to six subtypes (Table 4.10).

Table 4.8: Accession number and isolates of *tpi*

Number of genotype	Accession number	Isolates
Assemblage A		
Reference	L02120	WB
Reference	U57897	JH
1	KC313923	TB11.6, TG2.5, TG13.2, TB13.3, SB14.1, ST15.1, KK16.4, KK16.5, TB7.3, SE10.1, SE17.1, TB15.3, TG9.2, PD1.1, PD11.2, SE29.3, KK11.3, KM4.1, TB3.3, TB13.2, SE25.2, KK1.6, CK1.2, CK10.2, SB1.1, ST8.1, KN7.2, SM6.2, KM3.2, KM12.3, TB11.2, SB15.1, UK15.2, UK12.2, UK6.3, PG3.1, PP1.1, UK9.2, UK15.3, UK6.7, DK28.3, UK17.3, PM31.2, DB11.7, PB11.1, SB3.3, PG10.3, PS7.3, UL3.3, PB14.5, DK3.3, UK15.5, DK10.3, PG26.2, DB9.1, PM13.1, PM25.2, PM53.1, DK19.10, PG22.1, SP11.1, UK15.4, BP1.5
2	KT124815	UK17.1, DK29.1
3	KT124816	SW16.3
Assemblage B		
Reference	AF069560	Ad 19
Reference	L02116	GS/M
Reference	AY368167	7115
Reference	AY368165	2434
Reference	AY368163	2436
1	KC313907	CK7.1, SE8.4, TB5.1, DK28.2, JJ7.2, UK6.5, DK11.2, UL3.4, UK6.2, NT6.2, DK17.6, UL13.2
2	KC313908	SE24.2, KN7.4,TB4.1
3	KC313909	TB2.3
4	KC313910	TB15.1
5	KC313911	KM13.2
6	KC313912	CK20.2
7	KC313913	TB9.2

Table 4.8: (Continued)

Number of genotype	Accession number	Isolates
8	KC313914	SE9.3
9	KC313915	SE13.1
10	KC313916	UM3.1
11	KC313917	KN6.4
12	KC313918	SE12.1, SE27.6, TB9.1, SE7.1
13	KC313919	KK2.3
14	KC313920	SE27.7
15	KC313921	SM1.2
16	KC313922	KM15.1
17	KT124817	DK17.3
18	KT124818	DK10.4
19	KT124819	BP14.2
20	KT124820	SW14.3
21	KT124821	DB2.1
22	KT124822	JJ11.5, PM53.2, UL1.7
23	KT124823	JJ11.4
24	KT124824	UL19.6
25	KT124825	NT4.4
26	KT124826	SW10.1
27	KT124827	PG2.7, PG12.2
28	KT124828	PG5.1
29	KT124829	UL1.11
30	KT124830	UL4.4
31	KT124831	DK27.1
32	KT124832	UL1.3

Table 4.9: Multiple alignments of *tpi* sequences from this study with reference sequences obtained from GenBank, representing sub-assemblages of assemblages A and B

Isolates	GenBank accession no.	Nucleotide position from the start of the gene																														
Assemblage A		**129**	**184**	**399**	**445**																											
AI	**L02120**	T	A	C	G																											
AII (A2)	**U57897**	C	.	T	.																											
AII (A2)	KC313923	C	.	T	.																											
AII (A2)	KT124815	C	G	T	.																											
AII (A2)	KT124816	C	.	T	A																											
Assemblage B		**39**	**45**	**91**	**100**	**109**	**121**	**162**	**165**	**168**	**210**	**216**	**242**	**247**	**255**	**263**	**264**	**271**	**280**	**297**	**312**	**354**	**402**	**426**	**429**	**438**	**471**	**483**	**492**	**516**		
BIV	**AF069560**	A	T	T	C	G	G	G	T	T	A	C	T	A	G	G	G	C	A	A	C	G	A	G	A	T	A	A	C	?		
BIV	**L02116**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G		
BIV-Like	**AY368167**	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G		
BIII	**AY368165**	G	.	C	.	.	.	.	C	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	A	.	G		
BIII-Like	**AY368163**	G	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G		
	KC313907	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G		
	KC313908	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G		
	KC313909	G	C	C	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G		
	KC313910	G	C	C	.	.	.	.	.	.	G	.	.	.	.	.	.	t	.	.	.	.	.	.	G	.	.	.	.	G		
	KC313911	G	C	.	.	.	.	.	.	.	G	.	.	.	.	.	.	t	g	.	.	.	.	.	G	.	.	.	.	G		
	KC313912	G	.	C	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	G	.	.	g	.	G	.	.	G	.	G		
	KC313913	G	C	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G		
	KC313914	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	G		

Table 4.9: (Continued)

Isolates	GenBank accession no.	Nucleotide position from the start of the gene
	KC313915	. . . . . C . . . . . G . . G . G
	KC313916	. C . . . . . C G . . . . . G . . . . G
	KC313917	. . . . . C G . . . . . g . G . . . . G
	KC313918	. . C . . . . . C G . . . . . G . . . . G
	KC313919	G C C . . . . . C G . . . . . G . . . . G
	KC313920	. . C . . . . C C G . . . . . G . . . . G
	KC313921	G . C . . . . C C . . . g . . . . G . . . . G
	KC313922	. . . t . a . C C G . . . . . a . . G . . . . G
	KT124817	G C C . . . . C C G . . . . . G . . . T R
	KT124818	R C C . . . . C C G . . . . . G . . . . G
	KT124819	G C . . . . . C C G . . . . . G . . . . G
	KT124820	G C . . . . . A . . . . G . C G . G
	KT124821	G C . . . . . G . . . . G . . . . G
	KT124822	G C . . . . . G . . . . G . . . T A
	KT124823	G . C . . . . C C G . . . . . G . . . . G
	KT124824	G . C . . . . C C G . . . . . T . . . G . . . . G
	KT124825	R Y . . . . . Y Y R . . . . . G . . . . G
	KT124826	. Y C . . . . Y Y G . . . R . R . . . . G . . . . G
	KT124827	. . . . . C . . . . . G . . . . G
	KT124828	. . . . . A G . . . . G
	KT124829	R . Y . . . . Y Y R . . . . . G . . . . G
	KT124830	R Y Y . . . . Y Y . . . . . G . . . . G
	KT124831	. . . . . R . . . . . G . . . . G
	KT124832	G C . . . . . G . . . T R

Table 4.10: Distribution of *tpi* subtypes in different states

States	Types	Isolates	Assemblages
Sabah	KC313923	PG3.1, PG10.3, PM13.1, PG22.1, PM25.2, PM53.1, PG26.2, PM31.2, PB11.1, PB14.5, SP11.1	A
	KC313907	NT6.2	B
	KT124825	NT4.4	B
	KT124827	PG2.7, PG12.2	B
	KT124828	PG5.1	B
	KT124822	PM53.2	B
Selangor	KC313923	DB9.1, DB11.7	A
	KT124821	DB2.1	B
Pahang	KC313923	TB11.6, TG2.5, TG13.2, TB13.3, SB14.1, ST15.1, KK16.4, KK16.5, TB7.3, SE10.1, SE17.1, TB15.3, TG9.2, PD1.1, PD11.2, SE29.3, KK11.3, KM4.1, TB3.3, TB13.2, SE25.2, KK1.6, CK1.2, CK10.2, SB1.1, ST8.1, KN7.1, SM6.2, KM3.2, KM12.3, TB11.2, SB15.1, PP1.1, PS7.3	A
	KC313907	CK7.1, SE8.4, TB5.1	B
	KC313908	SE24.2, KN7.4,TB4.1	B
	KC313909	TB2.3	B
	KC313910	TB15.1	B
	KC313911	KM13.2	B
	KC313912	CK20.2	B
	KC313913	TB9.2	B
	KC313914	SE9.3	B
	KC313915	SE13.1	B
	KC313916	UM3.1	B
	KC313917	KN6.4	B
	KC313918	SE12.1, SE27.6, TB9.1, SE7.1	B
	KC313919	KK2.3	B
	KC313920	SE27.7	B
	KC313921	SM1.2	B
	KC313922	KM15.1	B

Table 4.10: (Continued)

States	Types	Isolates	Assemblages
Negeri Sembilan	KC313923	UK6.3, UK9.2, UK12.2, UK15.2, UK15.3, UK6.7, UK17.3, UK15.4, UK15.5, DK28.3, DK3.3, DK10.3, DK19.10	A
	KT124815	UK17.1, DK29.1	A
	KC313907	DK11.2, DK17.6, DK28.2, UK6.2, UK6.5	B
	KT124817	DK17.3	B
	KT124818	DK10.4	B
	KT124831	DK27.1	B
Kedah	KC313923	UL3.3	A
	KC313907	UL3.4, UL13.2	B
	KT124824	UL19.6	B
	KT124829	UL1.11	B
	KT124830	UL4.4	B
	KT124832	UL1.3	B
	KT124822	UL1.7	B
Kelantan	KT124816	SW16.3	A
	KC313907	JJ7.2	B
	KT124820	SW14.3	B
	KT124822	JJ11.5	B
	KT124823	JJ11.4	B
	KT124826	SW10.1	B
Melaka	KC313923	SB3.3, BP1.5	A
	KT124819	BP14.2	B

4.2.2.2 Assessment of *gdh* sequences

The sequence data of the 91 *gdh* isolates were examined over consensus of 408 base pairs. Multiple alignment with the reference sequences of assemblage A (accession no. M84604 and L40510) and assemblage B (accession no. AF069059 and L40508) resulted in identification of two subtypes among the 19 isolates of assemblage A and 31 subtypes among the 72 isolates of assemblage B (Table 4.11). Two mixed templates (i.e. UK15.5 and DK3.3) were amplified with visible band at expected size in gel electrophoresis but were excluded from the analysis because of the highly noised sequences. Nonetheless, these two isolates were assigned to assemblage A according to *tpi* and *bg* markers.

It was found that *gdh* assay was less sensitive to sequences of assemblage A and showed preference in amplifying isolates of Assemblage B. This can be seen from the results which showed 16 sequences that typed as assemblage A by *tpi* and *bg* could not be amplified using *gdh*; nine isolates of assemblage B could only be amplified by *gdh* but not *tpi* and *bg*; 14 samples typed as assemblage A at *tpi* but were typed as assemblage B by *gdh* (Table 4.7).

On the other hand, of the 31 subtypes belonged to assemblage B, heterogeneous nucleotides were detected in the chromatograms of 15 subtypes (Table 4.12). Eight positions with heterogeneous nucleotides were identified and all of them occurred at positions that contained common nucleotide between assemblages A and B. All the isolates of assemblage A were homologous to subtype KC313924 except ST10.3 (Accession no. KC313925). This subtype was distributed in all states except Selangor and Kedah. It was also noted that these two states were absent of isolates of assemblage A. On the other hand, 18 of the total subtypes of assemblage B were represented by single representative sequence while others were represented by two to 10

representative sequences. While some of the subtypes were restricted to a state e.g. KC313928 and KC313929 were found only in Pahang, some were seen in several states e.g. KT124836 was found in Pahang, Kedah, Negeri Sembilan and Kelantan while KT124840 was found in Sabah, Negeri Sembilan and Selangor (Table 4.13).

Table 4.11: Accession number and isolates of *gdh*

Number of genotype	Accession number	Isolates
Assemblage A		
Reference	**M84604**	Portland1
Reference	**L40510**	Ad-2
1	KC313924	TB11.6,TG2.5,TG13.2, TB13.3, SB14.1, ST15.1, PG3.1, DK19.1, PG22.1, PM13.1, PM25.2, PM31.2, PM53.1, SB3.3, PG26.2, UK17.3, PB11.1, SW16.3
2	KC313925	ST10.3
Assemblage B		
Reference	**AF069059**	BAH-12
Reference	**L40508**	Ad-7
1	KC313926	SM1.2
2	KC313927	CK1.4
3	KC313928	KM7.3,TB11.2,KM8.1, SM6.2
4	KC313929	KM15.2,ST8.1,KM15.1, ST13.2,KM8.4,ST20.3, TB2.3, KN7.2
5	KC313930	SE13.1
6	KC313931	KM3.2
7	KC313932	TB15.1, SB15.1
8	KC313933	SE12.1, TB4.1
9	KC313934	TB5.1, SE7.1
10	KC313935	KM13.2,CK7.1,TB9.2, SE24.2,SE18.4, SW14.3
11	KC313936	PD20.1, KN7.4
12	KC313937	CK20.2
13	KC313938	KM12.3
14	KT124833	UL4.4
15	KT124834	JJ7.2
16	KT124835	BP1.5
17	KT124836	PP1.1, UL1.3, UK15.2, UL3.3, PS7.3, DK28.2, SW10.1,UK15.3, UK17.1, JJ11.5

Table 4.11: (Continued)

Number of genotype	Accession number	Isolates
18	KT124837	UL1.7
19	KT124838	NT6.2, JJ11.4, UK6.3
20	KT124839	UK6.2
21	KT124840	NT6.3, UK9.2, DB9.1, UK12.2, PG10.3
22	KT124841	NT4.4, DB2.1
23	KT124842	UL19.6, DK17.3, DK17.6, PG2.7, DB11.7
24	KT124843	DK27.1
25	KT124844	UL3.4
26	KT124845	DK10.4
27	KT124846	BP14.2
28	KT124847	UL13.2
29	KT124848	SW11.2, SW5.3, UK6.5
30	KT124849	UL1.11
31	KT124850	UL19.5

Table 4.12: Multiple alignments of *gdh* sequences from this study with reference sequences obtained from GenBank, representing sub-assemblages of assemblages A and B

Isolates	GenBank accession no	Nucleotide position from the start of the gene														
Assemblage A		**237**	**246**	**341**	**485**	**603**	**621**									
AI	**M84604**	C	C	A	C	T	C									
AII	**L40510**	C	C	.	.	C	T									
AII	KC313924	?	?	.	.	C	T									
	KC313925	?	?	G	T	C	T									
Assemblage B		**309**	**357**	**360**	**429**	**447**	**468**	**519**	**540**	**561**	**570**	**576**	**582**	**597**	**606**	**612**
BIII	**AF069059**	C	T	G	T	T	G	C	C	C	C	G	G	C	C	G
BIV	**L40508**	T	.	.	C	C	.	.	T	T	.	.	.	.	.	A
	KC313926	T	.	.	.	C	.	T	.	.	.	.	.	.	.	.
	KC313927	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.
	KC313928	T	.	.	C	C	.	T	.	.	.	.	.	.	.	.
	KC313929	T	.	.	C	C	.	.	.	.	.	.	.	.	.	.
	KC313930	T	C	.	.	C	.	T	.	.	.	.	.	.	.	.
	KC313931	T	C	.	C	C	.	T	.	.	.	.	.	.	.	.
	KC313932	T	C	.	C	C	.	.	.	.	.	.	.	.	.	.
	KC313933	T	C	.	C	C	.	.	T	.	.	.	.	.	.	.
	KC313934	T	C	.	C	C	.	.	.	.	.	.	.	.	.	A
	KC313935	T	C	.	C	C	.	.	T	.	.	.	.	.	.	A
	KC313936	.	.	.	C	C	.	.	T	.	.	.	.	.	.	.
	KC313937	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.
	KC313938	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.

Table 4.12: (Continued)

Isolates	GenBank accession no	Nucleotide position from the start of the gene													
	KT124833	Y	C	.	C	C	.	.	T	Y	.	.	.	.	T	A
	KT124834	.	C	.	C	C	.	.	T	T	.	.	.	.	.	A
	KT124835	.	.	.	C	C	.	T	C	.	.	.	.	.	.	.
	KT124836	T	Y	.	Y	C	.	.	T	.	.	.	.	.	Y	A
	KT124837	T	Y	.	C	C	.	.	T	.	.	.	.	.	.	A
	KT124838	T	Y	.	Y	C	.	.	T	.	.	.	.	.	T	A
	KT124839	T	C	.	C	C	A	.	.	.	.	.	.	.	.	.
	KT124840	T	Y	.	Y	C	.	.	T	.	.	.	.	.	T	A
	KT124841	T	Y	.	C	C	.	.	T	Y	.	.	.	.	Y	A
	KT124842	T	C	.	C	C	.	.	T	Y	.	.	.	.	.	A
	KT124843	T	C	.	C	C	.	.	T	Y	.	.	.	.	T	A
	KT124844	T	C	.	C	C	.	.	T	T	.	.	.	.	Y	A
	KT124845	T	C	.	C	C	.	.	T	Y	.	.	.	.	T	.
	KT124846	Y	Y	.	C	C	.	Y	Y	.	.	.	.	.	.	R
	KT124847	T	Y	.	C	C	.	.	T	T	.	.	.	.	Y	A
	KT124848	T	Y	.	C	C	.	.	T	T	.	.	.	.	.	A
	KT124849	T	Y	.	C	C	.	.	Y	.	.	.	.	.	.	R
	KT124850	T	Y	.	Y	C	.	.	T	.	.	.	.	.	Y	R

Table 4.13: Distribution of *gdh* subtypes in different states

States	Types	Isolates	Assemblages
Sabah	KC313924	PG3.1, PG22.1, PG26.2, PM13.1, PM25.2, PM31.2, PM53.1, PB11.1	A
	KT124838	NT6.2	B
	KT124840	NT6.3, PG10.3	B
	KT124841	NT4.4	B
	KT124842	PG2.7	B
Selangor	KT124840	DB9.1	B
	KT124841	DB2.1	B
	KT124842	DB11.7	B
Pahang	KC313924	TB11.6,TG2.5,TG13.2, TB13.3, SB14.1, ST15.1	A
	KC313925	ST10.3	A
	KC313926	SM1.2	B
	KC313927	CK1.4	B
	KC313928	KM7.3,TB11.2,KM8.1, SM6.2	B
	KC313929	KM15.2,ST8.1,KM15.1, ST13.2,KM8.4,ST20.3, TB2.3, KN7.2	B
	KC313930	SE13.1	B
	KC313931	KM3.2	B
	KC313932	TB15.1, SB15.1	B
	KC313933	SE12.1, TB4.1	B
	KC313934	TB5.1, SE7.1	B
	KC313935	KM13.2,CK7.1,TB9.2, SE24.2,SE18.4	B
	KC313936	PD20.1, KN7.4	B
	KC313937	CK20.2	B
	KC313938	KM12.3	B
	KT124836	PP1.1, PS7.3	B

Table 4.13: (Continued)

States	Types	Isolates	Assemblages
Negeri Sembilan	KC313924	DK19.1, UK17.3	A
	KT124836	UK15.2, UK15.3, UK17.1, DK28.2	B
	KT124838	UK6.3	B
	KT124839	UK6.2	B
	KT124840	UK9.2, UK12.2	B
	KT124842	DK17.3, DK17.6	B
	KT124843	DK27.1	B
	KT124845	DK10.4	B
	KT124848	UK6.5	B
Kedah	KT124833	UL4.4	B
	KT124836	UL1.3, UL3.3	B
	KT124837	UL1.7	B
	KT124842	UL19.6	B
	KT124844	UL3.4	B
	KT124847	UL13.2	B
	KT124849	UL1.11	B
	KT124850	UL19.5	B
Kelantan	KC313924	SW16.3	A
	KC313935	SW4.3	B
	KT124834	JJ7.2	B
	KT124836	SW10.1, JJ11.5	B
	KT124838	JJ11.4	B
	KT124848	SW11.2, SW5.3	B
Melaka	KC313924	SB3.3	A
	KT124835	BP1.5	B
	KT124846	BP14.2	B

4.2.2.3 Assessment of *bg* sequences

The 72 consensus sequences of *bg* which contained 486 base pairs were analysed with reference sequences assemblage A (accession no. X85958, AY072723 and AY072724) and assemblage B (accession no. AY072727). Seven subtypes within assemblage A representing 45 isolates and 14 subtypes within assemblage B representing 26 isolates were identified (Table 4.14). One of the isolates (i.e. UL4.4) cannot be distinctly typed to subtype due to underlying signals in the chromatogram but was assigned to assemblage B. One subtype of assemblage A and two subtypes of assemblage B were noted with the presence of heterogeneous nucleotides (Table 4.15). Only three positions with heterogeneous nucleotide were identified. All present with nucleotide common to both assemblages A and B in which one showed polymorphism within assemblage A and two other two showed polymorphism within assemblage B.

Although *bg* marker amplified the lowest number of isolates, the subtypes found within assemblage A was the highest when compared to *tpi* (i.e. three) and *gdh* (i.e. two). Subtypes KC313947, KT124851, KT124852 and KT124853 were represented by only one isolate whereas the other three subtypes were represented by more than two isolates in which KC313945 was the most common subtype and was found in Pahang, Kelantan, Negeri Sembilan, Sabah and Melaka. On the other hand, the frequency of representative sequence for each of the 14 assemblage B subtypes varied from one to four with KC313941 being the most common subtype and was seen in Pahang and Negeri Sembilan (Table 4.16).

Table 4.14: Accession number and isolates of *bg*

Number of genotype	Accession number	Isolates
Assemblage A		
Reference	**X85958**	Portland 1
Reference	**AY072723**	KC8
Reference	**AY072724**	ISSGF7
1	KC313945	KK16.4, TB11.6, KK16.5, TB7.3, SE17.1, TB15.3, TG9.2, PD11.2, SE29.3, TB12.3, SE29.4, ST8.1, JJ11.4, DK10.3, BP14.2, DK19.10, PB11.1, PG22.1, PG26.2, PM13.1, SB3.3, SP11.1, UK9.2, UK15.5, UK17.3, UK15.2
2	KC313946	KK7.5, TG2.5, DK29.1, DK3.3, PG3.1, PM25.2, PM31.2, PM53.1, PM53.2, SW16.3, DB2.1, UK12.2
3	KC313947	SE10.1
4	KC313948	PD20.1, PD1.1, TG13.2
5	KT124851	BP1.5
6	KT124852	UK15.4
7	KT124853	NT6.3
Assemblage B		
Reference	**AY072727**	BAH8
1	KC313939	SB4.1, SE7.1
2	KC313940	TB2.3, NT4.4, UL1.11
3	KC313941	SE9.3, DK17.6, DK27.1, UK6.2
4	KC313942	TB4.1
5	KC313943	SE24.2
6	KC313944	TB5.1, CK20.2, SE12.1
7	KT124854	UL1.7, DK28.2, PG2.7
8	KT124855	DK17.3, DB11.7
9	KT124856	UL3.3, UL1.3
10	KT124857	UK6.5
11	KT124858	SW11.2
12	KT124859	SW14.3, DK10.4
13	KT124860	NT4.4
14	KT124861	KM15.1

Table 3: Multiple alignments of *bg* sequences from this study with reference sequences obtained from GenBank, representing sub-assemblages of assemblages A and B

Isolates	Genbank accession no	Nucleotide position from the start of the gene											
Assemblage A		**208**	**410**	**412**	**434**	**437**	**450**	**451**	**460**	**468**	**530**	**606**	**729**
AI-A1	**X85958**	C	C	A	C	T	C	C	C	T	A	C	A
AII-A2	AY072723	.	.	.	.	.	.	.	.	.	.	T	G
AII-A3	AY072724	.	.	.	.	.	.	.	T	C	.	T	A
A3	KC313946	.	.	.	.	.	.	.	T	C	.	T	?
A3	KC313947	.	.	.	.	.	.	.	Y	C	.	T	?
A3	KC313948	.	.	.	.	.	.	.	.	C	.	T	?
AII-A2	KC313945	.	.	.	.	.	.	.	.	.	.	T	?
A2	KT124851	T	.	.	T	.	.	.	.	.	.	T	?
A2	KT124852	.	.	.	.	C	.	.	.	.	.	T	?
A2	KT124853	.	T	G	.	.	.	-	.	.	G	T	?

Table 4.15: (Continued)

Isolates	Genbank accession no	Nucleotide position from the start of the gene											
Assemblage B		**210**	**228**	**285**	**354**	**378**	**411**	**438**	**456**	**498**	**531**	**540**	**564**
B	**AY072727**	C	A	T	C	C	C	C	C	C	G	A	T
	KC313939	T	.	.	T	.	.	.	A	.	.	.	.
	KC313940	T	.	.	.	.	.	.	.	.	.	.	.
	KC313941	.	.	.	.	.	.	.	.	.	.	.	.
	KC313942	Y	.	.	T	.	.	.	.	.	.	.	.
	KC313943	.	.	.	T	.	.	.	.	.	.	G	.
	KC313944	.	.	.	T	.	.	.	.	.	.	.	.
	KT124854	T	.	.	T	.	.	T	.	.	.	.	.
	KT124855	T	.	.	T	.	.	.	.	.	.	.	.
	KT124856	T	.	.	.	.	T	.	.	.	A	.	.
	KT124857	.	G	.	T	.	.	.	.	.	.	.	.
	KT124858	.	.	.	T	.	.	.	.	T	.	.	.
	KT124859	Y	.	.	Y	.	.	.	.	.	.	.	.
	KT124860	Y	.	.	.	.	.	.	.	.	.	.	.
	KT124861	.	.	C	T	.	.	.	.	.	.	.	.

Table 4.16: Distribution of *bg* subtypes in different states

States	Types	Isolates	Assemblages
Sabah	KC313945	PB11.1, PG22.1, PG26.2, PM13.1, SP11.1	A
	KC313946	PG3.1, PM31.2, PM53.1, PM53.2	A
	KT124853	NT6.3	A
	KC313940	NT4.4	B
	KT124854	PG2.7	B
	KT124860	NT4.4	B
Selangor	KC313946	DB2.1	A
	KT124855	DB11.7	B
Pahang	KC313945	KK16.4, TB11.6, KK16.5, TB7.3, SE17.1, TB15.3, TG9.2, PD11.2, SE29.3, TB12.3, SE29.4, ST8.1	A
	KC313946	KK7.5, TG2.5	A
	KC313947	SE10.1	A
	KC313948	PD20.1, PD1.1, TG13.2	A
	KC313939	SB4.1, SE7.1	B
	KC313940	TB2.3	B
	KC313941	SE9.3	B
	KC313942	TB4.1	B
	KC313943	SB24.2	B
	KC313944	TB5.1, CK20.2, SE12.1	B
	KT124861	KM15.1	

Table 4.16: (Continued)

States	Types	Isolates	Assemblages
Negeri Sembilan	KC313945	DK10.3, DK19.10, UK9.2, UK15.5, UK17.3, UK15.2	A
	KC313946	DK29.1, DK3.3, UK12.2	A
	KT124852	UK15.4	A
	KC313941	DK17.6, DK27.1, UK6.2	B
	KT124854	DK28.2	B
	KT124855	DK17.3	B
	KT124857	UK6.5	B
	KT124859	DK10.4	B
Kedah	KC313940	UL1.11	B
	KT124854	UL1.7	B
	KT124856	UL3.3, UL1.3	B
Kelantan	KC313945	JJ11.4	A
	KC313946	SW16.3	A
	KT124858	SW11.2	B
	KT124859	SW14.3	B
Melaka	KC313945	BP14.2, SB3.3	A
	KT124851	BP1.5	A

4.2.3 Molecular epidemiology

4.2.3.1 Mixed infections

The *tpi*-based PCR protocol for mixed infection using assemblage- specific primers was conducted on the 154 *Giardia*-positive isolates. Of these, 120 isolates showed amplicons for *G. duodenalis* assemblages; 49 isolates had mixed infections with assemblages A and B, 15 isolates had single infection with assemblage A and 56 cases were identified as assemblage B (Table 4.6 and 4.7). Pertaining to 27 isolates with discordant genotype result, nine were identified as mixed infection, 12 were amplified as assemblage B while six did not show amplification for both assemblages A and B. There were also amplicons amplified using the three standard assays without discordant genotypes but were tested as mixed infections, this including 10 isolates typed as assemblage A and 12 isolates typed as assemblage B. The specificity of this protocol was confirmed by sequencing selected cases representing assemblages A and B. When the chromatogram was scrutinized at the positions where nucleotide polymorphism could differentiate assemblage A and B, double peaks were observed in 16 of the mixed isolates.

4.2.3.2 Association of genotypes with risk factors

To access possibility association of risk factors with genotypes, 27 of the isolates with discordant genotype result were sorted into assemblages A and B by the following criteria: 1) When discordant result occurred in three markers, genotype that was agreed in any two of the three markers was selected. 2) When discordant result occurred in two markers, genotype identified using *tpi* will be given precedence followed by *gdh*. After the reclassification, both assemblages have the equal percentage of infection rate i.e. 47.6%. The distribution of both assemblages was almost similar between the West and East Malaysia (P = 0.613).

When the demographic and socioeconomic variables were inspected using univariate analysis (Table 4.17), all the three occurrences of assemblage A, B and mixed infection were significantly higher in indigenous people from Peninsular Malaysia. Younger individual with the age of 12 and below have significantly higher prevalence of assemblage A (6.4%; 95% CI = 5.0, 8.1 vs 2.1%; 95% CI = 1.1, 4.1) and mixed infection (5.1%; 95% CI = 3.8, 6.7 vs 0.3%; 95% CI = 0.05, 1.5) than those who aged above 13 while individuals from large family households (with family members of more than 7) was significantly associated with assemblage B (7.2%; 95% CI = 5.1, 10.0 vs 4.2%; 95% CI = 3.1, 5.8) and mixed infection (5.3%; 95% CI = 3.6, 7.9 vs 2.9%; 95% CI = 2.0, 4.2) when compared those from smaller households.

With respect to environment factors, the lack of toilet facility at household and exercising indiscriminate garbage disposal significantly exposed the individuals in contracting *Giardia* infection of assemblage A, B and mixed of both assemblages. On the other hand, individual who defecate at indiscriminate places such as river and bath in river had higher rate of getting assemblage B and mixed infection. Use of water tank for water storage appeared to be associated with mixed infection (4.9%; 95% CI = 3.5, 6.8) when compared with participants that do not use (2.5%; 95% CI = 1.5, 4.0).

In assessment of the personal practices and hygiene conducts, it was shown that not washing vegetables/ fruits before consumption and going out with barefoot were the variables that associated with infection of assemblage A, B and mixed assemblage. Individuals that drink water without boiling were at higher risk of acquiring assemblage A (OR = 2.2; 95% CI = 1.3, 3.9) and mixed infection (OR = 2.5; 95% CI = 1.3, 4.7). Not washing hands before eating and play with soil showed higher rate of infection with assemblage B and mixed assemblage. Individual that had close contact with animals and not washing hands after playing with animals had higher likelihood of infected with

assemblage A. The former was also associated with mixed infection while the latter was associated with assemblage B.

Gastrointestinal symptoms were reported in 18.8% (13/69) of the individuals infected with assemblage A; 12 of the cases had diarrhoea. Likewise, 14.5% (10/69) of the individuals infected with assemblage B were symptomatic and had diarrhoea. That said, among *Giardia*-positive and symptomatic individuals (i.e. 23 individuals), the prevalence of assemblages A and B was 56.5% and 43.5% respectively, and the difference was not significant (OR = 1.37; 95% CI = 0.56, 3.38). Gastrointestinal symptoms occurred in 10/49 of individuals with mixed infection and nine had diarrhoea (18.4%).

Table 4.17: Univariate analysis of factors associated with Giardia infection according to assemblages

Variables/ Assemblage	Assemblage A			Assemblage B			Mixed assemblage		
	Infected n (%)	**OR (95% CI)**	***P*-value**	**Infected n (%)**	**OR (95% CI)**	***P*-value**	**Infected n (%)**	**OR (95% CI)**	***P*-value**
Location									
Peninsular Malaysia	59 (6.0)	2.1 (1.1, 4.2)	0.027	61 (6.2)	2.8 (1.3, 5.8)	0.005	45 (4.6)	4.1 (1.5, 11.4)	0.004
Sabah (East Malaysia)	10 (2.9)	1		8 (2.3)	1		4 (1.2)	1	
Age group									
<=12 Years	61 (6.4)	3.2 (1.5, 6.8)	0.001	55 (5.8)	1.6 (0.9, 2.9)	0.112	48 (5.1)	20.2 (2.8, 147.7)	<0.001
> 12 Years	8 (2.1)	1		14 (3.7)	1		1 (0.3)	1	
Gender									
Male	35 (5.3)	1.0 (0.6, 1.7)	0.891	37 (5.6)	1.2 (0.7, 1.9)	0.528	21 (3.2)	0.7 (0.4, 1.3)	0.313
Female	34 (5.1)	1		32 (4.8)	1		28 (4.2)	1	
Size of household									
>7 members	25 (5.8)	1.2 (0.7, 2.0)	0.494	31 (7.2)	1.8 (1.1, 2.9)	0.023	23 (5.3)	1.9 (1.1, 3.3)	0.028
<=7 members	44 (4.9)	1		38 (4.2)	1		26 (2.9)	1	
Household income									
< RM500	37 (5.5)	1.1 (0.7, 1.8)	0.642	40 (5.9)	1.4 (0.8, 2.2)	0.228	31 (4.6)	1.7 (0.9, 3.1)	0.078
>= RM500	32 (4.9)	1		29 (4.4)	1		18 (2.8)	1	

Table 4.17: (Continued)

Variables/ Assemblage	Assemblage A			Assemblage B			Mixed assemblage		
	Infected n (%)	**OR (95% CI)**	***P*-value**	**Infected n (%)**	**OR (95% CI)**	***P*-value**	**Infected n (%)**	**OR (95% CI)**	***P*-value**
Educational level									
Secondary education	2 (1.6)	1		4 (3.1)	1		0 (0)	1	
Primary education	44 (6.2)	4.2 (1.0, 17.6)	0.033	39 (5.5)	1.8 (0.6, 5.2)	0.255	30 (4.2)	NA	0.017
Non educated	23 (4.7)	3.1 (0.7, 13.4)	0.109	26 (5.3)	1.7 (0.6, 5.1)	0.305	19(3.9)	NA	0.024
Educational status									
Secondary education	2 (1.6)	1		4 (3.1)	1		0 (0)	1	
Primary education/non educated	67 (5.6)	3.8 (0.9, 15.5)	0.050	65 (5.4)	1.8 (0.6, 5.0)	0.261	49 (4.1)	NA	0.019
Employment status									
Not working	65 (5.6)	2.5 (0.9, 6.9)	0.070	64 (5.5)	2.0 (0.8, 4.9)	0.148	49 (4.2)	NA	0.006
Working	4 (2.3)	1		5 (2.9)	1		0 (0)	1	
Source of drinking water									
Unsafe water (river, well, rain)	28 (5.2)	1.0 (0.6, 1.7)	0.94	27 (5.1)	1.0 (0.6, 1.6)	0.859	14 (2.6)	0.6 (0.3, 1.1)	0.092
Piped water	41 (5.2)	1		42 (5.3)	1		35 (4.4)	1	

Table 4.17: (Continued)

Variables/ Assemblage	Assemblage A			Assemblage B			Mixed assemblage		
	Infected n (%)	**OR (95% CI)**	***P*-value**	**Infected n (%)**	**OR (95% CI)**	***P*-value**	**Infected n (%)**	**OR (95% CI)**	***P*-value**
Use of water tank									
Not using	36 (5.5)	1.1 (0.7, 1.8)	0.604	37 (5.7)	1.2 (0.7, 2.0)	0.444	16 (2.5)	0.5 (0.3, 0.9)	0.019
Using	33 (4.9)	1		32 (4.7)	1		33 (4.9)	1	
Presence of toilet at household									
No	25 (7.6)	1.8 (1.1, 3.0)	0.022	28 (8.5)	2.2 (1.3, 3.6)	0.002	26 (7.9)	3.7 (2.1, 6.5)	<0.001
Yes	44 (4.4)	1		41 (4.1)	1		23 (2.3)	1	
Boiling water before consumption									
No	18 (9.4)	2.2 (1.3, 3.9)	0.005	13 (6.8)	1.4 (0.8, 2.6)	0.285	14 (7.3)	2.5 (1.3, 4.7)	0.004
Yes	51 (4.5)	1		56 (4.9)	1		35 (3.1)	1	
Bathing place									
River	20 (7.2)	1.6 (0.9, 2.7)	0.095	23 (8.2)	2.0 (1.2, 3.3)	0.010	21 (7.5)	3.0 (1.7, 5.3)	<0.001
Bathroom	49 (4.7)	1		46 (4.4)	1		28 (2.7)	1	
Indiscriminate defecation									
Yes	29 (6.6)	1.5 (0.9, 2.5)	0.099	41 (9.4)	3.2 (1.9, 5.2)	<0.001	31 (7.1)	3.7 (2.0, 6.7)	<0.001
No	40 (4.5)	1		28 (3.1)	1		18 (2.0)	1	

Table 4.17: (Continued)

Variables/ Assemblage	Assemblage A			Assemblage B			Mixed assemblage		
	Infected n (%)	**OR (95% CI)**	***P*-value**	**Infected n (%)**	**OR (95% CI)**	***P*-value**	**Infected n (%)**	**OR (95% CI)**	***P*-value**
Eating using hands									
Yes	65 (5.6)	2.5 (0.9, 6.8)	0.076	65 (5.6)	2.5 (0.9, 6.8)	0.076	48 (4.1)	7.6 (1.0, 53.0)	0.022
No	4 (2.4)	1		4 (2.4)	1		1 (0.6)	1	
Washing hands before eating									
No	13 (4.0)	0.7 (0.4, 1.3)	0.251	25 (7.6)	1.8 (1.1, 3.0)	0.022	21 (6.4)	2.4 (1.3, 4.3)	0.003
Yes	56 (5.6)	1		44 (4.4)	1		28 (2.8)	1	
Washing hands after defecation									
No	10 (3.7)	0.7 (0.3, 1.3)	0.220	18 (6.7)	1.4 (0.8, 2.5)	0.217	12 (4.5)	1.3 (0.7, 2.5)	0.454
Yes	59 (5.6)	1		51 (4.8)	1		37 (3.5)	1	
Consumption of raw vegetables									
Yes	21 (4.6)	0.8 (0.5, 1.4)	0.505	24 (5.3)	1.0 (0.6, 1.7)	0.908	3 (0.7)	0.1 (0.0, 0.4)	< 0.001
No	48 (5.5)	1		45 (5.1)	1		46 (5.3)	1	

Table 4.17: (Continued)

Variables/ Assemblage	Assemblage A			Assemblage B			Mixed assemblage		
	Infected n (%)	**OR (95% CI)**	***P*-value**	**Infected n (%)**	**OR (95% CI)**	***P*-value**	**Infected n (%)**	**OR (95% CI)**	***P*-value**
Washing vegetables/fruits before consumption									
No	26 (7.8)	1.9 (1.1, 3.1)	0.014	28 (8.4)	2.1 (1.3, 3.5)	0.003	25 (7.5)	3.3 (1.8, 5.8)	< 0.001
Yes	43 (4.3)	1		41 (4.1)	1		24 (2.4)	1	
Wearing shoes when outside									
No	25 (7.9)	1.9 (1.1, 3.1)	0.013	26 (8.2)	2.0 (1.2, 3.3)	0.005	22 (7.0)	2.7 (1.5, 4.9)	< 0.001
Yes	44 (4.4)	1		43 (4.3)	1		27 (2.7)	1	
Playing in river									
Yes	25 (6.4)	1.3 (0.7, 2.1)	0.337	25 (6.4)	1.6 (1.0, 2.8)	0.068	17 (4.3)	1.2 (0.7, 2.2)	0.572
No	44 (5.1)	1		35 (4.0)	1		32 (3.7)	1	
Playing with soil									
Yes	44 (5.9)	1.2 (0.7, 2.0)	0.444	43 (5.7)	1.8 (1.0, 3.2)	0.047	38 (5.1)	2.4 (1.2, 4.8)	0.008
No	25 (4.9)	1		17 (3.3)	1		11 (2.1)	1	
Garbage disposal									
Indiscriminate	31 (6.9)	1.6 (1.0, 2.6)	0.050	32 (7.1)	1.7 (1.1, 2.8)	0.027	32 (7.1)	3.8 (2.1, 7.0)	< 0.001
Proper disposal	38 (4.3)	1		37 (4.2)	1		17 (1.9)	1	

Table 4.17: (Continued)

Variables/ Assemblage	Assemblage A			Assemblage B			Mixed assemblage		
	Infected n (%)	**OR (95% CI)**	***P*-value**	**Infected n (%)**	**OR (95% CI)**	***P*-value**	**Infected n (%)**	**OR (95% CI)**	***P*-value**
Presence of domestic animals									
Yes	51 (5.4)	1.2 (0.7, 2.0)	0.592	52 (5.5)	1.3 (0.7, 2.2)	0.419	37 (4.0)	1.4 (0.7, 2.8)	0.307
No	18 (4.7)	1		17 (4.4)	1		12 (2.9)	1	
Close contact with animals									
Yes	47 (7.2)	2.3 (1.4, 3.9)	0.001	35 (5.4)	1.1 (0.7, 1.7)	0.776	32 (4.9)	2.0 (1.1, 3.6)	0.020
No	22 (3.3)			34 (5.0)			17 (2.5)		
Washing hands after playing with animals									
No	21 (7.9)	1.8 (1.1, 3.1)	0.027	21 (7.9)	1.8 (1.1, 3.1)	0.027	14 (5.3)	1.6 (0.9, 3.1)	0.129
Yes	48 (4.5)	1		48 (4.5)	1		35 (3.3)	1	

4.2.4 Phylogenetic analyses and subtyping

4.2.4.1 Subtyping of *G. duodenalis* assemblages A

While the positions of the nucleotide substitutions in the sequence alignments has provided an overall pattern and assemblage information, phylogenetic trees were constructed using Bayesian inference (BI) and neighbour-joining (NJ) algorithms to show the genetic structure, relationship and subtyping of the isolates. Based on analysis targeting *tpi* gene, 64 of the assemblage A isolates (Table 4.6) were classified as subtype A2 based on the phylogenetic analysis (Figures 4.1a and 4.2a) and the substitution pattern (Table 4). The phylogenetic tree placed the three representative sequences (KC313923, KT124815 and KT124816) in one cluster with AII sequence references with high bootstrap support. At *gdh* gene, all sequences of the assemblage A showed complete homologous to the reference sequence of subtype A2 (Accession No. L40510) except for isolate ST10.3 (Accession No. KC313925) where substitutions at two positions were observed (A–G at position 341 and C– T at position 485) (Table 4.12), (Figures 4.1b and 4.2b). Subtype A2 based on *tpi* or *gdh* belongs to sub-assemblage AII (Caccio et al., 2008; Feng and Xiao, 2011). At *bg* gene, 45 isolates were identified as assemblage A. Twenty nine were distinguished as A2 from A1 at position 606 (C–T) and from A3 at positions 460 (C–T) and 468 (T–C). Twelve were typed as A3 and the remaining four isolates have the subtype similar to A3 but different by one position at 460 (C–T/Y) (Table 4.15). This subtyping, however, was not clearly demonstrated in the phylogenetic trees (Figures 4.1c and 4.2c). Isolates of assemblage A were clustered into subtypes A2 and A3 in the NJ tree. Besides, subtype A1 was also clustered in one group with A2 perhaps due to the only one nucleotide at 606 that distinguish these subtypes. It should also be noted that subtypes A2 and A3 belong to sub-assemblage AII (Caccio et al., 2008; Feng and Xiao, 2011).

4.2.4.2 Subtyping of *G. duodenalis* Assemblage B

At *tpi* gene, the 32 subtype sequences had heterogeneous nucleotides and inconsistency nucleotide substitution pattern at positions defining the sub-assemblages, thus making proposing specific sub-assemblages for assemblage B isolates not possible (Table 4.9). Phylogenetic analysis confirmed the monophyletic group of assemblage B. Minor sub-structuring was seen in the cluster but it was not supported by bootstrap value (Figure 4.1a and 4.2a). At *gdh* gene, 31 sequences representing 72 isolates had high nucleotide variations, limiting the classification of assemblage B to sub-assemblages (Table 4.12). Similar to *tpi* gene, phylogenetic analysis formed a monophyletic group for assemblage B (Figures 4.1b and 4.2b). Similar results were observed in *bg* gene where all the 14 subtypes and references sequences of assemblage B were placed in one cluster while sub-clustering was not obvious (Table 4.15, Figures 4.1c and 4.2c).

4.2.4.3 Multilocus genotyping

In the above phylogenies, the topology of the above phylogenetic trees was similar across all loci and between BI and NJ analysis (Figures 4.1 and 4.2). When phylogenetic relationship were analysed using only isolates that were successfully sequenced at all there loci, the analysis by some means yielded trees of different genetic structure between the loci. Phylogenetic trees reconstructed using *tpi* and *gdh* isolates formed two distinct clusters (i.e. assemblage A and B) while three distinct clusters were observed in the phylogenetic trees of *bg* (i.e. assemblage A, subtypes A2 and A3, and assemblage B).

Besides, the indication of discordant genotyping can be seen in these trees. For example, seven isolates (i.e. UL3.3, DB11.7, UK12.2, UK15.2, UK9.2, ST8.1, and BP1.5) were clustered as assemblage A under phylogenetic tree of *tpi* but were grouped

with assemblage B in *gdh*. Among these isolates, UK15.2, UK9.2, ST8.1, BP1.5 were clustered as assemblage A2, UK12.2 as assemblage A3 while UL3.3 and DB11.7 as assemblage B at the *bg* tree (Figure 4.3).

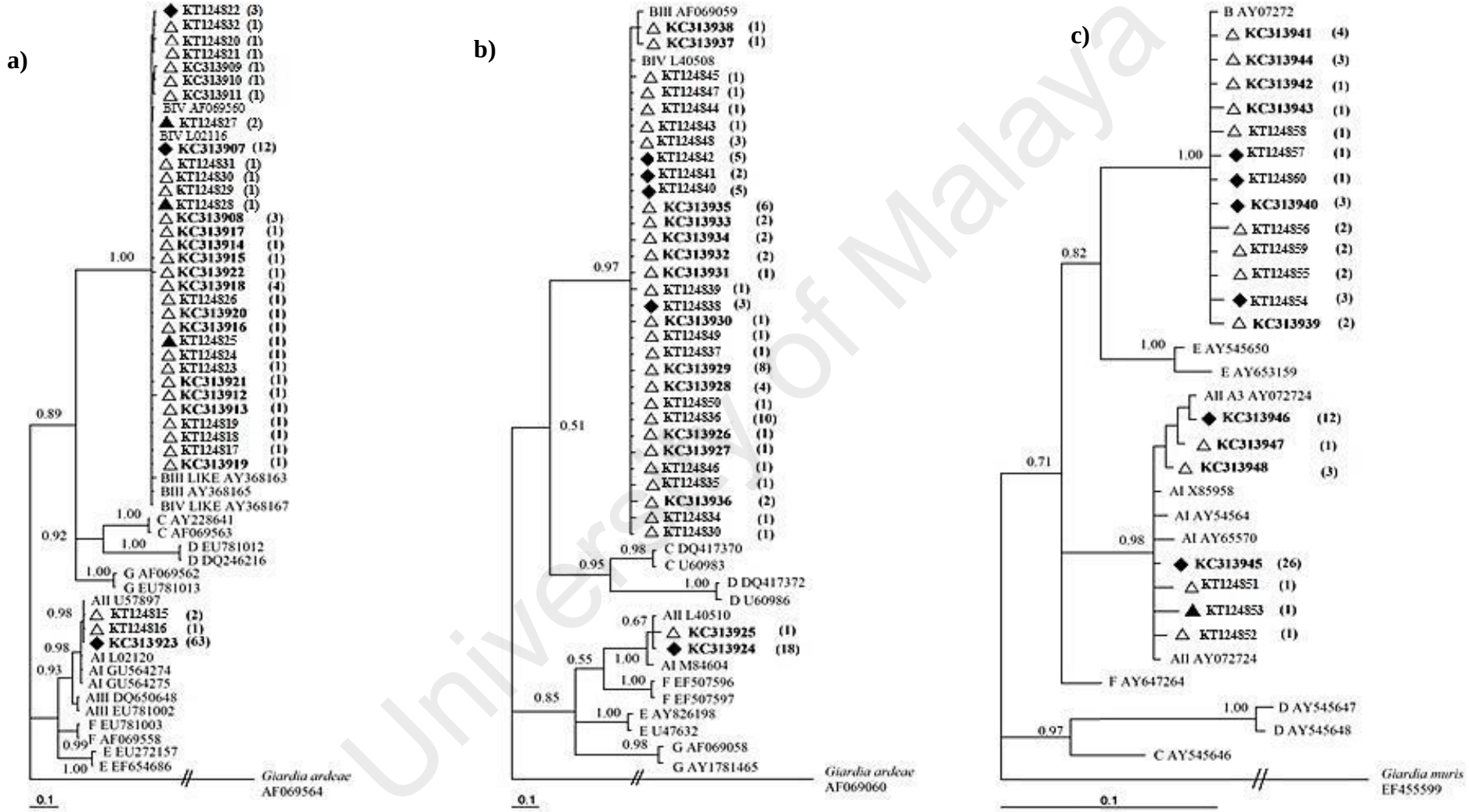

Figure 4.1: Phylogram constructed by BI analysis, based on the representative sequences of a) *tpi*; b) *gdh*; and c) *bg* (in bold type) compared with reference sequences from Genbank. The numbers of isolates with particular sequence types are in parentheses. The posterior probability (pp) is indicated for the main nodes. △ Represents isolates from West Malaysia only. ▲Represents isolates from East Malaysia only; ♦ Represents isolates from both East and West Malaysia.

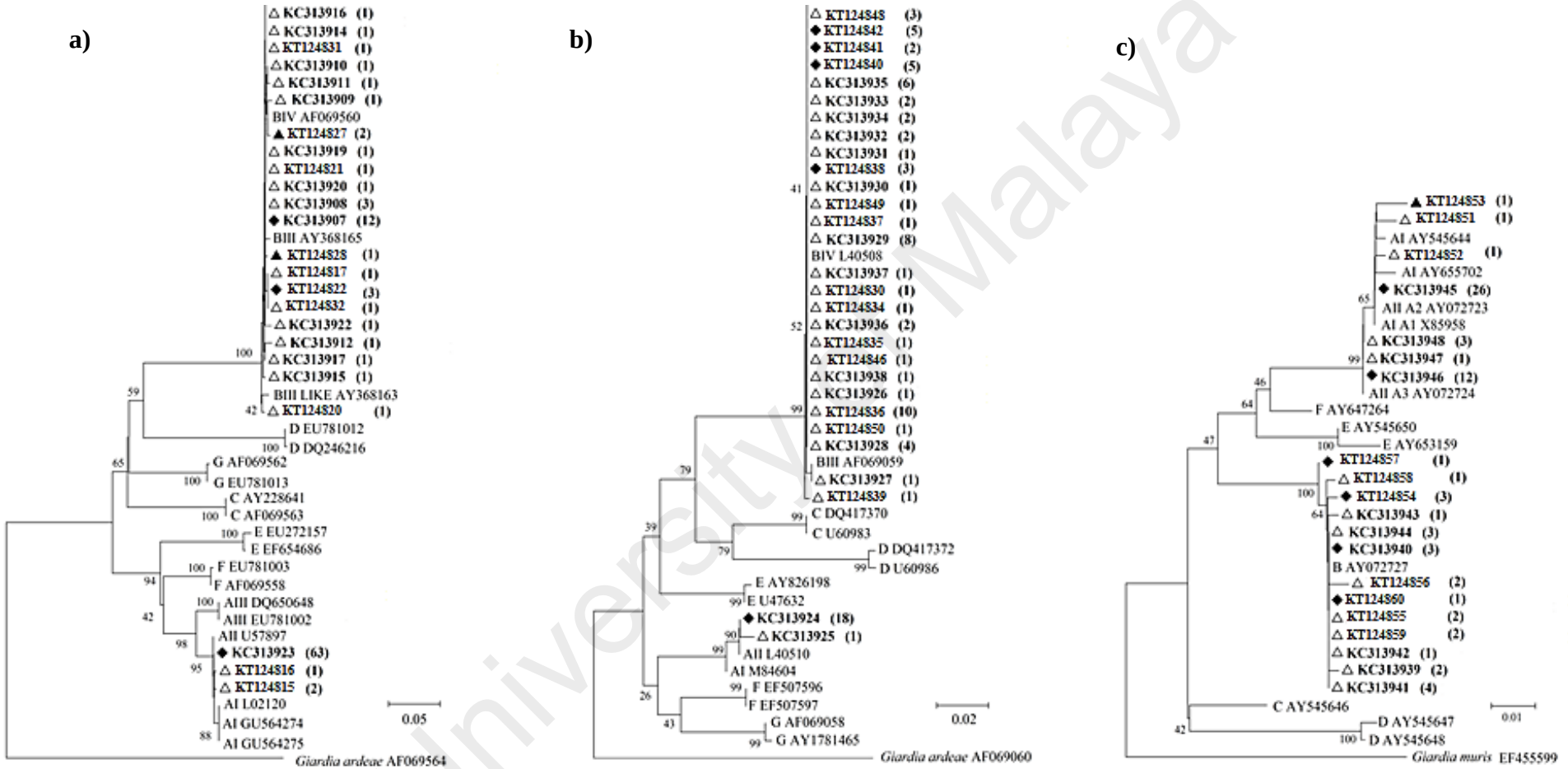

Figure 4.2: Phylogram constructed by NJ analysis, based on the representative sequences of a) *tpi*; b) *gdh*; and c) *bg* (in bold type) and reference sequences from Genbank. The numbers of isolates with particular sequence types are in parentheses. Bootstrap values obtained from 1000 replicates are indicated on branches in percentage. △ Represents isolates from West Malaysia only. ▲Represents isolates from East Malaysia only; ♦ Represents isolates from both East and West Malaysia.

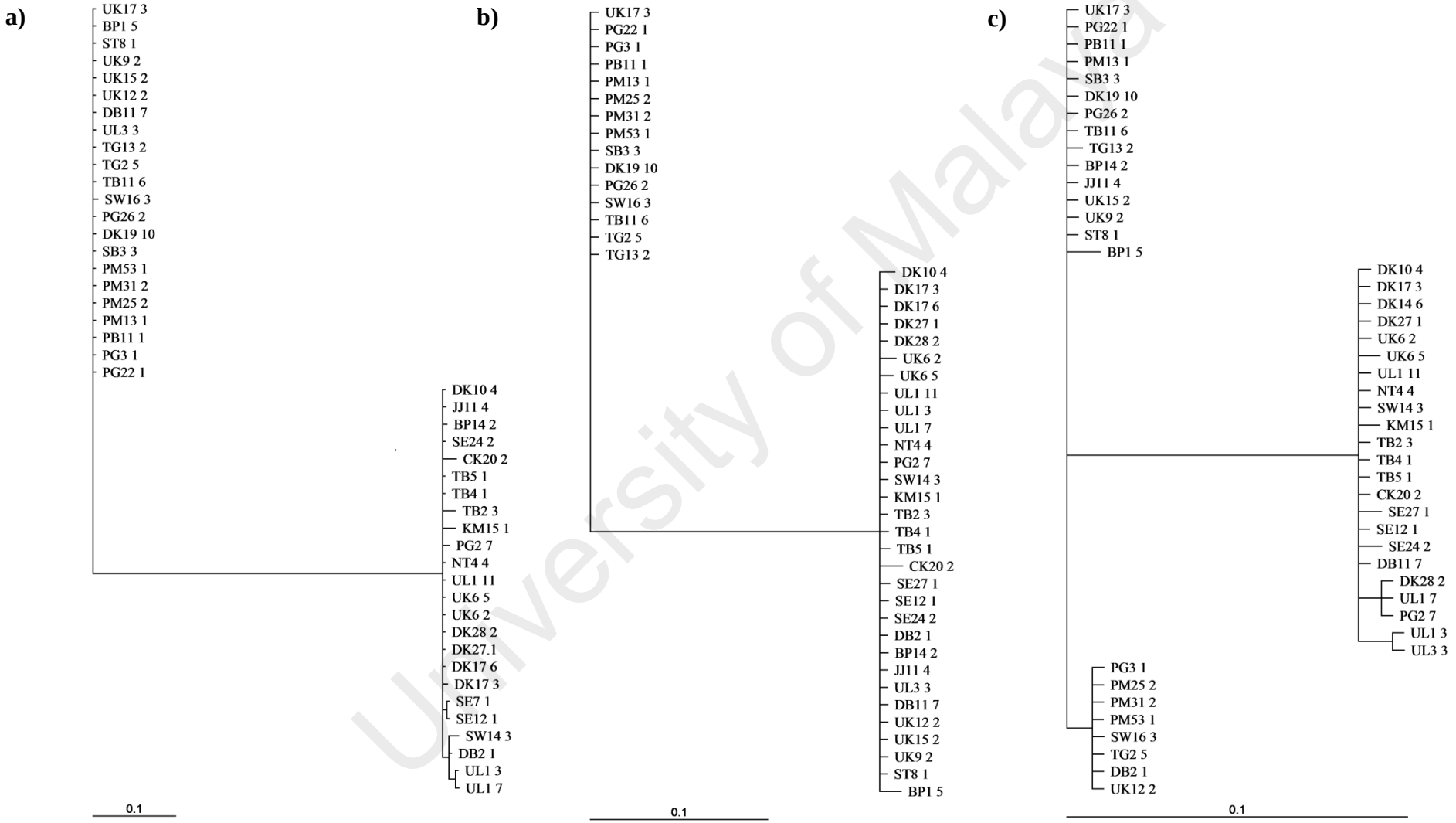

Figure 4.3: Phylogram constructed by BI analysis, based on the sequences amplified at all three loci a) *tpi*; b) *gdh*; and c) *bg*

4.2.5 Population genetic study

4.2.5.1 Genetic diversity and haplotype networks of *G. duodenalis* of the Malaysian population

Genetic diversity assessment of Malaysian isolates using different genes i.e. *tpi*, *gdh* and *bg* showed that all the three markers produced consistent results, with sequences of assemblage B having the higher level of both haplotype diversity (Hd) and nucleotide diversity (Pi) compared to assemblage A (Table 4.18). Comparison of nucleotide diversity of the different genes revealed highest level of diversity in *tpi* gene followed by *gdh* and *bg* genes. However, in regards to the haplotype diversity, *bg* gene had the highest diversity followed by *tpi* and *gdh* genes.

The 119 *tpi* sequences from Malaysia in which assemblage A comprised eight haplotypes among 72 isolates and assemblage B comprised 27 haplotypes among 47 isolates) were included in the Median Joining Network analysis. Haplotype network of assemblage A formed two distinct networks. The first and the largest network comprised all the haplotypes except three haplotypes that were included in the second network and were isolated from HIV patients (Figure 4.4). Inspections of the SNPs and phylogenetic analysis revealed that these three haplotypes belonged to sub-assemblage AI while the rest of the haplotypes in the first network belonged to sub-assemblage AII. However, for assemblage B, no distinct networks were formed and clustering according to the sub-structuring of assemblage (e.g. sub-assemblage BIII and BIV) were not seen (Figure 4.5). Both assemblages A and B did not show clear geographical clustering in the distribution of the haplotypes.

Table 4.18: Genetic diversity of *G. duodenalis* isolates from Malaysia among three loci

Locus	Data set	No of sequences	No of sites	S	h	Hd	Pi
gdh	Assemblage A	19	408	2	2	0.10500	0.00052
	Assemblage B	34	408	8	15	0.90600	0.00599
bg	Assemblage A	44	486	8	6	0.58400	0.00235
	Assemblage B	23	486	10	11	0.92900	0.00433
tpi	Assemblage A	72	476	10	8	0.23500	0.00098
	Assemblage B	47	489	30	27	0.90700	0.00830

Number of polymorphic sites (S), number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (Pi)

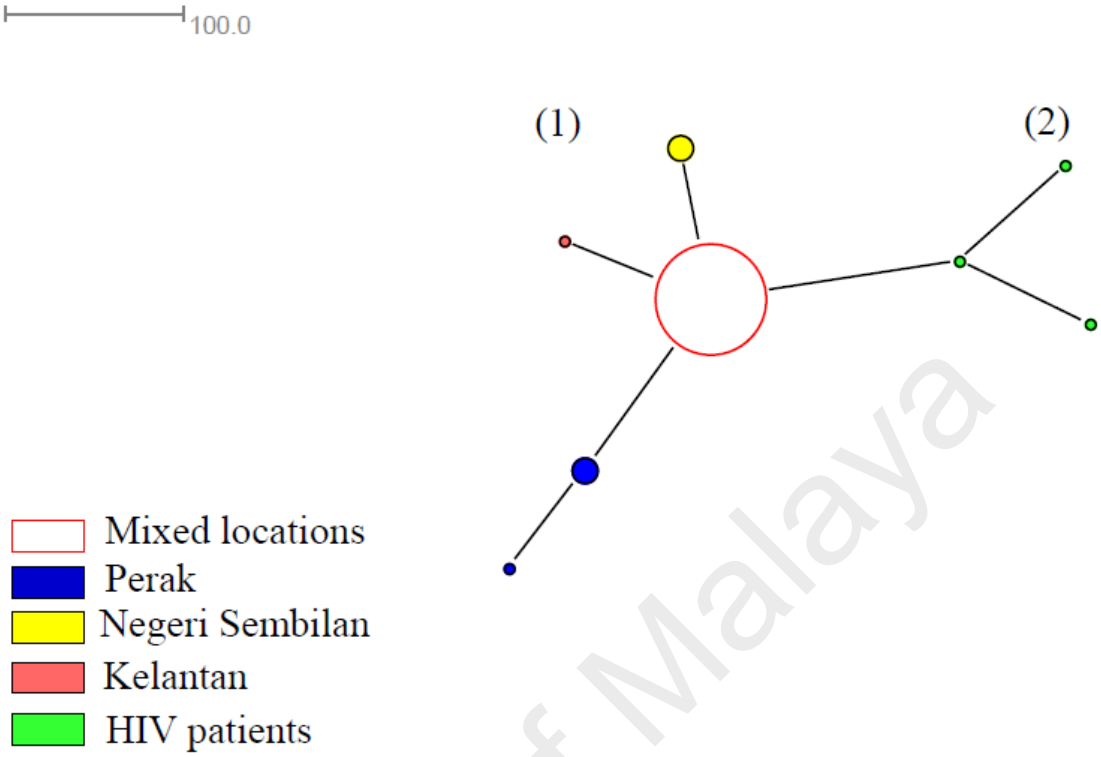

Figure 4.4: Median joining network of assemblage A sequences from Malaysian population

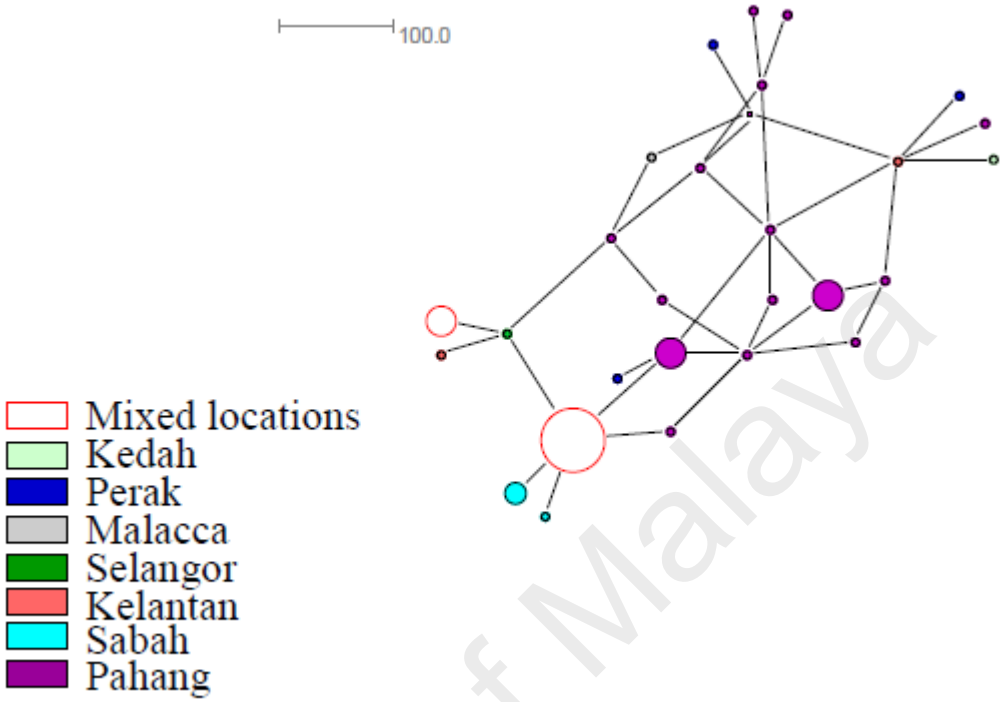

Figure 4.5: Median joining network of assemblage B sequences from Malaysian population

4.2.5.2 Genetic diversity and haplotype networks of *G. duodenalis* of the worldwide population

With respect to the worldwide data set, a total of 67, 155 and 66 haplotypes were inferred from sequences of assemblages A, B and E respectively. In terms of genetic diversity, assemblage B had the highest level of both haplotype diversity and nucleotide diversity, followed by assemblage E and assemblage A (Table 4.19).

All of the assemblages across the continents had large haplotype diversity, with an overall diversity ranging from Hd= 0.78 to Hd= 0.97. The European population displayed highest nucleotide diversity for assemblage A (0.01279) and assemblage E (0.1119) whereas the Asian population exhibited the highest nucleotide diversity for assemblage B (0.01216). Three distinct networks from 284 sequences of assemblage A were demonstrated in the median joining network analysis (Figure 4.6). Distinct SNPs pattern could be seen in the sequences of these networks. Nevertheless, phylogenetic analysis did not clearly show clustering correspond with the networks and with the sub-structuring of assemblage A (i.e. Sub-assemblages AI, AII and AIII). Instead, haplotypes of sub-assemblage AI and AIII shared the same network 1. Separation according the continents was not seen in the networks of assemblage A. However, it was shown that median joining network of assemblage B for 324 sequences formed a network that was exclusively isolated from Asia (yellow) while other haplotypes were well dispersed across the continents (Figure 4.7). For median joining network analysis of the 205 sequences of assemblage E, no distinct network and sub-structuring with the assemblage were observed (Figure 4.8).

The haplotype with the largest circle in the haplotype network of Malaysian population were predicted to be the ancestral haplotype of assemblages A (Figure 4.4) and B (Figure 4.5) in Malaysia. However, the ancestral haplotype cannot be identified

in the global populations as there were many high and low frequency haplotypes disseminated throughout the network.

4.2.5.3 Test for neutrality

Both of the statistical tests of neutrality, Tajima's *D* and Fu and Li's *D* produced consistent negative values for all assemblages and for all populations (Table 4.19). In most cases, the results of the test were statistically significant with the exception of Australian populations of all assemblages. This could be indicative of population size expansion and the hypothesis was supported by mismatch distribution test, which showed low values of the Raggedness index ($P>0.05$) and R_2 ($P<0.05$) and unimodal mismatch distributions (Figure 4.9).

Table 4.19: Genetic diversity and test of neutrality of *G. duodenalis* for the worldwide population

Population	No of sequences	No of sites	S	h	Hd	Pi	D	D*
Assemblage A								
Australia	36	476	51	31	0.98254	0.00704	-2.63963*	-4.81625*
America	74	476	21	14	0.58312	0.00314	-1.98395*	-4.24360*
Asia	104	476	18	14	0.46004	0.00218	-2.04851*	-3.40876*
Europe	33	476	30	10	0.80682	0.01279	-0.63810	-0.60189
Africa	37	476	23	14	0.84234	0.00599	-1.65001	-1.89922
Overall	284	476	100	67	0.78072	0.00564	-2.51918*	-7.40589*
Assemblage B								
Australia	28	489	25	26	0.99471	0.00882	-1.18578	-2.34957
America	65	489	51	33	0.93365	0.00931	-1.92659*	-4.16927*
Asia	131	489	86	86	0.97510	0.01216	-2.01663*	-5.90951*
Europe	60	489	48	39	0.91073	0.00867	-1.97667*	-4.54163*
Africa	40	489	42	30	0.96795	0.00858	-2.06524*	-4.28121*
Overall	324	489	155	176	0.96600	0.01083	-2.40897*	-8.98197*
Assemblage E								
Australia	32	468	34	29	0.99194	0.01059	-1.55162	-1.98732
America	21	468	11	11	0.81905	0.00435	-1.16746	-1.24885
Asia	79	468	30	30	0.91853	0.00539	-1.82865*	-3.99122*
Europe	11	468	17	11	1.00000	0.01119	-0.43806	-0.62712
Africa	62	468	42	10	0.84234	0.00563	-2.36766*	-5.72396*
Overall	205	468	85	66	0.82443	0.00709	-2.39843*	-8.00707*

Number of polymorphic sites (S), number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (Pi), Tajima's *D* (D), Fu and Li's *D* (D*) tests. Value with asterisks (*) indicate statically significant result (p-value < 0.05)

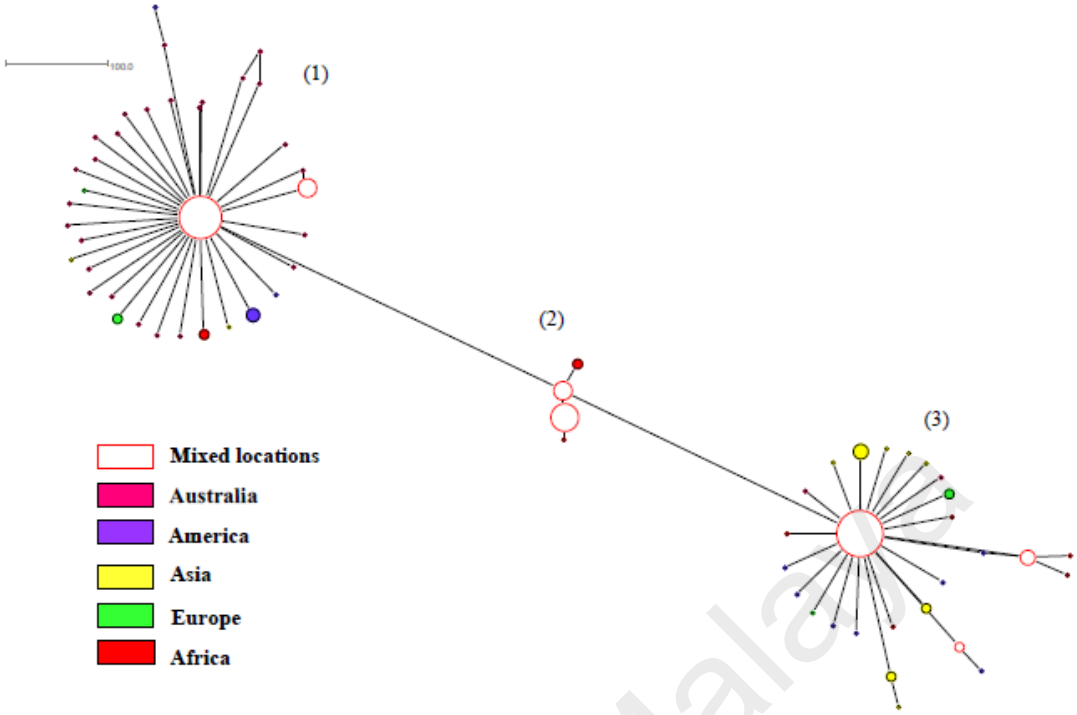

Figure 4.6: Median joining network of assemblage A sequences of the worldwide population

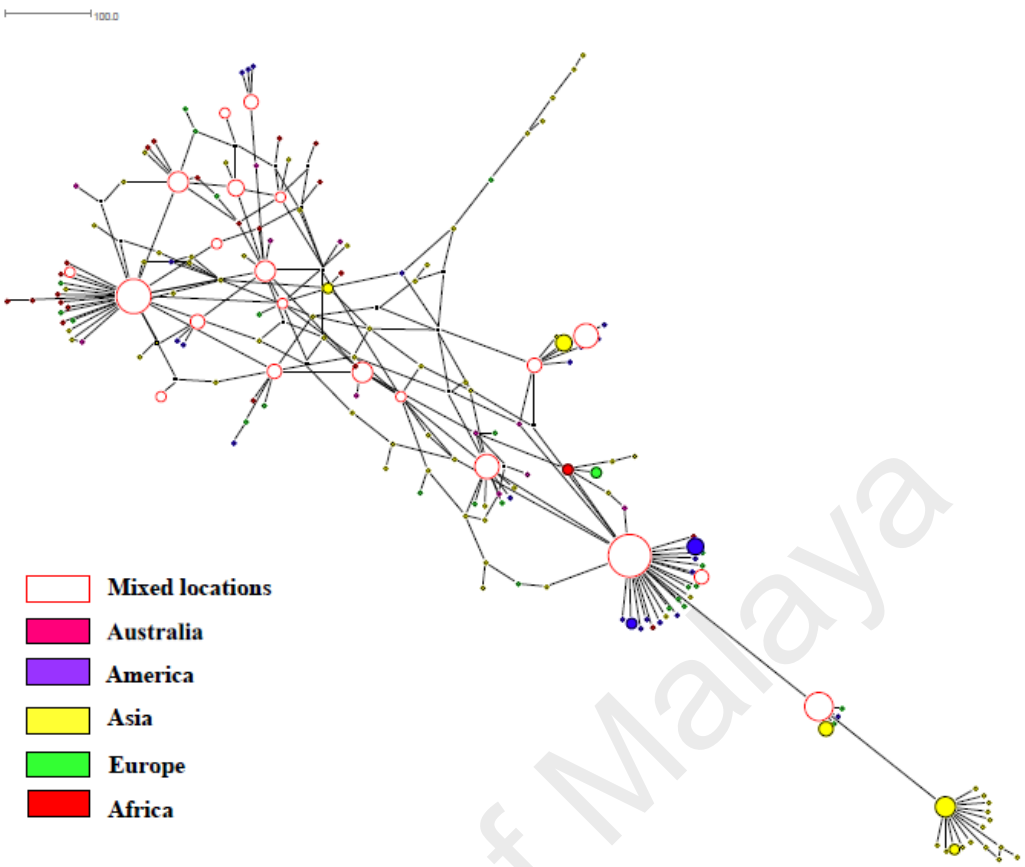

Figure 4.7: Median joining network of assemblage B sequences of the worldwide population

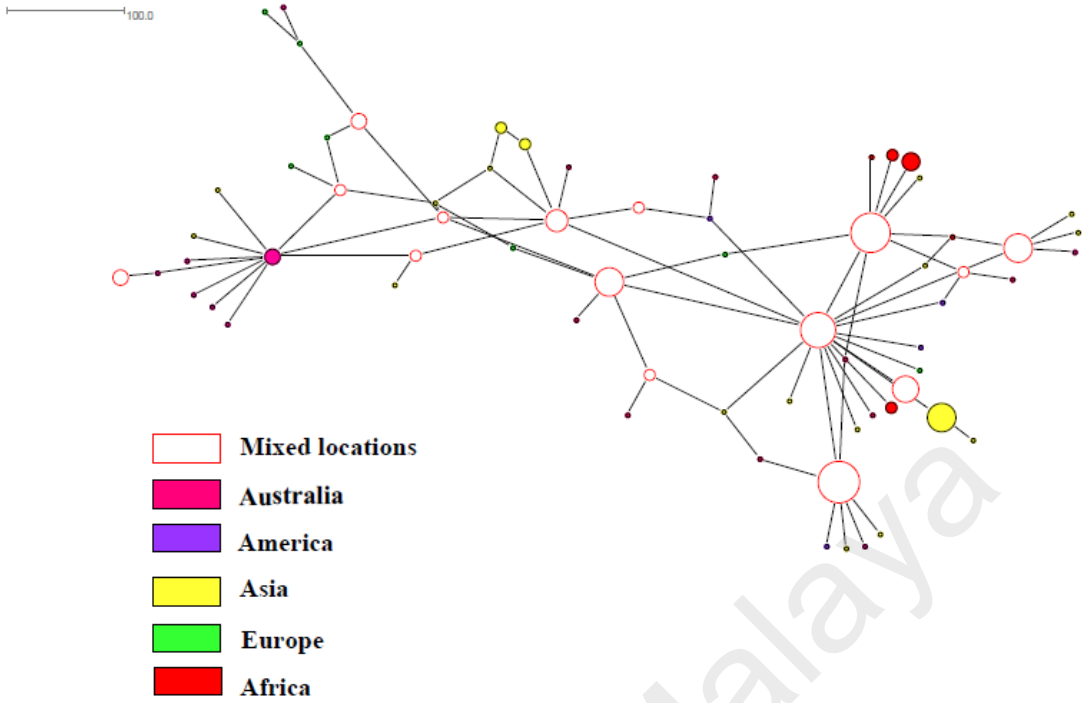

Figure 4.8: Median joining network of assemblage E sequences of the worldwide population

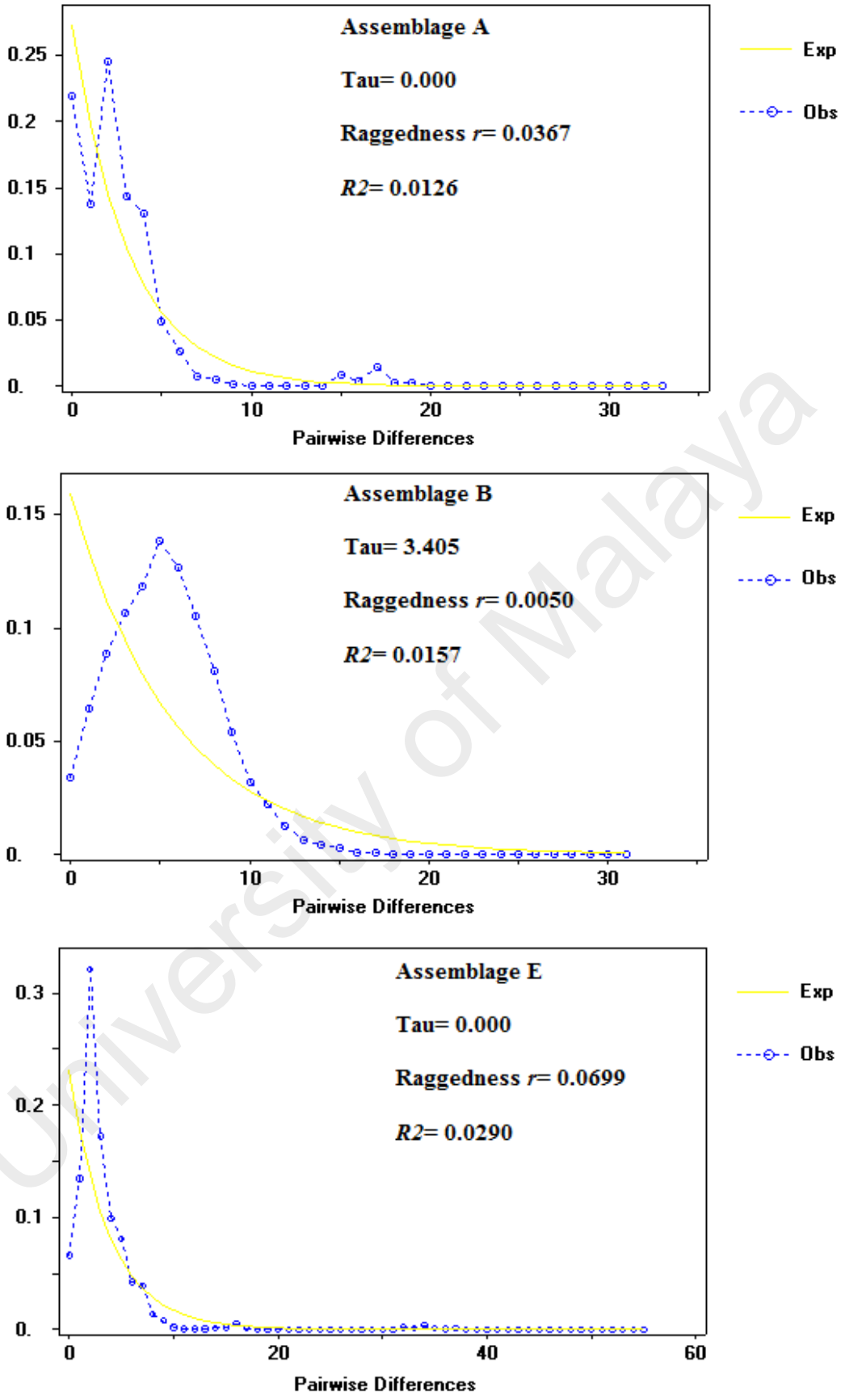

Figure 4.9: Observed and expected mismatch distribution for *Giardia duodenalis* based on *tpi* gene

4.2.5.4 Genetic differentiation and gene flow

The level of genetic differentiation measured using the Wright's test were determined for populations between each continent according to assemblage (Table 4.20). The gene flow or migration index (Nm) transformed from F_{ST} was also accessed and presented in Table 4.20.

For assemblage A, huge differentiation levels were recorded between Asian population with Australian (F_{ST}= 0.35464, Nm= 0.45) and with American (F_{ST}= 0.38293, Nm= 0.40) populations. Genetic differentiation of negligible to moderate differentiation was estimated among the populations for assemblage B except that populations between America-Africa recorded level of differentiation of 0.25445 (Nm= 0.73). The level of genetic differentiation for assemblage E was above 0.15 and significant for populations between Europe-Africa (Nm= 0.69) and between Asia-Europe (Nm= 0.91) whereas the rest of the population pairs had negligible to moderate differentiation. The overall results showed moderate divergence among the populations with 25/30 of the population pairs had F_{ST} less than 0.25 and half of them were statistically significant. These results reflected the spread of the same population of *Giardia* isolates across the geographical regions. In general, gene flow levels were high (i.e. Nm > 1). The highest gene flow was observed in assemblage B (2.41) followed by assemblage A (1.50) and assemblage E (1.48).

Table 4.20 Genetic differentiation (FST) and gene flow (Nm)

Population 1	Population 2	Fst	Nm
Assemblage A			
Australia	America	0.01828*	13.43
Australia	Asia	0.35464*	0.45
Australia	Europe	0.13342*	1.62
Australia	Africa	0.17621	1.17
America	Asia	0.38293*	0.40
America	Europe	0.11833*	1.86
America	Africa	0.13786*	1.56
Asia	Europe	0.08993*	2.53
Asia	Africa	0.06020*	3.90
Europe	Africa	0.03860*	6.23
Total			1.50
Assemblage B			
Australia	America	0.09306	2.44
Australia	Asia	0.02193	11.15
Australia	Europe	0.05506	4.29
Australia	Africa	0.06653	3.51
America	Asia	0.05330*	4.44
America	Europe	0.01962	12.49
America	Africa	0.25445*	0.73
Asia	Europe	0.02529	9.63
Asia	Africa	0.11234	1.98
Europe	Africa	0.19843	1.01
Total			2.41
Assemblage E			
Australia	America	0.14462	1.48
Australia	Asia	0.10296*	2.18
Australia	Europe	0.02651	9.18
Australia	Africa	0.14812*	1.44
America	Asia	0.04029	5.96
America	Europe	0.25382	0.73
America	Africa	0.05736*	4.11
Asia	Europe	0.21494*	0.91
Asia	Africa	0.04215*	5.68
Europe	Africa	0.26526*	0.69
Total			1.48

Value with asterisks (*) indicate statically significant result (p-value < 0.05)

CHAPTER V: DISCUSSION

5.1 PREVALENCE AND EPIDEMIOLOGY OF *GIARDIA* INFECTION

Despite intensive efforts to improve the quality of life in rural Malaysian communities, intestinal parasitic infections and diseases including giardiasis, amoebiasis and soil-transmitted helminthiasis are still highly prevalent, especially among aboriginal and rural populations. The present study provides information on the status of *Giardia* infection among different indigenous people in rural Malaysia, including the *Orang Asli* (aboriginal) population in West Malaysia and other indigenous groups in East Malaysia. Findings of the present study revealed that the overall prevalence of *Giardia* infection was 11.6%, with a significantly higher prevalence among the participants from West Malaysia when compared to those from East Malaysia (13.6% vs 5.8%). This is in agreement with a previous study among 716 rural individuals from five states of West Malaysia which found that the prevalence of *G. duodenalis* was 10.4% and was the highest among the protozoa infections i.e. *E. histolytica/dispar* (10.2%) and *Cryptosporidium* sp. (2.1%) (Ngui et al., 2011). Furthermore, several small-scale studies have been conducted among the aboriginal population in West Malaysia which showed that the prevalence of *Giardia* ranges between 4.0% and 29.2% (Noor Azian et al., 2007; Mahdy et al., 2008; Mahdy et al., 2009a; Ngui et al., 2011; Sinniah et al., 2012; Anuar et al., 2014a). In addition to which, a recent study among three aboriginal tribes in three different states of West Malaysia revealed a high prevalence (20.0%) of *Giardia* infections (Anuar et al., 2012a).

In West Malaysia, the present study showed that the prevalence of *Giardia* infection was highest among participants from Pahang state (15.9%) followed by Negeri Sembilan (14.9%) and Kedah (13.4%), while the lowest levels of prevalence were found among the participants from Malacca 4.6% and Selangor 6.1%. This could be attributed

to the differences in the culture, environment and population of *Orang Asli* communities in these areas. *Orang Asli* villages in Pahang, Kedah and Negeri Sembilan are located deep in the jungle, with inadequate sanitary facilities and away from healthcare facilities. In contrast, Selangor and Malacca are in peripheral areas with better sanitation and environmental factors, as well as being located near to the region's main health facilities. The present findings further revealed that the Senoi group (Semai, Jahut and Temiar tribes) had the lowest prevalence of infection amongst its population (9.6%), while the prevalence among the Negrito group (Kensiu) was higher than the Proto-Malay group (Temuan) (13.4% vs 10.8%).

These findings are consistent with previous studies conducted among the different ethnic groups of *Orang Asli* in West Malaysia, which reported a higher prevalence of *Entamoba* species and STH among Negrito groups, followed by Senoi and Proto-Malay groups (Anuar et al., 2012b; Anuar et al., 2014b). Similarly, an earlier study among 1,273 individuals from different ethnic groups showed that the Negritos harboured more intestinal parasites species when compared with other ethnic tribes (Dunn, 1972). *Orang Asli* belonging to the Negrito group live in remote areas, with their villages being made of wood or bamboo, which means they suffer from poor housing conditions, a lack of proper sanitation and no provisions for a clean water supply when compared to the Senoi or Proto-Malay groups who live in suburban areas under better conditions.

With regards to East Malaysia (Sabah and Sarawak states), supporting information on the prevalence of intestinal parasitic infections is lacking. The present study showed that the prevalence of *Giardia* infection in Sabah was much lower (5.8%) than in West Malaysia, with a significantly higher prevalence among participants from the Dusun tribe compared to those from Murut and Bajau tribes. Unfortunately, the only previous report from East Malaysia was conducted solely in Sarawak, revealing a very

low prevalence of *Giardia* infection (2.0%) among 355 individuals from five interior communities (Sagin et al., 2002).

The government has made intensive efforts to improve the quality of life of indigenous people throughout the country, with their main strategy being to the reallocate those living in remote areas to new settlements at the periphery of towns. Many of the villages/households were recipients of housing assistance under various government implementations such as Housing Assistance Programme (Program Bantu Rumah, PBR) and Structured Placement Programme (Program Penempatan Tersusun, PPT) with the latter being specially administered by JAKOA for the *Orang Asli*. The programs provide financial and management services with the aims of improving quality of life of the disadvantaged communities, relocating communities to safer and more comfortable locations or in the case when their original settlement were displaced due to various reasons e.g. disaster, acquisition of lands by private companies for commercial purposes or give way for development projects. Majority of the villages in West Malaysia were full or partial programme participants of the programs. For example, Kg. Sangwai, Kg. Jekjok and Kg. Dusun Kubur were full programme participants where the whole village was a new settlement comprising standard brick houses installed with piped water, electricity and in-house toilet facility. Villages with only partial programme participants such as Kg. Paya Sendayan and Kg. Ulu Kelaka have only certain percentage of villagers live in brick houses built by the government while the rest live in their traditional wooden or bamboo houses built by themselves.

There are two villages in Sabah participated in government resettlement scheme. One of them is Kg. Penimbawan which is a Bajau fishing village located at the Sulaman Lake near to the river tributary of Surusup in Tuaran. The traditional architecture of the village was originally stilt houses raised above the water and linked by timber boardwalk. However, when a financial allocation was provided by the government

under PBR for rehabilitation of the village, most of the residents were resettled in 2010 to proper-planned double-storey housing equipped with piped water and electricity and amenities such as school and dispensary on the land adjacent to the old setting.

Kampung Salarom Taka is another village in Sabah that was relocated in 2006 under Society Forestation Programme (Program Perhutanan Masyarakat, PPM) and PBR when the area in the interior forest where their old village used to be was designated as a part of Pensiangan Reserve Forest area (kawasan Hutan Simpanan Pensiangan).The current setting at Sepulut consists of 73 single-storey houses and a modern long house that was specially built in the compound with mountain water as source of water supply and electricity produced by generator.

New houses provided to the settlers which were ensured access to basic amenities, has helped greatly reduce the prevalence of many parasitic infections amongst the indigenous communities in Sabah. On the other hand, the adherence of the *Orang Asli* people in West Malaysia to their jungle habitats has constrained the overall effectiveness of this strategy. Therefore, providing new houses within the same locality will not help reduce the prevalence of intestinal parasitic infections in this area due to the heavily contaminated environment.

The present study investigated the possible risk factors associated with *Giardia* infection among the participants, revealing that children under 13 years old (1 month to 12 years old) were significantly associated with higher *Giardia* infection rates when compared to adults and children over 12 years old (13 to 84 years old) , and this is in agreement with previous studies (Norhayati et al., 1998; Mahdy et al., 2008; Mahdy et al., 2009a). This could be attributed to the higher exposure of young children to the source of a wide range of infections, which could be due to having lower standards of personal hygiene and specific habits such as putting objects into the mouth and sharing

the objects among friends when compared to the adults and older children (Lim et al., 2008).

In addition, the research revealed that not boiling drinking water before consumption was reported as a significant predictor of *Giardia* infection. It is well documented that *Giardia* and *Cryptosporidium* have been the most common causes of waterborne diseases outbreaks worldwide (Baldursson & Karanis, 2011). The present study further showed that living in houses without functioning toilets increased the odds of the observed population acquiring *Giardia* infections, and this is consistent with many previous studies in Malaysia and elsewhere (Cifuentes et al., 2000; Ahmed et al., 2011a; Ngui et al., 2011). It was further found that defecating in indiscriminate places, such as in rivers and bushes was a common practice in communities with inadequate toilet facilities.

In general, *Orang Asli* have a habit of building their villages beside rivers where water can be conveniently collected for multiple purposes, including drinking and cooking, whilst also conducting daily activities such as bathing and washing clothes in the river water. Besides which some inhabitants, especially children, prefer defecating at the site of the stream (Ahmed et al., 2011a). As a result, it is likely that the environment of the local rivers are heavily contaminated, becoming a source of infection for *Giardia* and other intestinal parasites/ bacteria/viruses (Al-Mekhlafi et al., 2005; Ahmed et al., 2011a). This presumption is supported by a finding from the present study, in which bathing in the rivers appeared as one of the significant risk factors. A recent study that involved sampling of river water reported presence of *Giardia* cysts in 51.3% of the 39 samples collected from five villages located in three states of West Malaysia with an overall mean concentration ranged from 0.10 to 25.80 cysts per litre (Lee et al., 2014b). In the same vein, an interesting finding was uncovered when the univariate analyses were stratified according to location, in which a

significantly higher prevalence of *Giardia* infection was found among *Orang Asli* who drink piped water when compared to those collected drinking water from unsafe sources (i.e. rivers, wells and rain) (15.9% vs 11.5%; χ^2 = 4.181; *P* = 0.041). In contrast, participants from East Malaysia who use piped water for drinking had a lower prevalence of infections when compared to their counterparts who used unsafe water (5.2% vs 15.8%; χ^2 = 3.655; *P* = 0.056). Drinking piped water has been identified as a significant risk factor among the aboriginal population in Pahang, Malaysia (Ngui et al., 2011). However, results from previous studies conducted in these communities showed that treated water is free from faecal coliforms, *Giardia* and *Cryptosporidium* contamination (Lim & Ahmad, 2004; Lim et al., 2008). Hence, it is believed that the contamination of the piped water is occurring after the treatment process has taken place, and could be attributed to the usage of containers and utensils that may have been previously soiled with *Giardia* cysts when handling and storing drinking water. Furthermore, it was observed that the *Orang Asli* people in West Malaysia who used tanks or other containers for drinking water collection left these containers uncovered, which is in direct contrast to the clean and covered tanks in East Malaysia.

The present study also showed that not washing hands before eating or after handling/playing with animals were significant risk factors for *Giardia* infection among the study population. Although previous studies have suggested zoonotic transmission for *Giardia* infection, studies among Malaysian aborigines have previously found no such association (Mahdy et al., 2008; Anuar et al., 2012a). *Giardia* cysts can remain infective in the environment for a very long period of time, meaning they could easily get picked-up on the fur of animals such as cats and dogs, who were observed as moving about in the contaminated environment while also mixing freely with the members of their households. Hence, not washing hands after handling or playing with these animals could facilitate the spread of the infections. Similarly, not wearing shoes

when outside the house may also contribute to the contamination of houses with cysts, and this was also identified as a significant predictor of *Giardia* infection by the present study.

It is also worth noting that a significantly higher prevalence of *Giardia* infection was reported among participants with low educational levels (either non educated or with only primary levels of education), as well as in areas in which indiscriminate defecation was common, in areas with indiscriminate garbage disposal systems, in large households (> 7 members), and when not washing vegetables/fruits before consumption. However, these associations were not retained by the logistic regression model. Previous studies among aboriginal communities in West Malaysia have identified these variables as significant predictors of *Giardia* and other intestinal parasitic infections (Mahdy et al., 2009a; Ngui et al., 2011; Anuar et al., 2012a; Nasr et al., 2013).

In view of this, when analysing the risk factors according to location, it is remarkable to find out that all of the variables were retained as significant risk factors of *Giardia* infection in West Malaysia, while only one significant risk factor was retained among the participants from East Malaysia (that is not washing vegetables/fruits before consumption). It is also interesting to note that while eating raw vegetables and fruits has been identified as significant risk factors of *Giardia* infection among *Orang Asli* in West Malaysia (Mahdy et al., 2009a; Mahdy et al., 2009b), the present study revealed that the significant association is actually in the practice of not washing vegetables or fruits prior to consumption, which appeared to be a more tenable reason when compared to the simple consumption of this nutritious food. Most of the fruits in these communities are tropical peeled fruits like rambutan (*Nephelium lappaceum*), longan (*Dimocarpus longan*) and mangosteen (*Garcinia mangostana*). It was observed that people collected dropped fruits (rambutan, longan) from the ground, opened the soft shell with their mouths and ate the fruit directly without first washing it. Thus, fruits and

vegetables could be the medium of transmission in cases where the surface carries the parasites infective stages, especially when the fruit has been in contact with the contaminated ground followed by the direct consumption of the fruit itself, both of which increase the chances of transmission from contaminated hands.

Concerning the presence of *Giardia* cysts in the environment, Lim et al. (2008) has reported the detection of cysts in 0.7% of the 138 soil samples taken from house compounds of *Orang Asli*. Besides, considering indiscriminate garbage disposal was retained by the present study as a significant risk factor of *Giardia* infection among the *Orang Asli* communities, Gabbad and Elawad (2014) have also found unsafe disposal of solid waste as a significant factor in the transmission of intestinal parasites. This practice not only contributes to the contamination of food and water but also encourages the breeding of houseflies and cockroaches which serve as vectors of some intestinal parasites. Flies have been reported to be capable of carrying *Giardia* cysts on their exoskeletons and in their digestive tracts and can be vehicles in the mechanical transmission of this parasite (Doiz et al., 2000; Szostakowska et al., 2004; Conn et al., 2007; Zhao et al., 2014). Flies were frequently seen around the households in the studied communities, especially when there was food left lying around.

The present study found a significant association between *Giardia* infection and diarrhoea among the studied population, with significantly higher frequency of diarrhoeal cases among *Giardia*-infected participants from West Malaysia when compared to their counterparts from East Malaysia. However, about two-thirds of the *Giardia* cases were mixed infection with at least one parasite species. Therefore, it was not possible to confirm the causal relationship between *Giardia* and diarrhoea in the present study, due to the limitation of the cross-sectional design used to gather results.

In most of the studied states, co-infection of *Giardia* with other intestinal parasites was a common scenario in areas where STH infection was high (67.5%) and the prevalence of STH especially *Trichusis* and *Ascaris* was consistently higher than *Giardia* infection. However, this was not reported for Kelantan and Sabah states where the overall prevalence of STH was low and the *Giardia* infection was relatively high (Table 4.2). In view of the insignificant association of *Giardia* with low income group and examples demonstrated by these particular groups (Kelantan and Sabah), it should be emphasized that low income does not necessarily have to link with parasitic infections, and it is especially true in this context where good household hygiene are practiced and low parasitic infections were observed. By identifying the route of transmission, health care promotion of targeting household hygiene including personal and food hygiene can be implemented in order to break the cycle of transmission and to effectively prevent infection.

Besides, another study has investigated helminths-*Giardia* co-infections pattern in 3275 participants and revealed that participants infected with helminths were less likely to become infected with *Giardia* (Blackwell et al., 2013). The authors attributed that to the antagonism between parasites that may reflect competitive inhibition or cross-immunity (Blackwell et al., 2013). The present study found lower odds in hookworm (OR=0.8) and *Ascaris* (OR=0.7) infections; however the results were not statistically significant. When stratified according to location, *Giardia* infection was significantly associated with *Ascaris* infection among the *Orang Asli* population in East Malaysia (OR=0.5; 95% CI = 0.3, 0.7). Findings by Blackwell et al. (2013) were obtained using longitudinal analysis as opposed to the present cross-sectional data. This could be the reason that the obtained findings were less comparable. However, another concerning point made by the authors was that removal of helminths with treatment may increase *Giardia* susceptibility. Hence, interaction between *Giardia* and helminth,

co-infection and treatment policies are important issues that worth proper attention especially in areas where these parasites are endemic.

The present study provides a community-based picture on the epidemiology of *Giardia* infection among the indigenous people in rural Malaysia. Overall, the findings show that *Giardia* infection in these communities was mostly associated with poor hygienic practices that were often coupled with poor sanitary facilities as well. Although provision of housing and upgrading of water and sanitary infrastructures are deemed the right strategy in improving the general health of indigenous communities, the amount of money and effort invested are always not meeting the expected outcome. In their report, McDonald et al. (2008) have considered this as a failed approach in developing countries. The highlighted issues are rapid introduction of new infrastructure in an ad hoc manner that is unparalleled with changes of behaviours to adapt new housing environment. Combination factors of heavily contaminated environment with multiple sources of disease transmission, poor personal hygiene, under-nutrition and low resistance have also contributed to the slow progress.

From the general characteristics of both groups, it is clear that the indigenous people in East Malaysia are more educated, have a higher monthly household income, live in better housing conditions and have a cleaner environment when compared to the indigenous people in West Malaysia. Hence, the lifestyle interventions already implemented in East Malaysia might be the explanation as to why there is a significantly lower prevalence of *Giardia* reported in that region.

There were some limitations of the present study. In many cases only a single faecal sample was collected, instead of the ideal three consecutive samples, because of a limitation of resources and the cultural belief of some *Orang Asli* against giving their faecal samples. Therefore, the prevalence of *Giardia* infection might be underestimated

due to the variation in cyst shedding per days. Many indigenous villages are located in deeply remote areas, with no road access, and therefore were not involved in the present study, though it is worth noting that even higher prevalence rates of intestinal parasitic infections have been previously reported in these remote areas when compared to the villages involved in the present study (Noor Azian et al., 2007).

Overall, the present findings can be generalised to other rural indigenous children in other states. However, it is possible that these findings may not be generally applicable to the entire Malaysian rural population, as ethnic groups other than the indigenous people tend to have better socioeconomic and environmental situations. Hence, further studies are required in order to confirm these conjectures.

5.2 Molecular characterization of *Giardia duodenalis*

5.2.1 Molecular epidemiology

Giardia-positive samples were further analysed using multilocus genotyping to study the assemblage and sub-assemblage of *Giardia* isolates.

Giardia has a lifecycle that involves encystation of the trophozoite before passing out in faeces in which ultimately enable the parasite to survive in the environment outside the host. The present study emphasized the environmental factors and personal hygiene practices that expose the communities to different type of assemblage and their effect in the transmission the parasites. In accordance with majority of studies, only assemblages A and B were isolated from the human samples. The molecular findings of the current study showed that assemblages A and B were present at equal frequency (69/138). Indeed, these findings contradict previous genotyping data using a single locus (i.e. *tpi* gene) (Mahdy et al., 2009a; Anuar et al., 2014a). A recent community-based study identified two-thirds of 98 *Giardia*-positive isolates as assemblage A and the rest were assemblage B (Anuar et al., 2014a). By contrast, a previous study using SSU rRNA locus identified only one specimen as assemblage A in 42 specimens while the rest were assemblage B (Mahdy et al., 2009a). This is, however, different from the proportion of assemblages A and B reported globally where assemblage B (~58%) has a higher prevalence than assemblage A (~37%) (Ryan & Caccio, 2013).

With regard to genotypes in symptomatic cases, gastrointestinal symptoms such as diarrhoea, vomiting, abdominal pain and dysentery were reported in 18.8% and 14.5% of assemblage A and B respectively. No significant difference was found in the prevalence of both assemblages among symptomatic infections. Similar findings were also reported in other studies (Kohli et al., 2008; Lebbad et al., 2008; Ajjampur et al.,

2009; Yang et al., 2010a). By contrast, a previous study among *Orang Asli* in Malaysia reported a strong association between the clinical symptoms of gastroenteritis and assemblage B (Mahdy et al., 2009b). To date, there is still a lack of clear association between the assemblage and the clinical outcome, with contradictory results. While previous studies conducted in Bangladesh, Australia and Spain have reported a significant association between assemblage A and the presence of symptoms (Read et al., 2002; Haque et al., 2005; Sahagun et al., 2007), other studies from various regions also suggest a correlation between the presence of symptoms and infection with assemblage B (Gelanew et al., 2007; Minvielle et al., 2008; Pelayo et al., 2008; Molina et al., 2011a; Puebla et al., 2014). Hence, conclusive inference with regard to the genotype-dependent pathogenecity may only be drawn after further well designed studies (Buret, 2008).

Besides that, the present study showed that the majority of *Giardia*-positive individuals were asymptomatic (83.8%). This high percentage of asymptomatic infections should be taken with apprehension from the public health standpoint as the infected individual can act as carrier and excrete infective cysts in faeces. Infection is acquired by ingestion of the resilient cysts in contaminated food, water or hands. It could have an adverse impact especially to the family and the community, if the symptoms-free cases go unnoticed and contaminate the environment. The cysts have been reported to have low infectious dose of as few as ten cysts to establish an infection and become immediately infectious upon being released in stool (Rendtorff, 1954). More significantly, this health hazard could be long lasting because the transmissive stage of this parasite can persist between 7 to 18 days in faeces, 7 weeks in soil and up to 3 months in water (deRegnier et al., 1989; Deng & Cliver, 1992; Thiriat et al., 1998; Olson et al., 1999). Yet, there has been no study conducted to investigate what genotype of *Giardia* infection sheds higher number of cyst or has higher level of resistance to

withstand the external environment and maintain longer period of infectivity. If these factors are genotype-related, one will expect that a particular assemblage will exist in greater amount in the environment and potentially lead to higher chance of transmission. One relevant study by Haque et al. (2005) reported that assemblage B infections produced higher load of DNA and higher overall prevalence whilst in a cyst quantification test, Kohli et al. (2008) found children infected with both assemblages A and B shed more cysts than children infected with either assemblage A or B alone.

All the three occurrences of assemblages A, B and mixed infections were reported among *Orang Asli* in Peninsular Malaysia. Despite continued efforts to improve the quality of life of *Orang Asli* people, the communities were observed to be poor in terms of personal and surrounding hygiene and children were seen wandering around without proper footwear. The *Orang Asli* has the penchant of engaging in unhygienic practices such as playing with soil, indiscriminate defecation and bathing in rivers. The latter two are common among the *Orang Asli* where toilet facilities are not available or not well maintained/ built. In some villages, the toilets were built outside of the house and shared publicly. Infection with assemblage B and mixed infection and not with assemblage A alone were significantly associated with the practice and habit of the indigenous people (i.e. indiscriminate defecation, bathing in river and playing with soil). This would suggest that the environments (river and soil) have more abundance of cysts of assemblage B , either due to excretion of higher number of cyst or long persistency compared to A and exposure to the these environments will lead to higher chance of getting *Giardia* infection.

The current findings showed that individuals from large household (family members of more than 7) were more likely to acquire assemblage B and mixed infections. A previous study by Anuar et al. (2014a) stated that humans might be emerged as the source of assemblage B infection and direct transmission within the

household can occur as a result of the presence of infected family members. Moreover, the present study showed that not washing hands before eating was also significantly associated with assemblage B and mixed infection. This is consistent with previous observation that eating using hands appeared to be a common practice among indigenous people and could be one of the modes of transmission among family members especially during meals and food preparation.

Findings of the present study showed that the occurrence of mixed infections was 31.8%. The global molecular data had shown higher prevalence of mixed infections in developing countries (9.2%) compared to developed countries (3.3%) (Table 2.7). Mixed infections are caused by the ingestion of genetically dissimilar cysts by the host especially in the endemic areas and may imply the involvement of multiple sources in human infections and the complex transmission of *G. duodenalis* in the environment (Gelanew et al., 2007; Caccio & Ryan, 2008; Sprong et al., 2009; Feng & Xiao, 2011; Lebbad et al., 2011). In a study among returning travellers, Broglia (2013) reported that tourists travel to endemic areas often acquired mixed infections. In the present study, the prevalence of mixed infections was 38.5% in symptomatic individuals and 30.5% in asymptomatic individuals. The difference was not significant and this is in agreement with a previous study from Egypt which reported the prevalence of mixed infections in 16.4% of the symptomatic individuals and 13.6% of asymptomatic individuals (El-Shazly et al., 2004). The high prevalence of mixed infections may reflect the frequent incidence of re-infections within the communities which is common in areas of high endemicity and poor faecal-oral hygiene (Gardner & Hill, 2001). Kohli et al. (2008) reported that children with mixed infection had higher chance of developing subsequent infections than those with single infection. This is of great concern particularly to the children because *Giardia* infections are known to have association with malabsorption, impaired growth and poor cognitive function (Cole & Parkin, 1977; Farthing et al.,

1986; Farthing, 1996; Berkman et al., 2002; Gendrel et al., 2003). In accordance with the significant association of mixed infections with indigenous people in Peninsular Malaysia and individuals aged 12 and younger, several previous reports have also indicated *Giardia* infection as a significant predictor of severe protein-energy malnutrition and vitamin A deficiency among *Orang Asli* children (Al-Mekhlafi et al., 2005; Al-Mekhlafi et al., 2010; Al-Mekhlafi et al., 2013). In Brazil, giardiasis was significantly correlated with impeded growth even the affected children were asymptomatic (Prado et al., 2005). Another study showed that school children were found to have higher rate of mixed infections compared to pre-school children (Yason and Rivera, (2007). School children have more active interaction with people and greater exposure to the environment and these may enhance the chances of getting various infections

5.2.2 Distribution of genotypes in different localities

With regard to the finding that a few of the *Giardia* isolates from different localities share a common subtype (e.g. KC313923, subtype A of *tpi*, was found in all states except Kelantan; NT6.3, subtype B of *gdh* was found in Sabah, Negeri Sembilan and Selangor), no correlation between subtype and geographical location (either between the different states or between East Malaysia and West Malaysia) was determined. The subtypes had a fairly dispersed distribution in all of the studied localities from both East Malaysia and West Malaysia (Tables 4.10, 4.13, and 4.16). As the isolates were obtained from indigenous people living in rural and some in isolated areas, cross-transmission between different regions was assumed to take place at minimum rate. The presence of common subtypes could reflect the ubiquitous presence of *Giardia* isolates especially among the subtypes of assemblage A in local and as well as global context. In addition, phylogenetic trees showed that the subtypes tend to cluster according to the assemblage and/or sub-assemblages. No specific geographical clustering patterns were

seen from the analysis of the *Giardia* variants obtained at all loci (Figures 4.1 and 4.2). Even though the occurrence of some subtypes was restricted to only one or two locations, there was not enough evidence to infer geographical isolation. Most of these subtypes belonged to assemblage B and were mostly represented by one or two isolates. High nucleotides variation among isolates of assemblage B as well as effect of sampling size and prevalence of *Giardia* infection at different sampling sites could be the explanations for the appearance of subtypes in limited locations. In conjunction, it was noted that the locality (i.e. West Malaysia) and the state (i.e. Pahang) which had larger sampling size and higher rate of infection contained greater number of subtypes.

5.2.3 Subtyping and its implication for zoonotic transmission

There is a lot of concern over the specific subgroups within the zoonotic assemblages A and B based on the pattern of nucleotide substitution and its consistency at intra-assemblage level across loci as these subgroupings might potentially link with the hosts (Wielinga & Thompson, 2007). To date, investigation of the molecular data of assemblage A had revealed three sub-assemblages designated as AI, AII and AIII with only AI and AII had been isolated from humans (Feng & Xiao, 2011; Liu et al., 2014a). In the present study, the isolates with assemblage A were all identified as A2 and A3, which belong to sub-assemblage AII. This is similar with other studies where AII was the predominant sub-assemblage in human (Hussein et al., 2009; Bonhomme et al., 2011; Lebbad et al., 2011). The sub-assemblages within isolates of assemblage A appear to have host-specificity. Sub-assemblage AI which was absent from this study was reported to have preference to cause infection in livestock or companion animals as opposed to AII which was found mainly in human cases and occasionally in animals (Sprong et al., 2009); whereas AIII has only been found circulated in wildlife (Feng & Xiao, 2011). Based on this aspect, AI is regarded as a major subtype of zoonotic concerns (Gomez-Puerta et al., 2014).

On the other hand, the classification of sub-assemblages BIII and BIV were originally determined by electrophoretic analyses but these subgroupings were not reproducible in recent molecular analyses and had produced conflicting results at different loci (Wielinga & Thompson, 2007). Similar with the finding in the current study shown by the substitution patterns and phylogenetic trees, sub-assemblages were not able to assign to isolates belonged to assemblage B due to high degree of nucleotides variation and this was not uncommon among other molecular studies (Caccio et al., 2008; Lalle et al., 2009; Levecke et al., 2009; Bonhomme et al., 2011; Lebbad et al., 2011). The specific substitutions that define the subgroups were not established and it was assumed that more subgroups other than BIII and BIV might be present (Wielinga & Thompson, 2007). Although isolates of assemblage B cannot be distinctly typed into sub-assemblages, its high intra-assemblage variations could be viewed as an advantage in outbreak and zoonotic transmission investigations.

The isolation of anthroponotic assemblages and sub-assemblages B and AII in this study implicates human as a potential source of the infection. In accordance with the findings of the present study, previous studies conducted in *Orang Asli* communities in Malaysia found that the presence of other family members infected with *G. duodenalis* was either the only or the main risk factor for giardiasis (Norhayati et al., 1998; Anuar et al., 2012a). However, the postulation of human-to-human transmission is limited by the fact that animals were not included for analysis in this study.

5.3 General opinions and interpretation of the molecular data obtained using multilocus genotyping

5.3.1 Variation in efficiency of amplification by different loci

With the application of the highly discriminatory MLG analysis, it was hoped that genetic variability among the samples isolated from the indigenous people from different regions can be extensively studied. However, amplifications of the three PCR assays (*tpi*, *gdh*, and *bg*) were performed differently at different loci and yielded negative impact on the analysis. The *tpi* locus achieved the highest percentage of amplicons produced (79.2%), followed by *gdh* (59.1%) and *bg* (46.8%). Similar occurrences were also reported in previous studies (Lalle et al., 2009; David et al., 2011; Broglia et al., 2013; Liu et al., 2014a). Apart from that, not all microscopy-positive samples were amplified despite several attempts had been done (amplifications were repeated at least once for isolates that were negative at any of the markers for the first time and this included some adjustments in the PCR conditions). Although PCR has been recognized for its sensitivity and specificity, the limitations in the performance of PCR can also be found in a number of reports (Breathnach et al., 2010; Yang et al., 2010b).

Several explanations have been suggested for the formal and latter problems. First, low rate of amplification could be due to nucleotide mismatches in the primer sequences especially in the case of high genetic variation. Excessive mismatches may result in low efficiency of the annealing between primers and DNA templates (Liu et al., 2014a). Second, when the copy number of genes is considered, amplifications of single copy genes such as *tpi, gdh* and *bg* tends to be more erratic and less sensitivity compared to multi-copy gene i.e. *rDNA*. In addition, marker such as *rDNA* is thought to have lower chances of primer mismatch because of its high sequence conservation

compared to the highly variable genes (Caccio & Ryan, 2008). In the present study, however, as all the markers used belong to single-copy genes, the difference in copy number is not applicable in the current context to explain the difference in the efficiency of amplification rate. Other factors that could influence the amplification are low number of cyst or low concentration of DNA and presence of inhibitors (David et al., 2011). Better result could be obtained by concentrating and purifying the cysts before proceeding to DNA extraction and optimizing PCR conditions and reagents concentration. Despite, even when optimal conditions are thought to have been achieved (as in the PCR conditions described in the methodology session), several adjustments are needed to improve the amplifications of the samples isolated from faecal material that might contain considerable variants in content.

5.3.2 Discordant genotyping results

Discordant genotyping results at the three loci have been observed in 18.6% of the isolates in this study, which is in agreement with previous reports (Caccio et al., 2008; Sprong et al., 2009; Thompson et al., 2009; Yang et al., 2010a; Broglia et al., 2013; Helmy et al., 2014). Of note, it has been a common phenomenon found in both human and animal samples. Some samples were typed as host-adapted assemblages (e.g. C and E) using one marker while another marker typed the same samples as assemblages with zoonotic potential (i.e. A and B). An analysis of sequences from four genetic loci (*tpi*, *gdh*, *bg* and *rDNA*) has been conducted on 61 human isolates and 29 animal isolates of *G. duodenalis* (Caccio et al., 2008). In this study, Caccio et al. (2008) reported incongruent assignment of five human isolates and one macaque isolates of *G. duodenalis* assemblages. Yang et al. (2010a) genotyped 124 human isolates of *G. duodenalis* at *gdh* and *rDNA* genes, and reported incongruent genotyping results in five isolates, where three isolates were classified as assemblage A at *rDNA* gene and assemblage B at *gdh* gene, and two isolates were classified as assemblage B at *rDNA*

gene and assemblage A at *gdh* gene. Inconsistency in genotyping results of *G. duodenalis* has also been reported among dogs and cats isolates, where 7 isolates (3 dogs and 4 cats) were classified as assemblage D at *rDNA* gene, and as assemblage B (3 isolates), assemblage C (3 isolates) and assemblage E (1 isolate) at *gdh* gene. One dog isolate was classified as assemblage C at *rDNA* and assemblage B at *gdh* gene (Read et al., 2004). Thompson et al. (2009) genotyped *G. duodenalis* isolated from 70 cayotes at *rDNA* and *gdh*, and reported four incongruent genotyping results.

The implication of the inconsistency results has provoked questions about the strength and validity of genotyping relying on single locus and hence, the true picture of *Giardia* diversity has a high chance of being masked by the data generated from analysis using single marker (Feng & Xiao, 2011). Although the underlying mechanisms remain uncertain, Caccio and Ryan (2008) had reviewed and attributed the incongruent assignment of assemblages by different makers to a number of factors including retention of ancestral polymorphism, introgression, meiotic recombination and mixed infections.

Genetic markers are used to identify genetic variations among sequences. These sequences variations can be used to infer the relationships between the taxa under study (Anderson, 2001). Considering the species complex concept of *G. duodenalis* which consists of eight distinct assemblages (i.e. A-H), some of which are host-specific, and each of them represents different taxon diverged from a common ancestor. In the event of retention of ancestral polymorphism, identical alleles in genetically distinct populations (e.g. *G.duodenalis* of different assemblages) can happen when time elapse is insufficient for two closely related taxa of a common ancestor to fix alleles differences through mutation and genetic drift. Contrariwise, introgression which involves gene flow through back-crossing between closely related taxa and one of the parental populations can cause divergence in the alleles of the same population (e.g.

G.duodenalis of the same assemblage). Both of these evolutionary processes can contribute to the presence of specific alleles of an assemblage in the genome of another assemblage (e.g. assemblage A in B) and resulting in incongruent assignment of assemblages by different markers (Anderson, 2001; Caccio & Ryan, 2008). While the explanations using evolutionary events seem probable, there are areas needs to be deliberately considered. In the question on the length of time required for alleles of two sister taxa to be reciprocal monophyletic (i.e. differentiated to a monophyletic group in which alleles of one taxon are more closely related to each other than to alleles of the other taxa), Anderson (2001) explained that it depends on generation time and demographic features such as effective populations size (Ne). Incidentally, phylogenetic studies on different isolates has shown that assemblages of *G. duodenalis* containing evolutionary distances comparable to those segregating different genera of protists, suggesting that these assemblages have undergone long periods of independent evolution (Mayrhofer et al., 1995; Monis et al., 2003; Wielinga & Thompson, 2007). Likewise, introgression can only happen if sexual reproduction takes place in the life cycle of *Giardia*. The postulation of sexual reproduction (or meiotic recombination) and another contributing factor i.e. mixed infections in discordant genotype would be discussed further in the later context. There were other puzzling but important observations that might lead to confusion and difficulty in interpretation of the genotyping data.

5.3.3 High polymorphism and heterogeneous nucleotides in assemblage B

In the present study, sub-assemblages were not able to assign to isolates belonged to assemblage B due to high degree of nucleotides variation which was not uncommon among other molecular studies (Caccio et al., 2008; Lalle et al., 2009; Levecke et al., 2009; Bonhomme et al., 2011; Lebbad et al., 2011). Furthermore, analysis of the sequences at the three markers revealed heterogeneous nucleotide at various positions

primarily in the isolates of assemblage B. It was observed in 6.6% of the *tpi* sequences (n=122), 40.7% of the *gdh* sequences (n=91) and 5.6% of the *bg* sequences (n=72). The heterogeneous nucleotides were relatively high among the *gdh* sequences. This could be related to higher percentage of assemblage B being identified by the marker (i.e. 79.1% by *gdh* vs.43.4% by *tpi* and 20.9% by *bg*) as almost all of the heterogeneous nucleotides were found among isolates of assemblage B except one *bg* isolate of assemblage A. In a multilocus study, Caccio et al. (2008) also noted the highest percentage of heterogeneous sequences in the *gdh* marker (i.e. 75% compared to 50% in *tpi* and 36% in *bg*). In the same study, no ambiguous positions in the sequences of assemblage A were reported and this outcome also implied that the heterogeneous nucleotides were less likely the consequence of amplification errors or artefacts as this phenomenon occurred only in the sequences of assemblage B. These observations also reinforced other studies that reported the absence of heterogeneous nucleotides in the isolates of assemblages A, F and G but were frequently detected in isolates of assemblages B, C, D and E (Fava et al., 2013).

5.3.4 Allelic sequence heterozygosity and mixed infection

The two aforementioned phenomenon (i.e. high degree of nucleotides variation and high heterogeneous nucleotide found in sequences of assemblage B) led us back to the focus of mixed infection, which is known to take place at both the inter-assemblage (e.g. assemblages A and B) and at the intra-assemblage levels (e.g. sub-assemblages AI and AII or among subtypes of assemblage B) (Caccio & Ryan, 2008). Mixed infection can happen when a host ingests *Giardia* cysts of different genetic profiles or subsequent infection of an infected host by genetically different *Giardia* cysts. This is especially common in areas where giardiasis is endemic (Caccio & Ryan, 2008; Sprong et al., 2009; Lebbad et al., 2011). With the use of the assemblage-specific assays in the present study, high prevalence (40.8%) of mixed infections was detected. This further proven

the biasness of using a standard PCR assay alone as the most plentiful assemblage would be amplified in preference (Wielinga & Thompson, 2007). However, in the present study, the feature of mixed infections could only be used to explain fraction of the inconsistent genotype results because only nine of the 27 discordant genotype isolates were verified as mixed infection using this assay.

In respect of heterogeneous nucleotides, regardless at which loci they were detected, majority of them were found at polymorphism site that either restricted to assemblage B only or at positions that contain nucleotide common to both assemblages A and B but show polymorphism within assemblage B. In view of the absence of heterogeneous nucleotides at polymorphism sites that define assemblages A and B as well as overall observation of the multiple sequence alignments which showed more intra-assemblage variations in assemblage B than A, it could be inferred that the heterogeneous nucleotides were occurred more likely due to intra-assemblage polymorphism/ mixed infection rather than inter-assemblage mixed infections, however it could not be determined due to the nature of this study.

Correspondingly, as the DNA was extracted directly from faecal samples without cysts isolation, the sequences variations isolated within a host might represent genetic profile of multiple cysts or a single cyst with divergent alleles. The first scenario could reflect inter-assemblage mixed infection that contributes to discordant genotypes and intra-assemblage mixed infections (mixed subtype infections) which can stand as a factor that causes the high polymorphism in assemblage B. As for the second scenario, given that a *Giardia* cyst contains two nuclei with full genome content, therefore, theoretically saying, a single gene could be presented by up to four alleles that carry either similar or different genetic settings (Helmy et al., 2014). And so, allelic sequence heterozygosity (ASH) that occurs between the two nuclei of single cyst could also represent genetic polymorphism of the parasite found in the present study.

In order to resolve the question whether the mixed genotype is due to mixed infections or ASH, Ankarklev et al. (2012b) had conducted an investigation on single *Giardia* trophozoite and cyst. The result based on molecular analysis positively exhibited the occurrence of ASH at single cell level. However, high numbers of mixed subgenotypes infections (demonstrated by variable sequence patterns) were also observed in different cysts isolated from the same sample indicating that mixed infection is also playing a role for the high heterogeneity.

There were currently three assemblages with full genome sequences i.e. assemblage A (WB), assemblage B (GS) and assemblage E (P15). When ASH at the genomic level is taken to account, it was found that genome sequence of the isolate GS contains relatively high degree of ASH i.e. 0.5% contradicts with WB and P15 which have ASH less than 0.01% (Jerlstrom-Hultqvist et al., 2010a; Jerlstrom-Hultqvist et al., 2010b). Considering the fact that *Giardia* has two nuclei cycle between ploidy of 2N and 4N, many deem the low level of allele sequence heterozygosity (ASH) i.e. less than 0.01% in the genome sequence WB and PG15 isolates as something surprising (Carpenter et al., 2012).

5.3.5 Debate related to genetic recombination

Giardia has long been thought to be primitively asexual due to the mode of trophozoite replication by binary fission and no observation of cell fusion reported. Since both nuclei contain full copy of the genome and are transcriptionally active, if a tetraploid *Giardia* reproduces asexually without recombination, neutral mutations are expected to accumulate independently over evolutionary time in alleles of different chromosome sets (Figure 5.1a) and generate high sequence divergence (Meselson's effect) (Andersson, 2012). Hence, the low ASH in isolates of WB (assemblage A) and PG 15 (assemblage E) which also indicates strong resemblances of alleles within the same nucleus and between sister nucleus could be an evidence that implies homogenization of genome by sexual recombination (Cooper et al., 2007; Andersson, 2012). Consistent with the identification of genes homologous to higher eukaryotes known to be involved in meiosis (Ramesh et al., 2005) and various forms of evidences from recent studies, it is thought that cryptic sexual reproduction or recombination might occur in certain stage of *Giardia*'*s* life cycle (Birky, 2010; Adam, 2011).

Sexual reproduction involves unification of chromosomes from two nuclei within the same cell (selfing) or two cells (outcrossing) to generate genotypes with alleles of new combinations (Birky, 2010). Of these two types of reproduction, outcrossing can occur between individuals of the same assemblage or different assemblages (Takumi et al., 2012). In 2008, Poxleitner et al. (2008) reported nuclear fusion in cyst of *G. duodenalis* and proposed the process which termed as diplomixis may function to exchange genetic material. The observations were further supported by Jirakova et al. (2012) who found linkage in nuclei of daughter cells and permit nuclear content to communicate. In addition, high rate of chromosomal genetic exchange between nuclei was observed by Carpenter et al. (2012). The authors also demonstrated that cysts are formed through incomplete mitotic division rather than fusion of two

trophozoites. Even though, outcrossing was not shown, these studies have presented evidences that low levels of heterozygosity could potentially be caused by genetic exchange during diplomixis in the cyst.

Reports from molecular epidemiological studies have also shown several evidences of recombination in the level of genetic diversity. In a population study that determine at what extent the variation occur within a lineage/single isolate and within a population, Teodorovic et al. (2007) examined nine isolates represents three medical relevant lineages (i.e. A1, A2 and B) at 10 loci and revealed extreme low intra- and inter-isolate genetic diversity in both coding and non-coding region especially among isolates group A1 and A2. In another study by Cooper et al. (2007), five samples isolated from an area of highly endemic for giardiasis were analysed at four loci from three chromosomes. All the isolates belonged to genotype A2 yet phylogenetic trees constructed based on different loci exhibited dissimilar clustering of the isolates. This could be an indication that the genes on different chromosome did not evolve in parallel resulting in different inheritance pattern. Both of these population genetics studies suggest that some form of sexual recombination exists in the organism contributed to the unexpected low level of genetic diversity and disparity in inheritance pattern of loci.

In the present multilocus study, sequences of 45 isolates that were amplified at all three loci also produced phylogenetic trees with different isolate clustering. For example isolates UL3.3 and DB11.7 were in one group with the cluster of assemblage B in *gdh* and *bg* but were formed one group with the cluster of assemblage A in *tpi.* Of note, both *tpi* and *gdh* trees yielded only two big clusters i.e. assemblages A and B while *bg* tree formed three clusters i.e. assemblages A2, A3 and B (Figure 4.3). Additionally, Teodorovic et al. (2007) has also identified alleles of assemblage A within assemblage B. This result suggests that occurrence of discordant genotypes and identification of mixed infection of assemblages A and B in the present and other

studies (Lasek-Nesselquist et al., 2010; Helmy et al., 2014) could possibly be due to inter-assemblage genetic exchange. It was assumed that rates of recombination could be higher in endemic areas because it is more likely for a single mammalian host to be infected by more than one genotype of *Giardia* and thus increases the chance of recombination (Cooper et al., 2007).

A model which is contradicted to the common assumptions has been put forward. With regards to the epidemiological data that shown ASH reflected in the form of heterogeneous nucleotides particularly among the isolates of assemblage B, Andersson (2012) proposes ameiotic crossing that has taken place in a more recent time either within nucleus (Figure 5.1b) or between nuclei (Figure 5.1c) before achieving homogenization is responsible for these observations. On the other hand, the low level of ASH among the isolates of assemblage A was regarded as a result of asexual reproduction that have been carried out long enough to purge out mutations and lose the allelic variation. Alternatively, instead of sexual recombination, a single clone that has reproduced and dispersed successfully in a population can potentially cause high resemblance between isolates.

The observations and questions such as the following raised by discordant genotypes, mixed infection, ASH and recombination have been summarized in Figure 5.2: (1) What is the frequency of the discordant genotyping results caused by ASH and true mixed infection (inter-assemblage)? Similarly, (2) What is the frequency of the heterogeneous nucleotides caused by ASH and true mixed infection (intra-assemblage)? (3) If ASH is introduced by independent accumulation of mutations of asexual reproduction (Meselson's effect), can low ASH be an indicator for sexual reproduction? Or (4) How true is that ASH which is especially high in isolates of assemblage B found in most of the molecular epidemiology studies are resulting from recent genetic

exchange as proposed by Andersson (2012)? (5) If recombination cannot be ruled out, how often does it occur at inter-assemblage and intra-assemblage levels?

Answers to these questions are important for understanding the epidemiology and aetiology of *Giardia* infection as it could impact the development of vaccines and management of the disease. In parasites that reproduces asexually, development of resistance to host immune response can only be introduced by new mutations, gene or chromosome rearrangements, or horizontal gene transfers (Birky, 2010). On the other hand, besides all the features mentioned above, sexual reproduction has the advantage of recombination which allows alleles re-assortment and reduction of deleterious mutations resulting in progeny that better adapt to adverse environment (Birky, 2010). The event of recombination could produce virulence that is different from parental cells (Andersson, 2012) and could have an effect in altering *Giardia* host specificity given that assemblages have different host ranges (Helmy et al., 2014).

Understanding the level of recombination also provides useful framework for the taxonomy of this parasite. By using the full genome sequences of GS, WB and P15, Xu et al. (2012) analysed inter-assemblages recombination and advocated the suggestion that *G. duodenalis* assemblages should be regarded as taxonomically distinctly species. The authors found very low frequency of inter-assemblage recombination and came out with this statement based on the species concept by De Queiroz (2007) which defines assemblages that involve genetic exchange as biological species while suggests that assemblages should be viewed as separate species if frequent gene flow take place within but not between. In conjunction with this, Takumi et al. (2012) found absence of recombination between assemblages after inspecting sequences amplified by three loci (i.e. *tpi, gdh,* and *bg*). These results were contradicted with finding by Lasek-Nesselquist et al. (2009) that reported inter-assemblages recombination.

However, even if sexual reproduction between assemblages seems to be a rare event, Baruch et al. (1996) proposed significant biological differences accompanied by genetic differences should be taken into considerations when assigning different assemblages of *Giardia* that mainly carry out clonal reproduction into separate species. This also reinforces that quick assignment of *Giardia* isolates to the specific genotypes will aid in determining of whether important biologically properties between the groups present.

The observations of the present study are consistent with the notion that the occurrences of ASH and mixed infections can present misleading signals towards understanding status of taxonomy and epidemiology. Until recently, many studies have adopted MLG as a discriminatory tool to explore the epidemiology and possible zoonotic transmission of *Giardia*. However, the standard approach of applying MLG and interpretation of the data generated by MLG still yet to be clarified. This is indubitably making the extensive comparisons of findings from various sources difficult. Furthermore, the great polymorphic profiles especially in assemblage B accompanied by the emerging evidence of sexual recombination has prompted the re-evaluation of molecular genotyping approach and inevitably challenge the effort to achieve a systematic nomenclature and its application in epidemiology of *Giardia*.

a)

b)

c)

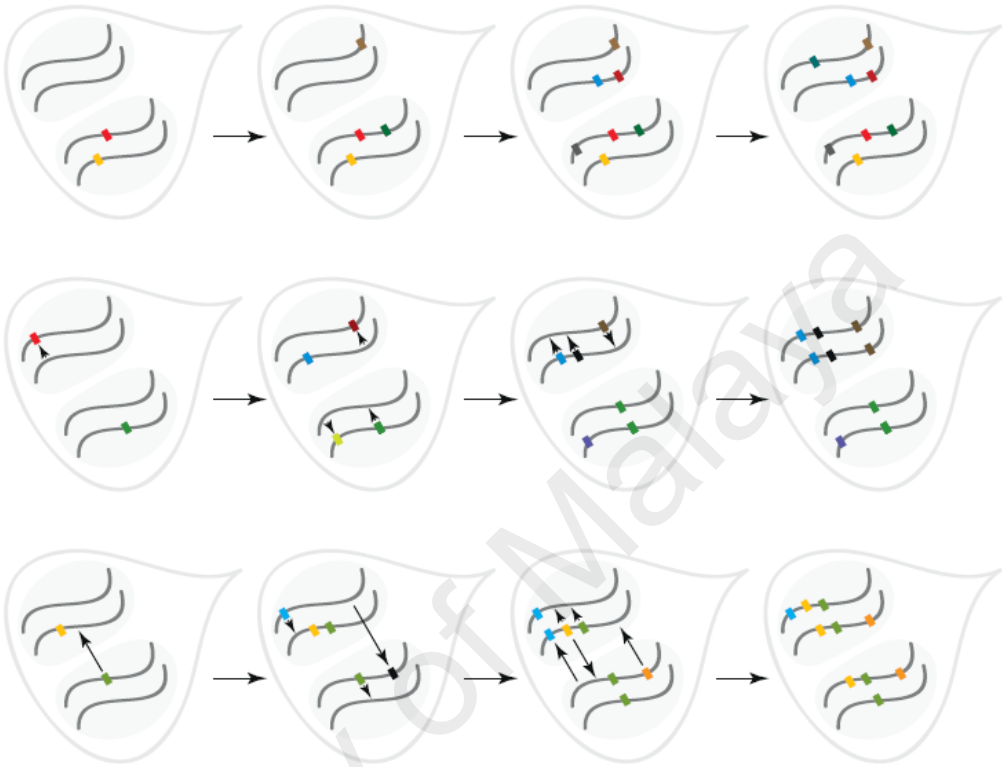

Figure 5.1: Events of recombination: a) Recombination is absent between alleles leading to high ASH. b) Ameiotic recombination between alleles of the same nucleus. c) Ameiotic recombination involving both of the nuclei.

Adapted from: Andersson (2012)

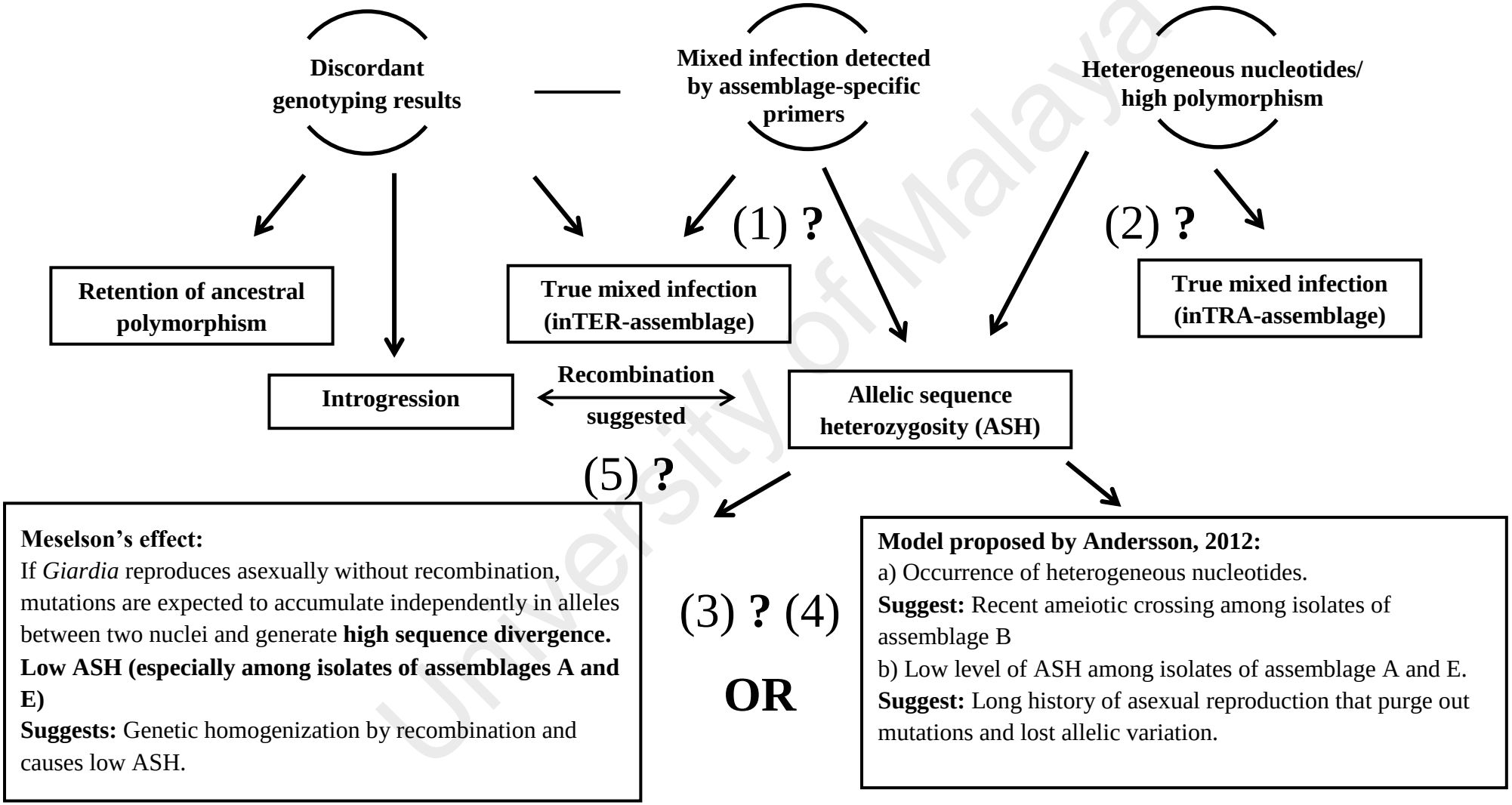

Figure 5.2: Relationship revolving the question of ASH and mixed infection

5.4 Population genetic study

The present study revealed that the level of genetic diversity was greater among the sequences of assemblage B compared to assemblage A for the Malaysian population. Comparison of Malaysian population with data from Thailand based on *bg* gene (Kosuwin et al., 2010) showed that showed that the nucleotide diversity was similar with Thailand i.e. 0.00235 vs. 0.00340 for assemblage A and 0.00433 vs. 0.00620 for assemblage B. Another study from Thailand based on *gdh* gene (Siripattanapipong et al., 2011) also showed significant higher nucleotide divergence (K) in assemblage B than assemblage A.

In the context of the overall worldwide comparison, assemblage B yielded the highest level of genetic diversity followed by assemblage E and assemblage A. Despite asexual reproduction having been generally regarded as the main reproduction mode for *Giardia*, the difference in genetic diversity are not clearly understood while some have suggested the different assemblages especially A and B to be considered as different species which also displayed some biological differences (Franzen et al., 2009; Xu et al., 2012).

In view of host specialisation, parasites with multiple host species are less likely to experience local extinction and susceptible hosts can be easily found in new areas, thus should have comparatively higher levels of genetic variation (Barrett et al., 2008). Although both assemblages A and B infect humans All of the three networks contained isolates derived from humans and a wide range of animal hosts, there is marked difference with regard to host-specificity within sub-structuring of assemblage. Assemblage A comprises sub-assemblages AI, AII and AIII with host preferences in livestock, humans and wildlife respectively (Sprong et al., 2009). Whereas for assemblage B, the classification of sub-assemblages BIII and BIV was not reproducible

and similar frequency was found in the distribution of the sub-genotype in human and animal hosts (Wielinga & Thompson, 2007; Sprong et al., 2009). While assemblage E infects only livestock animals, another possibility for the relative lower levels of genetic diversity in assemblage A and E could be due to the cycle of sub-assemblages A and assemblage E genotypes in limited hosts compared to assemblage B which has a broad host range and host-specificity in sub-assemblage that was not clearly exhibited.

The haplotype network analysis for assemblage A did not separate the isolates into networks according to geographical regions from where there were collected. It is worth noting that clustering of isolates into identified sub-assemblages (i.e. AI and AII) was obtained in the Malaysia population (Figure 4.4), but such clustering was not seen in the global population despite the formation of three distinct networks (Figure 4.6). All three of the networks contained isolates derived from human and animals. In addition, it was found that humans were sharing the same haplotype with a wide range of animals such as cat, dog, cattle, sheep, alpaca, gull, grey seal and dolphin.

A few studies (Chen et al., 2010; Villalobos et al., 2014) had shown that phylogenetic network analysis can recognize species and sub-clustering within species. Regarding why known sub-clustering was only seen in Malaysian population of assemblage A but not in the global population as well as in the network analysis of assemblage B, Hart and Sunday (2007) attributed the discordance between networks and taxa to several reasons such as inadequate sampling (e.g. sub-assemblage AIII), limited divergence, hybridization, cryptic speciation with undocumented phenotypic differences and incomplete lineage sorting. Similar to assemblage A, it was found that human and animal isolates of assemblage B were sharing the same haplotypes. On the other hand, animal isolates assemblage E did not share any haplotype with human. The sharing of the same haplotypes between animal and human may support the potential of zoonotic transmission of this parasite.

As one type of parasite, *Giardia* is closely tied with its host as it is only in the intestine of the host that this flagellate can proliferate by binary fission. Moreover, the dispersal ability of this food and waterborne parasites is dependent on the host in which the cysts are spread and continue the lifecycle at other places. From the distribution pattern of haplotypes in the network analysis, corroborated with observation of moderate gene differentiation and high gene flow across continents (Table 4.20), it was shown that *G. duodenalis* with similar genotype were well dispersed over the globe. In light of the rapid pace of globalization in sectors such as intercontinental travelling, migration, trading of livestock and agricultural activities, host dispersal can be a major determinant of parasite gene flow.

Blouin et al. (1995) had proposed host movement as the key role in gene flow of nematode parasites which will in turn provide great opportunity in the dispersal of rare alleles e.g. drug resistant or virulent mutant alleles. Assemblage B which had high genetic diversity also had higher level gene flow than assemblages A and E. Pathogens with high gene flow generally have higher genetic diversity than those with low level of gene flow because high gene flow increases the size of the population and the geographical area where the genetic material is present (Agrios, 2005). The survival strategy of *Giardia* that is the formation of resistant cysts, enables the spread of the parasite genome over long distances and large areas. This genome dispersal is a kind of gene flow known as genotype flow which occurs in asexual reproduction pathogen (with little or no recombination) where entire genotypes can be transmitted from one population to another (Agrios, 2005).

In the present study, the negative sign shown in the tests of neutrality, Tajima's *D* and Fu and Li's *D* signifies an excess of low frequency of polymorphism compared to the expectations under neutral processes such as mutation, genetic drift and population size equilibrium(Teodorovic et al., 2007). In addition, the unimodal displayed in the

mismatch distribution is an indication of populations that experience recent expansion. Populations that have been stable over time are expected to have a bimodal and multimodal mismatch distribution (Slatkin & Hudson, 1991; Rogers & Harpending, 1992; Schneider & Excoffier, 1999). However, the influence of selection could be locus-dependent as test for neutrality from other studies suggested that the *bg* gene was possibly influenced by ongoing purifying selection (Kosuwin et al., 2010) while the *gdh* gene was under neutral selection (Siripattanapipong et al., 2011).

On the other hand, if *Giardia* undergoes reproduction asexually as it is generally assumed, the population would be made up of independently evolving lineages and mutations are expected to confine to the lineage in which they began (Andras & Ebert, 2013), thereby the different rate of mutation with selection pressure might contribute to the excess polymorphism sites but low frequency of haplotype and limit its potential of evolution. Similar to the observation of high gene flow, recent expansion could have been promoted by human activities and host migrations.

As the population analyses were performed based on single gene, stronger inferences could be made if molecular data from multiple genes were included. In addition, it is well known that assemblages A and B can infect both humans and animals while assemblage E predominantly cause infection in livestock with a few rare cases reported in humans (Sprong et al., 2009; Helmy et al., 2014). Despite identical haplotypes being found shared by humans and animals, higher resolution of molecular data e.g. by combining sequences of different loci would be needed to provide sufficient evidence and understanding for the zoonotic potential of assemblages A and B. The complexity of life cycle, host specificity, modes of transmission and dispersal as well as the degree of recombination are the important history traits in shaping the population structure of microbial parasites (Tibayrenc et al., 1991; Criscione & Blouin, 2004; Barrett et al., 2008).

CHAPTER VI: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Indigenous communities particularly those living in Peninsular Malaysia (*Orang Asli*) are still vulnerable to parasitic infections in which *Giardia* infection is one of the most common protozoan infections reported among these communities. Studies since 1970s had reported a prevalence of 4.0-29.2%. However, diarrhoea caused by *Giardia* could have been underestimated in Malaysia either because the infected person does not seek medical care as the symptoms are mild and self-limiting or because of the non-availability of medical and laboratory facilities especially in rural areas. The present cross-sectional epidemiological study aimed at identifying and analysing human *Giardia* infections among indigenous communities in Malaysia.

In conclusion, the findings reveal that the prevalence of *Giardia* infection is still high (11.6%) and of public health concern among indigenous populations in rural Malaysia. The prevalence was found to be higher among the aboriginal population in West Malaysia (13.6%) when compared to the indigenous people in East Malaysia (5.8%). It was most commonly found among children and those living in poor sanitation conditions who also had poor standards of personal hygiene. The lower prevalence of *Giardia* among indigenous people in East Malaysia could be attributed to their better socioeconomic as well as hygiene status as they generally receive higher level of education, earn higher household income and live in better housing environment. Majority of *Giardia*-positive individuals were asymptomatic (83.9%) and they may contribute to difficulties in the disease prevention and control programmes.

Multilocus genotyping was conducted to analyse the genetic diversity of *Giardia* infection among the studied indigenous communities. The molecular findings of the

current study showed that assemblages A and B have equal frequency of infections (69/138). Gastrointestinal symptoms such as diarrhoea, vomiting, abdominal pain, and dysentery were reported by symptomatic individuals but the difference in prevalence that is 18.8% and 14.5% in assemblages A and B respectively was not significant. The occurrence of mixed infections was 31.8% and the prevalence of mixed infections was 38.5% in symptomatic individuals and 30.5% in asymptomatic individuals. The presence of anthroponotic assemblages and sub-assemblages (B and AII) in this study suggest that human as a potential source of the infection and the mode of transmission in Malaysia may be human-to-human. No specific geographical clustering patterns were seen from the analysis of the *Giardia* variants obtained at all loci as shown by the phylogenetic analysis as well as the haplotype network analysis. Genetic heterogeneity, mixed infection, allelic sequence heterozygosity (ASH) as well as the concern regarding potential of recombination had contributed to the complexity of *Giardia* epidemiology.

In general, high gene flow was observed in all of the assemblages and this could pose a threat in the transmission of the parasites especially the spread of the virulent alleles. The different genetic diversity observed in assemblages A, B and E could be due to their different evolutionary patterns especially between lineages of assemblages A and E with assemblage B. The departure from neutral expectations from the test results of Tajima's *D* and Fu and Li's *D* can be the consequence of population expansion after a bottleneck event or the presence of purifying selection. These findings provide fundamental evolutionary information of *G. duodenalis* and enhance our understanding of the dynamics and distribution of *Giardia* infection.

6.2 Recommendations

1) Different control measures are required in order to combat current levels of infection. These include health education pertinent to good personal hygiene and good sanitary practices, as well as education aimed at improving general awareness about parasitic infections. Besides which, if re-allocation to new settlements is not possible, providing proper sanitation, as well as making provisions for clean and safe drinking water, are crucial for maintaining the health of indigenous communities in West Malaysia.

2) However, instead of solving the problem by stopgap and piecemeal measures, ministries of different portfolio i.e. Ministry of Rural and Regional Development (particularly JAKOA), Ministry of Health and Ministry of Education should work in a highly collaborative way to put the aforementioned approaches in place, identify what works and what does not work and maximize the resources and research outcomes such as the present work in empowering the minority communities. The process to eradicate or to reduce parasitic infection might be slow but it should be planned systematically and done as a whole.

3) The general population in Malaysia still hold the perception that the *Orang Asli* are the underprivileged groups with low economic and educational level and tend to marginalize them in the society. The focus of the effort to improve health, social and economic status should not be limited to government and the indigenous people. The public should be made aware about the existence of this minority group and cognizant of their disadvantaged socio-economic condition. Instead of treating them with discrimination, the public should share the responsibility to work with the *Orang Asli* to achieve success in leading a life of better quality by accepting them to the society, offering job opportunities and promoting their unique culture. The indigenous people in Sabah and Sarawak (East Malaysia) are proud of showcasing their unique culture and

tradition. The way they benefit from this uniqueness in cultural tourism is an excellent example to follow.

6.3 Perspective future studies

Although no outbreak of *Giardia* infection was recorded in Malaysia, the prevalence of as high as 41% in one of the villages under study could imply a serious health threat and imperil the growth and development of people in the village. A comprehensive research liken the investigation of an outbreak could be carried out by using the existing information found by the present and other published studies as preliminary data. Several components were recommended below to be considered in the design of further epidemiological studies in order to improve the utility of data collected from the studies.

1) Investigation in communities where *Giardia* infection were found to be high can incorporate human samples with the environmental samples such as water, soil and animal samples from localized geographical areas to help us gain in-depth understanding about the dynamic of *Giardia* transmission.

2) Molecular analyses adopting multilocus genotyping such as the present study can be an appropriate approach to elucidate the source of *Giardia* infection and the potential or if present, the extent of zoonotic transmission occurs in the community. However, sensitivity, specificity and detection of mixed infection can be further improved by using a more advanced approach such as real time PCR.

3) Application of molecular study can also help in understanding the correlation between variations in genetic background with phenotypic variations. Studies to investigate the contribution of different genotypes to symptomatic giardiasis can be done by examining clinical samples, i.e. collect diarrhoeic samples from the local clinic if association of genotypes with symptomology is to be studied.

4) Besides, a longitudinal study e.g. multiple sampling from the same group of people can be considered to investigate virulence, drug sensitivity and infectivity of *Giardia* infection with regards to the occurrence of different genotypes and answer questions such as: How frequent are reinfection happened in the endemic areas? Does the reinfections caused by the same genotype or mixed infections? Are the infected persons always present with the same symptoms? Investigations to answer these questions reinforce the importance of using molecular tools to trace the relative frequency of each genotype and determine the modes of transmission.

5) To study whether *Giardia* infection and transmission is related to host factors, assessment about immunological and nutritional status of the hosts either human participants or animal hosts can be included. In addition, burden of the disease especially on the physical growth, cognitive development and impacts on economic and standard of life advancement is also an important area to be explored.

6) A study to evaluate the knowledge, attitudes and practices (KAP) of the community members towards the infection can be performed with a view that extensive evaluation to recognize the root cause of the problem would assist in formulating an effective prevention program. For instance, if the problem of the infection derived from their behaviour and lifestyle, greater emphasis should be put in providing practical and continuous education to raise awareness and encourage them to change their perception and practices. If the problem was due to other underlying social factors such as poverty, living environments and lacking of access to education and medical care, greater effort in providing basic facilities and employment opportunities should be prioritized.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Journal publications

Phd Project:

1) Seow Huey Choy, Hesham M. Al-Mekhlafi, Mohammed A. K. Mahdy, Nabil N. Nasr, Maria Sulaiman, Yvonne A. L. Lim, Johari Surin (2014). Prevalence and Associated Risk Factors of *Giardia* Infection among Indigenous Communities in Rural Malaysia. Scientific Reports, 4:6909

2) Choy Seow Huey, Mohammed A K Mahdy, Hesham M Al-Mekhlafi, Nabil A Nasr, Yvonne A L Lim, Rohela Mahmud, Johari Surin (2013). Multilocus genotyping of *Giardia duodenalis* in Malaysia. Infection, genetics and evolution, 17.

3) Seow Huey Choy, Mohammed A. K. Mahdy, Hesham M. Al-Mekhlafi, Van Lun Low, Johari Surin (2015). Population expansion and gene flow in *Giardia duodenalis* as revealed by triosephosphate isomerase gene. Parasites & Vectors, 8:454

Other Relevant Projects:

1) Soo Ching Lee, Mei San Tang, Yvonne A L Lim, Seow Huey Choy, Zachary D Kurtz, Laura M Cox, Uma Mahesh Gundra, Ilseung Cho, Richard Bonneau, Martin J Blaser, Kek Heng Chua, P'ng Loke (2014). Helminth colonization is associated with increased diversity of the gut microbiota. PloS Neglected Tropical Diseases, 8 (5):e2880.

2) Mohammed A K Mahdy, Yvonne A L Lim, Romano Ngui, M R Siti Fatimah, Seow H Choy, Nan J Yap, Hesham M Al-Mekhlafi, Jamaiah Ibrahim, Johari Surin (2012). Prevalence and zoonotic potential of canine hookworms in Malaysia. Parasites & Vectors, 5:88.

3) Abdulhamid Ahmed, Hesham M Al-Mekhlafi, Seow Huey Choy, Init Ithoi, Abdulelah H Al-Adhroey, Awatif M Abdulsalam, Johari Surin (2011). The burden of moderate-to-heavy soil-transmitted helminth infections among rural Malaysian aborigines: an urgent need for an integrated control programme. Parasites & Vectors, 4:242.

Manuscript:

1. NMN NikNadia, I-Ching Sam, Nasibah Khaidir, Romano Ngui, Yvonne A. L. Lim, Xiang Ting Goh, Seow Huey Choy, Yoke Fun Chan. Risk Factors for Enterovirus A71 Seropositivity in Indigenous Populations in West Malaysia Running title: Risk factors for Enterovirus A71 in indigenous populations

Manuscript under Review: The American Journal of Tropical Medicine and Hygiene

Conference proceedings

1) S.H. Choy, M.A.K. Mahdy, H.M. Al-Mekhlafi, N.A. Nasr, Y.A.L. Lim, J. Surin. Genetic characterisation of *Giardia duodenalis* among Malaysian aboriginal populations using a multilocus genotyping approach, 23rd European Congress of Clinical Microbiology and Infectious Diseases, European Society of Clinical Microbiology and Infectious Diseases, 27 Apr 2013 to 30 Apr 2013 (Poster)

2) Seow Huey Choy, Mohammed A.K. Mahdy, Romano Ngui, Johari Surin, Siti Fatimah Binti Muhd Radzi, Yap Nan Jiun, Hesham M. Al-Mekhlafi and Yvonne A.L. Lim. Molecular characterization of canine hookworm isolated from urban and rural dogs in Malaysia., 48th Annual Conference of the Malaysian Society of Parasitology and Tropical Medicine (MSPTM). 27 Mar 2012 to 28 Mar 2012 (Oral)