HUMAN GIARDIASIS IN MALAYSIA: CORRELATION BETWEEN THE PRESENCE OF CLINICAL MANIFESTATION AND *GIARDIA INTESTINALIS* ASSEMBLAGE

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Abstract. Clinical manifestations of giardiasis vary from asymptomatic infection to chronic diarrhea. A total of 611 stool samples from Aboriginal participants residing in Jelebu, Gerik and Temerloh States, Malaysia, ages 2 to 74 years were screened for *Giardia intestinalis* using microscopic examination and sequence analysis of a fragment of nested-PCR amplified triosephosphate isomerase (*tpi*) gene. Demographic data was collected through a structured questionnaire. *tpi* was successfully amplified from 98/110 samples microscopically positive for *G. intestinalis*, with 62 and 36 belonging to assemblage A and B, respectively. There is a significant correlation between assemblage A and symptomatic infection only in participants of < 15 years of age. In the other age group, host factors may have more effects on the presence of clinical signs and symptoms than *G. intestinalis* assemblage types.

Keywords: *Giardia intestinalis*, assemblage, children, diarrhea, Malaysia

INTRODUCTION

Giardia intestinalis is one of the major causes of diarrhea in humans worldwide, responsible for an estimated $2.8x10^8$ cases per year (Lane and Lloyd, 2002). The infection is transmitted by fecal-oral route through ingestion of infective cysts. Prevalence of the infection is higher in developing countries as poor sanitary conditions in these regions favor contamination of water and food with *G. intestinalis* cysts. However, factors determining the variability in clinical infection of giardiasis are still poorly understood. Approximately $2x10^8$ people have symptomatic giardiasis in Latin America, Africa and Asia, and about 500,000 new cases are reported each year (Pestechian *et al*, 2014). Although this disease is usually self-limiting and not life threatening, *G. intestinalis* infection

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in young children has been correlated to stunted growth and poor cognitive function (Al-Mekhlafi *et al*, 2005).

G. intestinalis is a complex species comprising of eight assemblages (A-H), of which assemblages A and B are considered potentially zoonotic strains as they infect several mammalian species, including humans, companion animals livestock, and wildlife (Caccio and Ryan, 2008). Assemblages C and D infect domestic and wild canids, and assemblage E, F, G and H infects hooved livestock, cat, rodents and seal, respectively (Feng and Xiao, 2011). Several genetic loci have been described for determining assemblages of *G. intestinalis* isolates including genes of triosephosphate isomerase (*tpi*) (Bertrand *et al*, 2005), glutamate dehydrogenase (*gdh*) (Read *et al*, 2004), β-giardin (*bg*) (Lalle *et al*, 2005) and small subunit ribosomal RNA (ssu-rDNA) (Hopkins *et al*, 1997). A multi-loci genotyping approach has been described to assign an assemblage type to a particular *G. intestinalis* isolate (Huey *et al*, 2013).

Studies on the association of *G. intestinalis* genotypes with the spectrum of symptoms associated with giardiasis have been inconclusive (Rafiei *et al*, 2013). Different spectra of symptoms are apparently associated with different assemblages in different populations (Robertson *et al*, 2010). A recent study from Cuba reported prevalence of positive cases in children is 40% for assemblage A and 42% for assemblage B using *tpi* as molecular marker, and that children in the latter group are more likely to have symptomatic infections than those in the former group (Puebla *et al*, 2014). In Malaysia, *G. intestinalis* has been identified as one of the most common intestinal parasites among the aboriginal population, with an overall prevalence of giardiasis of 20% (Anuar *et al*, 2012). Thus far, only one study has been conducted in Malaysia to assess *G. intestinalis* assemblages and clinical signs and symptoms (Mohamed Mahdy *et al*, 2009).

The present study determined whether there is any association between *G. intestinalis* assemblage and the presence of clinical manifestation in participants.

MATERIALS AND METHODS

Study areas

The study was carried out from June to December 2011 in three different states of Malaysia: Jelebu (2° 55′ N latitude, 102° 4′ E longitude), Gerik (5° 26′ N latitude, 101° 7′ E longitude) and Temerloh (3° 43′ N latitude, 102° 22′ E longitude). These states have a similar tropical rainforest climate, being hot and humid throughout the year.

Sample size and sampling strategy

The villages are occupied by Aboriginal people who belong to tribes, *eg*, Proto-Malay (Jelebu), Negrito (Gerik) and Senoi (Temerloh) (Anuar *et al*, 2012). Sample selection was achieved using a two-stage sampling method; namely, random selection of village and random selection of 10 to 15 households per village. Based on an expected minimum prevalence of *G. intestinalis* in the study area of 20% (Anuar *et al*, 2012), with 95% confidence interval and an absolute precision of 0.05 (Lwanga and Lemeshow, 1991), the appropriate sample size for the study was estimated to be 246 subjects. Within each village, subjects ≥ 2 years of age and those who provided consent to participate were included in this study. Exclusion criteria included children < 2 years old and refusal to participate. All village entry had been approved by the Ministry of Rural and Regional Development of Malaysia. Prior to data and stool collections, the study protocol (reference

no. UKM 1.5.3.5/244/FF-165-2011) was reviewed and approved by the Ethics Committee of Universiti Kebangsaan Malaysia Medical Centre (UKMMC) and permission for field works were obtained from the Ministry of Rural and Regional Development, Malaysia before the commencement of the study. At the conclusion of the study, each participant confirmed with *G. intestinalis* infection was treated, free of charge, with metronidazole according to guidelines of the Ministry of Health, Malaysia (MOH, 2014).

Questionnaire survey

The rationale and procedures of the study were explained to the participants and an informed consent sheet was signed by the head of the household or a designated literate substitute. A trained research team interviewed each subject using a previously tested, structured questionnaire that sought information on the following: (i) demographic data, (ii) socioeconomic background, (iii) behavioral risks, (iv) environmental sanitation and characteristics of living condition, (v) close contact with household pets, and (vi) health conditions with history of symptoms. Diarrhea was defined as unusually loose stool occurring ≥ 3 times/day for both children and adults (WHO, 1988). For children, the questionnaire was completed by interviewing their parents or guardians, who signed the informed consent form.

Stool samples collection and parasitological examination

All participants were requested to provide a stool sample (~10 g) in a wide mouth screw-capped container pre-labeled with their names and coded for both microscopic examination and molecular analysis. Approximately 5 g of each stool sample was stored in 3 ml of polyvinyl alcohol. A portion of stool sample was subjected to Wheatley's trichrome staining (Salleh *et al*, 2012), while another portion was kept unfixed and stored at 4°C upon arrival at the laboratory for PCR analysis. Samples were considered microscopically positive if *G. intestinalis* cysts and/or trophozoites were detected by at least one of the two techniques and negative if absent by both techniques.

Nested-PCR amplification of *G. intestinalis tpi*

Genomic DNA was extracted from stool samples using QIAamp Stool DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was stored in 70 µl of elution buffer at -20°C until used. A partial sequence of *tpi* (530-bp) was amplified using nested-PCR as described previously (Sulaiman *et al*, 2003). Primary PCR was performed using primers AL3543 (5'-AAA TIA TGC CTG CTC GTC G-3') and AL3546 (5'-CAA ACC TTI TCC GCA AAC C-3'), and secondary PCR with primers AL3544 (5'-CCC TTC ATC GGI GGT AAC TT-3') and AL3545 (5'-GTG GCC ACC ACI CCC GTG CC-3'). Primary and secondary PCRs were performed in a 50-µl solution comprising 2 µl of DNA, 0.2 µM each primer, 1 U HotStarTaq® *Plus* DNA polymerase (Qiagen, Hilden, Germany), 1X PCR buffer, 200 µM dNTPs, 1.5 mM $MgCl_2$ and 0.2 mg/ml bovine serum albumin. Thermocycling (in an Eppendorf Pro-S thermal cycler, Hamburg, Germany) conditions were as follows: 95°C for 5 minutes; followed by 35 cycles of 94°C for 45 seconds, 50°C (primary PCR) or 58°C (secondary PCR) for 45 seconds, 72°C for 60 seconds; and a final heating at 72°C for 10 minutes. Amplicons were electrophoresed in 1.5% agarose gel and stained with GelRed (Biotium, Hayward, CA). Genomic DNA positive for *G. intestinalis* was used as

Table 1
Microscopic examination and nested-PCR assay of *G. intestinalis* in stool samples from Aboriginal subjects, Malaysia.

Microscopic examination	Nested-PCR assay				
	Assemblage A	Assemblage B	Mixed infection	Negative	Total
Positive	62	36	0	12	110
Negative	0	0	0	501	501
Total	62	36	0	513	611

positive control and distilled water as negative control.

DNA sequencing

G. intestinalis-positive amplicons were purified using SolGent™ kit (Solgent, Daejeon, South Korea) and sequenced in both directions using the same primer sets of the secondary PCR assay in an ABI 3730XL sequencer (Applied Biosystems, Carlsbad, CA). Sequences were assembled using BioEdit Sequence Alignment Editor Program (http://mbio.ncsu.edu). Sequences were deposited in GenBank (accession nos. KT357495 and KT357496).

Phylogenetic analysis

Similarity comparisons of the sequences obtained with those in GenBank database were made using BLAST (http://blast.ncbi.nlm.nih.gov). Sequences were genotyped into assemblage using multiple alignments implemented by ClustalW (Thompson *et al*, 1994) with previously defined reference sequences retrieved from GenBank database (Sulaiman *et al*, 2003). Phylogenetic analysis was performed in MEGA 5 (www.megasoftware.net) using neighbor-joining algorithm with evolutionary distances calculated by Kimura-2-parameter method (Read *et al*, 2004) and 1,000 bootstrap value. Sequences of *G. ardae* (AF069564) and *G. muris* (AF069565) were used as out group as the construction on an unrooted tree showed them to be the most divergent members.

Statistical analysis

Data were analyzed using SPSS program for Window version 20.0 (IBM, Armonk, NY). Chi-square was used to assess the association between dependent and independent variables. Significance of the difference is defined as p-value < 0.05.

RESULTS

Characteristics of the study population and parasitological status

A total of 611 participants provided stool samples, of whom were 266 males and 345 females, and 277 (45%) were < 15 years of age and 334 ≥ 15 years old, with a median age of 18 years (interquartile range of 9-34 years). A total of 110 (18%) samples were microscopically positive for *G. intestinalis* cysts and/or trophozoites only (27 samples) and the remaining in combination with other intestinal parasites (Table 1). Of the *G. intestinalis*-positive stool samples, 59 (54%) were present with signs and symptoms of giardiasis, *eg*, diarrhea, abdominal pain, fever, nausea, vomiting, flatulence, loss of appetite and loss of weight. Five such subjects were co-infected with other pathogenic parasites, such as *Entamoeba histolytica/dispar*, *Trichuris trichiura*, *Ascaris lumbricoides* and hookworm.

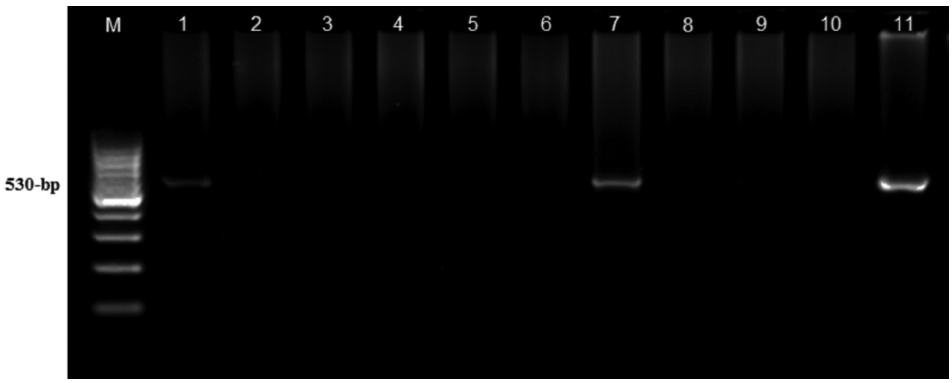

Fig 1–Electrophoretic separation of *G. intestinalis tpi* nested-PCR amplicons. Experimental protocols are described in Materials and Methods. Lane M, 100-bp DNA size markers; lane 1, *G. intestinalis*-positive control; lanes 2-7 and 11, stool samples; lanes 8-10, negative controls.

Genetic characterization and phylogenetic analysis

From 110 *G. intestinalis*-positive stool samples, nested-PCR assay showed the expected amplicon (530 bp) of *G. intestinalis tpi* from 98 (89%) samples (representative results shown in Fig 1). To determine the assemblage of *G. intestinalis*, *tpi* amplicons were sequenced and compared with all homologous sequences representing different *G. intestinalis* assemblages. Sixty-two samples belonged to assemblage A and 36 to assemblage B, with no mixed assemblages (Table 1). Neighbor-joining tree placed four representative sequences for assemblage A (AF069556, AF069557, L02120 and U57897) in one cluster with high bootstrap value. Phylogenetic analysis also confirmed the monophyletic group of assemblage B (bootstrap = 100%) (Fig 2).

Correlation between presence of clinical manifestations and assemblages

Clinical manifestations of *G. intestinalis* infection varied greatly, ranging from the absence to moderate, with diarrhea being the most common sign (35/59 subjects). For subjects < 15 years of age with symptomatic giardiasis, 77% of these were infected with *G. intestinalis* assemblage A and 23% with assemblage B ($p = 0.006$), but no correlation is seen with asymptomatic subjects (Table 2). There is no statistically significant correlation in symptomatic or asymptomatic giardiasis subjects from 15 to 74 years of age.

DISCUSSION

Human giardiasis is caused by two distinct genetic groups of *G. intestinalis* known as assemblages A and B, which are likely to represent distinct species (Monis *et al*, 2009; Jerlstrom-Hultqvist *et al*, 2010). In this study, the characterization of *G. intestinalis* from stool samples by nested-PCR and sequencing a fragment of *tpi* was successful in 89% of the samples. A number of factors can influence the success of DNA amplification, such as presence of DNA polymerase inhibitor(s) in stool, cyst quantity and quality, sample storage conditions, DNA isolation technique, type of gene target (number of copies, polymorphism) and choice of primers and thermocycling conditions (Wilke and Robertson, 2009).

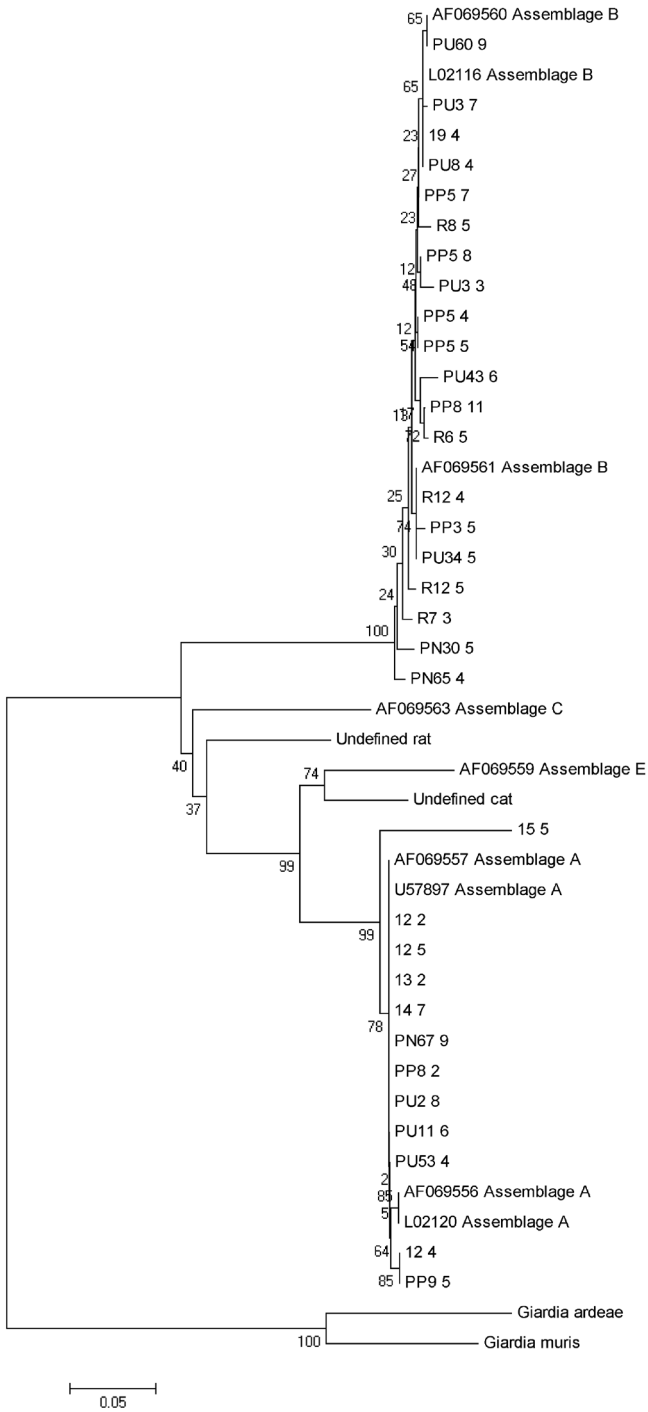

Fig 2–Phylogenetic tree of *G. intestinalis* assemblages constructed by neighbor-joining analysis, based on partial *tpi* nucleotide sequences from this study compared with reference sequences of known assemblages deposited in GenBank. Percent bootstrap values obtained from 1,000 replicates are indicated on the branches.

Table 2
Correlation between *G. intestinalis* assemblage, subject's age and symptomatic or asymptomatic infection among Aboriginal population, Malaysia.

Infection	Subject < 15 years old		Subject ≥ 15 years old	
	A[a]	B	A	B
Symptomatic	24[b]	7[b]	19	9
Asymptomatic	7	11	12	9
Total	31	18	31	18

[a]Assemblage. [b]Significant difference, $p = 0.006$.

The distribution of *G. intestinalis* assemblages A and B among the 98 *G. intestinalis*-positive samples in the present study differ considerably from that observed in previous study, in which assemblage B is more frequent than assemblage A (98% *vs*, 2%, respectively) among the Aboriginal populations in Pahang, Malaysia (Mohammed Mahdy *et al*, 2009). However, the two studies cannot be compared strictly as different target genes were used and the test populations were different.

However, the present findings confirmed data previously reported from Spain of a correlation between assemblage A and symptomatic giardiasis infection in patients < 5 years of age (Sahagun *et al*, 2008). These findings are in agreement with those of Read *et al* (2002) and Haque *et al* (2005), who reported the same types of correlation in children. The reason usually given to explain the susceptibility of this age group is the immaturity of the immune system (Katelaris and Farthing, 1992). We postulate that in adults, differences in participant status, such as degree of host adaptation, nutritional and immune conditions may have a greater impact on the clinical manifestations of *G. intestinalis* infection than assemblage types. There also exists the possibility of the role of other enteric pathogens not screened in this study.

In conclusion, to the best of our knowledge, this is the second study to provide information on assemblages associated with human giardiasis infection, but is the first study to compare the relationship between clinical manifestations and assemblage in *G. intestinalis* infections in participants under and ≥15 years of age. The association of *G. intestinalis* assemblage A infection with gastroenteritis symptoms has been confirmed as statistically significant only in children under 15 years of age. This has not been shown in any other study in Malaysia. However, further studies need to be carried out to explain these observations.

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