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Multilocus genotyping of *Giardia duodenalis* in Malaysia

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ABSTRACT

Giardia duodenalis is considered the most common intestinal parasite in humans worldwide. In Malaysia, many studies have been conducted on the epidemiology of giardiasis. However, there is a scarcity of information on the genetic diversity and the dynamics of transmission of *G. duodenalis*. The present study was conducted to identify *G. duodenalis* assemblages and sub-assemblages based on multilocus analysis of the glutamate dehydrogenase (*gdh*), beta-giardin (*bg*) and triose phosphate isomerase (*tpi*) genes. Faecal specimens were collected from 484 Orang Asli children with a mean age of 7 years and examined using light microscopy. Specimens positive for *Giardia* were subjected to PCR analysis of the three genes and subsequent sequencing in both directions. Sequences were edited and analysed by phylogenetic analysis. *G. duodenalis* was detected in 17% (84 of 484) of the examined specimens. Among them, 71 were successfully sequenced using at least one locus. Genotyping results showed that 30 (42%) of the isolates belonged to assemblage A, 32 (45%) belonged to assemblage B, while discordant genotype results were observed in 9 specimens. Mixed infections were detected in 43 specimens 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. The finding of assemblage B and the anthroponotic genotype AII implicates human-to-human transmission as the most possible mode of transmission among Malaysian aborigines. The high polymorphism found in isolates of assemblage B warrants a more defining tool to discriminate assemblage B at the sub-assemblage level.

1. Introduction

Giardia duodenalis (syn. *G. intestinalis*; *Giardia lamblia*) is a flagellate enteric parasite that infects the intestinal tract of humans and a wide variety of other mammals through ingestion of infective cysts (van der Giessen et al., 2006). It is a major cause of non-viral/bacterial diarrhea affecting both developed and developing countries and more frequently encountered in areas with substandard environment, poor hygienic practices and inadequate water treatment system (Daly et al., 2010; Robertson et al., 2009). *G. duodenalis* infections are most often asymptomatic. For symptomatic cases, the degree of symptoms and severity varies among different individuals (Buret, 2008).

Knowing the genetic diversity of *Giardia* is an essential component in enhancing our understanding on the taxonomy, epidemiology and population genetics of this parasite. Therefore, 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 and Xiao, 2011). Molecular analyses revealed that *G. duodenalis* isolated from humans and animals is a 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 these, 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 (Feng and Xiao, 2011; Nolan et al., 2010; Sprong et al., 2009; Thompson et al., 2000).

In Malaysia, giardiasis is an endemic disease and is associated with malnutrition among children in the rural areas resulting in stunting, wasting and vitamin A deficiency (Al-Mekhlafi et al.,

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2010, 2005). The prevalence of human *Giardia* infection varies between 0.2% and 29.2% (Lim et al., 2008; Noor Azian et al., 2007). Most of the epidemiological studies detected *Giardia* on the basis of microscopic examination without employing molecular approach. Data on genotypes of *G. duodenalis* up to the assemblage level remains scarce. In a previous genotyping study using SSU rRNA locus, one specimen was identified as assemblage A in 42 specimens and the rest were assemblage B (Mahdy et al., 2009). In a study on immunocompromised patients, assemblage A was identified in four of the microcopy-positive *Giardia* specimens using *tpi* gene (Lim et al., 2011). Assemblage A was also isolated from environmental samples including recreational lake water and water bodies in a zoo (Lim et al., 2009a,b). In addition, genotyping study was conducted on animals and assemblages A and E were detected among goats (Lim et al., 2013). However, subtyping of assemblages A and B in the previous studies was not conducted which limit our understanding on the transmission dynamics and the source of infection of giardiasis in this country. Thus, the present study was aimed to identify *G. duodenalis* assemblages and sub-assemblages based on multiloci genes which included *gdh*, *bg* and *tpi* genes to attain better understanding of the genetic diversity and transmission of giardiasis in Malaysia. The study also aimed at determining the occurrence of mixed infections using primers targeting *tpi* gene specific for assemblages A and B.

2. Materials and methods

2.1. Source of samples

A total of 484 children (51% males, 49% females) with the mean age of 7 years were involved in the present study. The sample collection was conducted from April to September 2011 in 13 Orang Asli villages located in Lipis district, Pahang state, Peninsular Malaysia. The study protocol was approved by The Medical Ethics Committee of University of Malaya Medical Center, Kuala Lumpur, Malaysia under the MEC Ref. No.: 788.74. Informed consents were obtained from the participants' parents or guardians prior to the collection. Single sample was collected from each participant. Upon receipt, the samples were transferred to the Department of Parasitology, University of Malaya, preserved in 2.5% potassium dichromate and stored in cold room (4 °C) until further analysis.

2.2. Microscopy

The faecal samples were concentrated based on formal-ether technique. Briefly, a small amount of stool samples (pea size) was emulsified with 7 ml of 10% formalin and sieved through a double-layered gauze and collected in a beaker. The suspension was transferred into 15 ml conical centrifuge tube and topped up with three ml of diethyl ether. The centrifuge tube with the suspension was capped and mixed by shaking before centrifuging for 5 min at 2500 rpm. The top three layers (ether, debris and formalin water) were removed and the sediment was examined by light microscope under x100 and x400 magnification for the presence of *Giardia* cysts and other intestinal parasites after staining with iodine.

2.3. Molecular analysis

DNA was extracted directly from samples positive for *Giardia* cyst using PowerSoil®DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, California) following the manufacturer's instructions. 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.

A partial sequence of *gdh* gene (≈432-bp) was amplified using semi-nested PCR as described by Read et al. (2004). For primary PCR, the forward primer GDHeF (5′-TCA ACG TYA AYC GYG GYT TCC GT-3′) and the reverse primer GDHiR (5′-GTT RTC CTT GCA CAT CTC C-3′) were used. In the secondary PCR, the forward primer GDHiF (5′-CAG TAC AAC TCY GCT CTC GG-3′) and the reverse primer GDHiR were used. Primary and secondary PCR reactions were performed in a 50 μl PCR master mix comprising 0.5 μM of each primer (Bioneer Q-Oligos, Korea), 2.5 U of HotStarTaq® *Plus* DNA Polymerase (Qiagen, Hilden, Germany),1× PCR buffer (Qiagen, Hilden, Germany), 200 μM of dNTP (Fermentas, Ontario, Canada), 1.5 mM $MgCl_2$ (Qiagen, Hilden, Germany), 5% dimethyl sulfoxide (Sigma–Aldrich, USA) and 0.4 mg/ml BSA (New England Biolabs, Ipswich, USA). 2 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 at 95 °C for 5 min, 1 cycle at 94 °C for 2 min, 56 °C for 1 min and 72 °C for 2 min, followed by 55 amplification cycles at 94 °C for 30 s, 56 °C for 20 s, 72 °C for 45 s, and a final extension at 72 °C for 7 min. For secondary PCR, the number of cycles was reduced to 33.

A partial sequence of *tpi* (≈530-bp) was amplified using nested-PCR protocol according to Sulaiman et al. (2003). 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. Primary and secondary PCRs were performed in a 50 μl PCR mix comprising 0.2 μM of each primer (Bioneer Q-Oligos, Korea), 1 U of HotStarTaq® *Plus* DNA Polymerase (Qiagen, Hilden, Germany), 1× PCR buffer (Qiagen, Hilden, Germany), 200 μM dNTP (Fermentas, Ontario, Canada), 1.5 mM $MgCl_2$ (Qiagen, Hilden, Germany), and 0.2 mg/ml BSA (New England Biolabs, Ipswich, USA). 2 μl of DNA template were used and the prepared master mix was incubated in the MyCycler thermal cycler (Bio-Rad, Hercules, USA) under the following conditions: Initial hot start at 95 °C for 5 min, 35 amplification cycles at 94 °C for 45 s, 50 °C for 45 s (58 °C for secondary PCR), 72 °C for 60 s and a final extension at 72 °C for 10 min.

In addition, the first PCR product of the reaction described by Sulaiman et al. (2003) underwent further amplification using a set of separate A (Geurden et al., 2007) and B (Geurden et al., 2009) assemblage-specific primers. Presence of mixed infection was detected by visualizing the occurrence of bands 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 consisted 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× PCR buffer (Qiagen, Hilden, Germany), 200 μM dNTP (Fermentas, Ontario, Canada), 1.5 mM $MgCl_2$ (Qiagen, Hilden, Germany) and 0.1 mg/ml BSA (New England Biolabs, Ipswich, USA) to a final volume of 25 μl. 1 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 hot start at 95 °C for 5 min, initial denaturation at 94 °C for 10 min, and 35 amplification cycles at 94 °C for 45 s, 64 °C for 45 s (62 °C for secondary PCR) and 72 °C for 45 s.

A partial sequence of *bg* gene (≈511-bp) was amplified using PCR protocols described by Caccio et al. (2002) and Lalle et al. (2005). 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