Molecular characterization of Giardia duodenalis isolated from Semai Pahang Orang Asli (Peninsular Malaysia aborigines)

fong mun yik, fongmy

Available at: http://works.bepress.com/fong_mun_yik/5/
Molecular characterization of *Giardia duodenalis* isolated from Semai Pahang Orang Asli (Peninsular Malaysia aborigines)

A. K. MOHAMMED MAHDY¹,²*, JOHARI SURIN¹, A. MOHD-ADNAN², K.-L. WAN² and Y. A. L. LIM¹

¹ Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
² School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor DE, Malaysia
³ Department of Parasitology, Faculty of Medicine, Sana’a University, Sana’a, Yemen

(Received 4 February 2009; revised 6 May 2009; accepted 6 May 2009; first published online 7 August 2009)

**SUMMARY**

This study was conducted to determine the genotypes of *Giardia duodenalis* isolated from human faecal samples at Pos Betau, Pahang, Malaysia. Faecal specimens were collected and examined for *G. duodenalis* cysts using Trichrome staining techniques. Molecular identification was carried out by the amplification of a region of the small subunit of the nuclear ribosomal RNA (SSU rRNA) gene using nested PCR and subsequent sequencing. The sequences from 15 isolates from *G. duodenalis* were subjected to phylogenetic analysis (including appropriate outgroups) using the neighbor-joining and maximum parsimony methods. The trees identified *G. duodenalis* assemblages A and B, with a predominance of assemblage B. The predominance of anthroponotic genotypes indicates the possibility of anthroponotic transmission of these protozoa in this Semai Pahang Orang Asli community.

Key words: *Giardia duodenalis*, assemblage B, genotypes, Semai Pahang Orang Asli.

**INTRODUCTION**

Human giardiasis has been recognized as the most common cause of protozoal diarrhoea worldwide, leading to significant morbidity and mortality both in developing and industrialized nations (Marshall et al. 1997). Although person-to-person transmission of *Giardia duodenalis* is the major route of transmission, transmission *via* the ingestion of contaminated water and foods as well as zoonotic transmission of these protozoa have also been noted (Marshall et al. 1997). *Giardia* has caused multiple waterborne outbreaks in developed and developing countries.

*G. duodenalis* comprises at least 7 lineages; designated as assemblages A–G. Assemblage A has a wide range of hosts and has been considered as zoonotic assemblage. Although assemblage B has been reported to be an anthropoic assemblage, it has also been isolated from animals. The other assemblages appear to be host-adapted; assemblage C and D (dog), assemblage E (cattle and other ungulates), assemblage F (cats) and assemblage G (rats), and have not represented a potential public health concern (Monis et al. 1999).

In Malaysia, giardiasis is an endemic disease, predominantly in children, with prevalences ranging from 2.6% to 25% (Lim et al. 2008). *Giardia* has also been isolated from animals and environmental samples (Lim et al. 2008). However, the prevalence of individual *G. duodenalis* assemblages has yet to be estimated in Malaysia. This study aimed at determining the genotypes of *Giardia*, which will assist Malaysian public health authorities to build a more effective control strategy based on a better understanding of the epidemiology of this parasite.

**MATERIALS AND METHODS**

**Samples**

Faecal samples were collected from the Orang Asli (Peninsular Malaysia aborigines) community at Pos Betau, Pahang, Malaysia ~200 km north of Kuala Lumpur. The Orang Asli in this community belong to the Semai Pahang ethnic subgroup. The Orang Asli houses are made of wood or bamboo and they are provided with basic amenities, such as electricity and piped water by the Malaysian government. Although they have piped water supply, living beside a river is crucial to them, as most of their daily activities, including bathing, washing clothes and household items, swimming, playing, are linked to the river. Some inhabitants may also defecate along or in the
river and use river water to wash themselves. A total of 400 stool containers were randomly distributed among 8 villages; faecal samples were collected randomly during several visits in 2 months from February to March 2006.

**Light microscopy, and purification of Giardia cysts using immuno-magnetic separation (IMS)**

For the identification of Giardia cysts, faecal smears were made from the preserved stool and stained with the Trichrome stain (Wheatley, 1951). The smears were then examined by light microscopy for Giardia cysts under 100× and 400× magnification respectively.

For purification of cysts from faecal specimens, a small portion of the faeces was mixed with 10 ml of distilled water and sieved through gauze. The suspension was then centrifuged at 1500 g for 10 min, and the supernatant was discarded. The pellet was re-suspended in 2 ml of water and stored at 4 °C until use. IMS was carried out using Dynabeads® GC-Combo kit (Dynal, Oslo, Norway) according to method of Xiao et al. (2004).

**DNA methods and analyses**

Genomic DNA was extracted from the IMS-isolated cysts using QIagen DNA Mini Kit (QIAGEN, Germany), according to Xiao et al. (2004). Briefly, IMS-isolated cysts were re-suspended in 180 μl of Buffer ATL provided in the kit, and then subjected to 5 consecutive cycles of freezing in liquid nitrogen for 1 min and thawing at 56 °C for 2 min, vortexing for 30 s following each cycle. DNA was then extracted, purified and stored at −20 °C until use.

Nested PCR assay was used to amplify a region (~200 bp) of the small subunit of the nuclear ribosomal RNA (SSU rRNA) gene, according to Read et al. (2002). Primary PCR was carried out with the primers RH11 (5′-CAT CCG GAT CCT GCC GAC-3′) and RH4 (5′-AGT CGA ACC CGT AT'T CTC CGC CAGG-3′) (Hopkins et al. 1997), and the secondary PCR with primers Giar-F (5′-GAC GTG CTC CCC AAG GAC-3′) and Giar-R (5′-CTG CGT CAC GCT GTC-3′) (Read et al. 2002). Both primary and secondary PCRs were performed in a 25 μl reaction volume containing 12:5 pmol of each primer (Research Biolab, Singapore), 2 U of Taq polymerase (New England Biolabs, Ipswich, USA),

<table>
<thead>
<tr>
<th>Assemblage</th>
<th>Host species</th>
<th>GenBank Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test samples representing samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3s</td>
<td>Human</td>
<td>FJ460002</td>
</tr>
<tr>
<td>10</td>
<td>Human</td>
<td>FJ460014</td>
</tr>
<tr>
<td>12</td>
<td>Human</td>
<td>FJ460013</td>
</tr>
<tr>
<td>17a</td>
<td>Human</td>
<td>FJ460003</td>
</tr>
<tr>
<td>25a</td>
<td>Human</td>
<td>FJ460004</td>
</tr>
<tr>
<td>26a</td>
<td>Human</td>
<td>FJ460005</td>
</tr>
<tr>
<td>29</td>
<td>Human</td>
<td>FJ460006</td>
</tr>
<tr>
<td>32a</td>
<td>Human</td>
<td>FJ460007</td>
</tr>
<tr>
<td>44</td>
<td>Human</td>
<td>FJ460009</td>
</tr>
<tr>
<td>45a</td>
<td>Human</td>
<td>FJ460008</td>
</tr>
<tr>
<td>47a</td>
<td>Human</td>
<td>FJ460010</td>
</tr>
<tr>
<td>51</td>
<td>Human</td>
<td>FJ460011</td>
</tr>
<tr>
<td>S</td>
<td>Human</td>
<td>FJ460012</td>
</tr>
<tr>
<td>Group I = (53, 58a, 64, 68, 73, 74, 87, 101, 115a, 119, 124, 138, 141, 147a, 49a, 162a, 197, 199, 200, 201, L12, L23, L24, L41, M1, M2, M6, M10)</td>
<td>Human</td>
<td>FJ460016</td>
</tr>
<tr>
<td>Reference sequences representing assemblages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>—</td>
<td>M54878</td>
</tr>
<tr>
<td>B</td>
<td>Human</td>
<td>U09491</td>
</tr>
<tr>
<td>C</td>
<td>Dog</td>
<td>AF113899</td>
</tr>
<tr>
<td>D</td>
<td>Dog</td>
<td>AF113900</td>
</tr>
<tr>
<td>E</td>
<td>Cattle</td>
<td>AF199448</td>
</tr>
<tr>
<td>F</td>
<td>Cat</td>
<td>AF199444</td>
</tr>
</tbody>
</table>
the PCR Thermopol buffer (New England Biolabs, Ipswich, USA), 200 μM of each dNTP (Fermentas, Ontario, Canada), 2 mM MgCl₂ (Fermentas, Ontario, Canada), 5% dimethyl sulfoxide (Sigma, USA), 0.2 mg/ml gelatin and 400 mg/ml BSA (New England Biolabs, Ipswich, USA). Two μl of DNA template was used in both primary and secondary PCRs. In both amplifications, samples were incubated in the MyCycler thermal cycler (Bio-Rad, Hercules, USA) under the following conditions: denaturing step at 95 °C for 2 min, followed by 35 cycles of denaturing for 20 s at 96 °C, annealing for 20 s at 59 °C and extension for 20 s at 72 °C, followed by a final extension at 72 °C for 7 min. The PCR products were subjected to electrophoresis in 2% agarose gels and stained with ethidium bromide (Nichols et al. 2003).

The DNA was purified using the QIAquick Gel Extraction Kit (QIagen, Germany), according to the manufacturer’s instructions. Cycle sequencing (bidirectional) was carried out using the ABI PRISM® BigDye™ terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA) in an 3700 DNA Analyzer (Applied Biosystems, USA). The sequences determined for all samples were aligned using the program MEGA4 (www.megasoftware.net). Similarity searches were carried out using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997). Neighbor-joining (NJ) and maximum parsimony (MP) analyses were performed using the MEGA4 (NJ) and maximum parsimony (MP) analyses were performed using MEGA4 using G. muris (GenBank Accession no. AF113895) and G. ardeae (GenBank Accession no. Z17210) as outgroups. NJ analyses were performed with distances calculated with the Kimura 2-parameter (Kimura, 1980). Unweighted parsimony analyses were performed using the the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000). To evaluate the support for inferred topologies, bootstrapping (Felsenstein, 1985) was carried out using 1000 replicates.

RESULTS AND DISCUSSION

Of 321 faecal samples examined microscopically, 76 samples (23.7%) contained Giardia. Amplicons (~200 bp) were produced for 42 of the 76 samples. All of these amplicons were successfully sequenced in both directions. Fifteen sequences representing all of the 42 isolates and 6 reference sequences representing all known G. duodenalis assemblages (A-F) obtained from the GenBank database (see Table 1) were used in the phylogenetic analyses (NJ and MP methods), utilizing 140 informative nucleotide positions shown previously to differentiate among genotypes (Hopkins et al. 1997). The NJ and MP trees were concordant in topology; the NJ tree is shown as a representative in Fig. 1. In this tree, all 21 G. duodenalis sequences grouped together with strong support (100%) and G. duodenalis isolates (3s, 10, 17a, 25a, 26a, 29, 32a, 44, 45a, 47a, 51, S and Group I) grouped with a representative of assemblage B (82%). G. duodenalis isolate 43 was placed with assemblages A, E and F (96%).

Genetic characterization was further confirmed by similarity search using BLAST. It was found that all the isolates that were clustered in assemblage B were similar to a small ribosomal subunit RNA sequence of assemblage B (GenBank Accession numbers AY826201, AY826202, AY826203, AY826207) from faecal samples from humans in the Netherlands (van der Giessen et al. 2006). The similarity was 100% for isolates 44 and S, 99% for isolate 10, 98% for isolates 25a, 29, 32a, 51 and group 1, 97% for isolates 3s, 45 and 17a, 95% for isolate 26a and 93% for isolate 47a. The isolate 43 was 98% similar to assemblage A (GenBank Accession number EU562195).

The present study showed that G. duodenalis assemblage B was the predominant assemblage in Semai Pahang Orang Asli communities in Malaysia.
The predominance of this assemblage has been documented in several studies in the UK (Caccio et al. 2005), Australia (Read et al. 2004), Albania (Berrilli et al. 2006), Turkey (Aydin et al. 2004), Peru (Sulaiman et al. 2004), the Philippines (Yason and Rivera, 2007) and India (Sulaiman et al. 2004). In contrast, assemblage B appeared to be less prevalent compared with assemblage A in the USA and Canada (van Keulen et al. 2002), Italy (Lalle et al. 2005), Mexico (Eligio-Garcia et al. 2005) and Ethiopia (Gelanew et al. 2007).

The 2 main assemblages responsible for human infections are assemblages A and B. Although assemblage B appears to be predominantly human specific, it has been isolated from dogs, calves, cats and monkeys (van Keulen et al. 2002; Itagaki et al. 2005). The greatest zoonotic risk is considered to be posed by assemblage A, which infects humans and a wide range of animals (Itagaki et al. 2005). The predominance of assemblage B in this Semai Pahang Orang Asli community indicates the possibility of anthroponotic transmission from human to human. Furthermore, the suggestion of anthroponotic transmission was supported by epidemiological findings indicating the rearing of pets is not a risk factor for giardiasis in this Orang Asli community (Mohammed Mahdy et al. 2008). Anthroponotic transmission may be facilitated by low hygienic practices which include consuming food with unwashed hands, consuming raw unwashed vegetables and storing drinking water in dirty containers which are not covered. Drinking tap water and eating raw vegetables were identified as significant risk predictors of giardiasis in this Orang Asli community (Mohammed Mahdy et al. 2008). Another study showed that contamination of *Giardia* in drinking water occurred after treatment in the Temuan Orang Asli community (Lim and Ahmad, 2004). In addition, it has also been reported that some of the Orang Asli children feel more comfortable defecating in an open space, thereby contaminating the environment. Unfortunately, there is a void of information regarding *Giardia* genotypes in aboriginal populations within Malaysia and in other countries, which currently makes the comparison of *Giardia* genotypes between communities that have similar behavioural, cultural background and share the same public health problems impossible.

In conclusion, assemblage B is the predominant assemblage of *G. duodenalis* in this Semai Pahang Orang Asli community, suggesting that human giardiasis is mainly linked to anthroponotic transmission. However, the inference of such transmission is limited by the fact that domestic animals were not included in the present investigation and that genetic characterization of *Giardia* isolates was based on a single locus. Therefore, further molecular studies of a range of *Giardia* isolates from humans, animals and the environment using multiple loci are warranted in the Semai Pahang Orang Asli community. Better insights into the genetic make-up of *Giardia* populations as well as improved sanitary measures with health education will be the key factors in the control of giardiasis in this community.

The authors would like to thank staff of the Department of Orang Asli Affairs and staff of Clinic Betau for their help during this study. We thank Wan Hafiz for his help in the field trips. This work was supported by research grants (P.J.P.) No. F0202/2005C, P0108/2006C and PS176/2007B, from the University of Malaya.

**REFERENCES**


comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16, 111–120.


Read, C. M., Monis, P. T. and Thompson, R. C. (2004). Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. *Infection, Genetics and Evolution* 4, 125–130.


