Yang, R., Lee, J., Ng, J. and Ryan, U. (2010) *High prevalence* *Giardia duodenalis* *assemblage B* *and potentially zoonotic subtypes in sporadic human cases in Western Australia*. International Journal for Parasitology, 40 (3). pp. 293-297.

http://researchrepository.murdoch.edu.au/4187/

Copyright: © 2009 Australian Society for Parasitology Inc.

It is posted here for your personal use. No further distribution is permitted.
High prevalence *Giardia duodenalis* assemblage B and potentially zoonotic subtypes in sporadic human cases in Western Australia

Rongchang Yang, Jeremy Lee, Josephine Ng, Una Ryan

PII: S0020-7519(09)00336-1
DOI: 10.1016/j.ijpara.2009.08.003
Reference: PARA 3018

To appear in: *International Journal for Parasitology*

Received Date: 30 June 2009
Revised Date: 6 August 2009
Accepted Date: 10 August 2009

Please cite this article as: Yang, R., Lee, J., Ng, J., Ryan, U., High prevalence *Giardia duodenalis* assemblage B and potentially zoonotic subtypes in sporadic human cases in Western Australia, *International Journal for Parasitology* (2009), doi: 10.1016/j.ijpara.2009.08.003

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
High prevalence Giardia duodenalis assemblage B and potentially zoonotic subtypes in sporadic human cases in Western Australia

Rongchang Yang\textsuperscript{a}, Jeremy Lee\textsuperscript{a}, Josephine Ng\textsuperscript{a} and Una Ryan\textsuperscript{a,*}

\textsuperscript{a}Division of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Western Australia, 6150.

*Corresponding author.

Tel.: +61 08 9360 2482; fax: +61 08 9310 4144

E-mail address: Una.Ryan@murdoch.edu.au
Abstract

*Giardia duodenalis* is a widespread parasite of mammalian species, including humans. Fecal samples from sporadic human clinical cases of giardiasis in Western Australia were analysed at two loci; 18S rRNA and glutamate dehydrogenase (*gdh*), and *G. duodenalis* assemblage B isolates were identified in 75% of isolates. Sequence analyses of 124 isolates at the 18S rRNA locus identified 93 isolates as assemblage B and 31 as assemblage A. Analyses of 109 isolates at the *gdh* locus identified 44 as B3, 38 as B4 and 27 were A2. Infection with *Giardia* was highest amongst children < 5 years of age, with > 56% of infections in this age group. The majority of the isolates were from rural areas (91/124) compared with urban areas (33/124). The assemblage A isolates were completely homogenous genetically at the *gdh* locus, while assemblage B isolates showed variability at the nucleotide but not at the amino acid level at this locus. Some of the assemblage B3 and B4 subtypes identified in humans were previously identified in marsupials in Australia and in a fox, indicating potential zoonotic transmission.

Keywords: *Giardia*, 18S rRNA, *gdh*, Assemblage A2, Assemblage B3, Assemblage B4, Zoonotic
1. Introduction

*Giardia duodenalis* is a widespread parasite of mammalian species, including humans, and has a global distribution causing an estimated $2.8 \times 10^8$ cases per year (Lane and Lloyd, 2002). In Asia, Africa and Latin America, about 200 million people have symptomatic giardiasis with some 500,000 new cases reported each year (WHO, 1996). There is considerable variation within *G. duodenalis* and several major genotypes/assemblages have been identified; with assemblages A and B associated with human and animal infections. The remaining assemblages (C to G) are likely to be host-specific, as assemblages C and D have been identified in dogs, cats, coyotes and wolves, assemblage E in cattle, sheep, goats, pigs, water buffaloes and muflons, and assemblages F and G in cats and rats, respectively (Caccio and Ryan, 2008). The prevalence of assemblage A and B varies considerably from country to country, although assemblage B seems more common (Caccio and Ryan, 2008).

Little data are available on the prevalence of *G. duodenalis* subtypes infecting humans in Western Australia, as only small numbers of isolates have been sequenced, usually at only one locus and the distribution of subtypes is unclear, although previous data suggests that assemblage B dominates (Hopkins et al., 1997; Read et al., 2002, 2004). The aim of the present study was to characterize a much larger number of sporadic *Giardia* isolates at two loci and determine the prevalence and distribution of *Giardia* subtypes in different age-groups, the extent of variation within subtypes and comparison of subtypes identified in humans with subtypes identified in animals.

2. Materials and methods

2.1. Faecal sample collection

Faecal specimens ($n = 124$), positive for *Giardia* by microscopy, from sporadic human
cases were collected from January 2005 to November 2005 from a diagnostic pathology laboratory in Western Australia. Patient epidemiological information (age, location, symptoms or clinical signs, collection date, etc.) for most of the human specimens were collected.

2.2. DNA isolation

Genomic DNA was extracted from 200 mg of each faecal sample using a QIAamp DNA Mini Stool Kit (Qiagen, Hilden, Germany) or from 250 mg of each faecal sample using a Power Soil DNA Kit (MolBio, Carlsbad, California, USA). A negative control (no faecal sample) was used in each extraction group.

2.3. PCR amplification

All samples were amplified at the 18S rRNA locus and Giardia-positive samples were genotyped by sequencing. Amplification of a fragment of the Giardia 18S rRNA gene was performed as described by Hopkins et al. (1997) and Read et al. (2002). A total of 109 isolates were also analysed at the Giardia glutamate dehydrogenase (gdh) locus as previously described (Read et al., 2004). PCR contamination controls were used including negative controls and separation of preparation and amplification areas. The amplified DNA fragments from the secondary PCR product were separated by gel electrophoresis and purified using the freeze-squeeze method (Ng et al., 2006).

2.4. Sequence and phylogenetic analysis

Purified PCR products were sequenced using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer’s instructions. Nucleotide sequences were analyzed using Chromas lite version

Phylogenetic trees were constructed for the \textit{gdh} locus with additional isolates from GenBank. Distance estimations were conducted using TREECON (Van de Peer and De Wachter, 1997), based on evolutionary distances calculated with the Tamura-Nei model and grouped using the Neighbour-Joining method. Parsimony analyses were conducted using MEGA version 3.1 (MEGA3.1: Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, Arizona, USA). Bootstrap analyses were conducted using 1,000 replicates to assess the reliability of inferred tree topologies. Maximum Likelihood (ML) analyses were conducted using the program PhyML (Dereeper et al., 2008) and the reliability of the inferred trees was assessed by the approximate likelihood ratio test (aLRT) (Anisimova and Gascuel, 2006).

3. Results

3.1. PCR and sequence analysis

From the 124 isolates received between January 2005 to November 2005, analysis of the 18S rRNA locus identified 93 isolates as \textit{G. duodenalis} assemblage B and 31 as assemblage A. Infection with \textit{Giardia} was highest amongst children < 5 years of age with > 56% of infections in this age group (Table 1). Analyses of 109 isolates at the \textit{gdh} locus identified 44 as B3, 38 as B4 and 27 were A2 (Table 1). There was good agreement between the two loci with the exception of five isolates, three of which (GH104, GH135 and GH172) typed as assemblage A at the 18S rRNA locus and typed as B3, B4 and B3, respectively, at the \textit{gdh} locus and two isolates (GH94 and GH179) which typed as assemblage B at the 18S rRNA locus and typed as A2 at the \textit{gdh} locus. The majority of the isolates (~73%) were from rural areas (91/124) compared with urban areas (33/124) (Table 2).
3.2. Phylogenetic analysis of Giardia isolates at the gdh locus

Phylogenetic analyses of the partial nucleotide sequence of the gdh locus using Distance, Parsimony and ML analyses produced similar results (data not shown) and identified that all of the 27 assemblage A2 isolates were 100% identical to the A2 reference isolate AD-2 (L40510) and revealed variation within the assemblage B3 and B4 isolates with the majority of variation in B3 (Fig. 1 - NJ Distance tree shown). Within B3 there were several different sub-groups, which received poor support using all methods of analysis. A total of 14 isolates matched 100% with the reference B3 isolate BAH12 (AF069059). The remaining isolates had between one and four single nucleotide polymorphisms (SNPs) from BAH12 (Table 3). Isolate GH78 was identical to a B3 variant sequence (DQ904425) from a Norwegian red fox (Hamnes et al., 2007).

Within B4, there were two sub-groups. Group one consisted of 23 isolates, which were identical to the B4 reference isolate Ad45 (AY178739). Group two consisted of 15 isolates, 14 of which were identical to an assemblage B isolate Vanc/89/UBC/059 (AY178750) from a dog and one isolate (GH16) which exhibited one SNP (Table 4). All of the variations in both B3 and B4 isolates were synonymous changes and therefore resulted in no amino acid differences.

3.3. Clinical symptoms and other concurrent infections

Data on clinical symptoms were available for 80 of the 124 samples received. Diarrhoea was the most common symptom reported in 74% of cases, abdominal pain in 7.5% of cases, nausea and vomiting in 5% of cases, failure to thrive in 5% of cases, fever in 2.5% of cases and weight loss in 2.5% of cases. Several of the Giardia isolates were co-infected with other pathogens. One isolate (GH73) from a patient suffering from anemia was co-infected with Endolimax nana, Hymenolepis nana and Blastocystis hominis. Co-infections with H.
Similarly co-infections with \textit{B. hominis} only were identified in three isolates, and one patient (GH35) had a concurrent \textit{H. nana} and \textit{B. hominis} infection. One isolate (GH25) was co-infected with \textit{Shigella sonni} and \textit{H. nana}. One isolate (H137) was co-infected with \textit{Isospora belli} and one (GH179) with \textit{Cryptosporidium}.

4. Discussion

To the best of our knowledge, this is the first study to sequence and analyse a large number of Australian human \textit{Giardia} isolates at two loci. Analyses of \textit{Giardia} isolates using at least two loci is essential due to ‘assemblage swapping’ or the assignment of \textit{Giardia} isolates to different assemblages using different markers, which has been frequently reported (Traub et al., 2004; Caccio and Ryan, 2008). Similar to \textit{Giardia} genotyping reports from humans from a wide range of geographic areas, (cf Caccio and Ryan, 2008), only assemblages A and B were detected. \textit{Giardia} infection was highest amongst children < 5 years of age but was present in 14-16% of samples from humans aged between 6-70 years (Table 1). Other studies have reported the highest prevalence amongst children aged < 1-9 years of age (cf. Xiao and Fayer, 2008).

Previous analyses have shown the existence of two sub-groups in assemblage A (A1 and A2) and in assemblage B (B3 and B4). In the present study, all of the assemblage A isolates were subtyped as A2 at the \textit{gdh} locus. Genotype A1 is generally found in animals, whereas genotype A2 has mainly been identified in humans. However, A2 has occasionally been detected in animals (cf. Caccio and Ryan, 2008).

The present study identified a high prevalence (75%) of \textit{G. duodenalis} assemblage B isolates amongst sporadic \textit{Giardia} cases in Western Australia. Previous studies in Australia, which sequenced small numbers of isolates at one locus, have also reported a high prevalence
of assemblage B in human isolates (Hopkins et al., 1997; Read et al., 2002, 2004). Sequence analysis of the 18S rRNA from *Giardia*-positive stool samples from humans living in isolated Aboriginal communities identified 92% (11/12) as assemblage B and 8% (1/12) as a mixed A and B infection (Hopkins et al., 1997). A population survey of 353 children under the age of 5 years and attending day-care centres in Western Australia conducted during 1998-2000 identified a prevalence of 7.6% (27/353) for *Giardia* (Read et al., 2002). Sequence analysis of the 18S locus of 23 isolates identified 70% (16/23) as assemblage B and 25% (7/23) as assemblage A (Read et al., 2002). Another study using sequence and restriction fragment length polymorphism (RFLP) analysis of the *gdh* locus, which included 17 Australian human isolates, identified assemblage A1/A2 in ~41% (7/17) of isolates and assemblage B3/B4 in ~59% (10/17) of isolates (Read et al., 2004). Few studies have compared the prevalence of *Giardia* subtypes in sporadic cases from urban and rural areas. In the present study, the majority of cases (~73%) were from rural areas and of these ~26% were A2, ~38% were B3 and ~36% were B4 (Table 2).

The prevalence of assemblages A and B in humans varies considerably from country to country, and from study to study, although assemblage B seems more common overall (Gelanew et al., 2007; cf. Caccio and Ryan, 2008; Pelayo et al., 2008; Ajjampur et al., 2009; Lalle et al., 2009). In New Zealand, ~76% of human isolates (*n* = 30) were typed as assemblage A and the remainder (~24%) were assemblage B (Winkworth et al., 2008). In Egypt, two different surveys based on RFLP analysis of the triose phosphate isomerase (*tpi*) locus have identified assemblage A as more prevalent than B (El-Shazly et al., 2004; Helmy et al., 2009), whereas another study based on sequence analysis of the *tpi* locus identified assemblage B in 80% of human isolates genotyped (Foronda et al., 2008). A recent study reported that assemblage B infections had a higher rate of cyst shedding in children, which may promote its spread (Kohli et al., 2008).
The genotyping of *G. duodenalis* assemblages in Australian humans was conducted using PCR amplification and sequencing of two commonly used markers, the 18S rRNA and *gdh* genes. The 18S rRNA locus is more conserved, whereas the *gdh* locus is more variable and permits subtyping within assemblages (Caccio and Ryan, 2008). Although the 18S locus is very conserved, it was included in this analysis as it is reported to easily detect mixed templates (Wielinga and Thompson, 2007). Of the 124 samples amplified at the 18S locus, only 109 (~88%) could be amplified at the *gdh* locus. The reason for this is unknown but may be due to the higher copy number of the 18S locus. There was > 95% agreement in assemblage assignment between the two markers with only five isolates assigned to different assemblages at the two loci. The assignment of *Giardia* isolates to different assemblages using different markers has been found in both human and animal isolates and has been found using different combinations of gene markers (Caccio and Ryan, 2008). There are various explanations for ‘assemblage swapping’ such as mixed infections and/or meiotic recombination (Caccio and Ryan, 2008). As mixed A and B infections were detected in two human isolates at the 18S locus (GH71 and GH61), the most likely explanation is that there were mixed assemblage infections present in the five isolates which typed differently at the two loci, and the different markers used (18S and *gdh*) amplified different assemblages from the same host. Mixed infections are quite common and have been identified in multiple hosts, including dogs, where both zoonotic and host adapted genotypes were identified in the same sample using different markers (Traub et al., 2004; Caccio and Ryan, 2008). However, analyses of the 18S sequence chromatograms for the five isolates (GH104, GH135, GH172, GH94 and GH179) indicated no evidence of multiple peaks.

Several studies have reported a correlation between assemblage A/A2 and diarrhoea (Read et al., 2002; Haque et al., 2005; Sahagún et al., 2008). In contrast, two other studies have reported a strong correlation between assemblage B and severe/persistent diarrhoea (Homan and Mank, 2001; Gelanew et al., 2007). Another study reported no correlation
between the type of assemblage and symptoms (Lebbad et al., 2008). In the present study, all of the isolates were obtained from a pathology laboratory and although clinical data were not available for ~35% of samples, diarrhoea was the most common symptom (~74%) for both assemblages A and B.

Recent molecular analyses of Giardia isolates at the gdh, tpi and the β-giardin (bg) genes indicate a high degree of genetic variability within both assemblages A and B (Wielinga and Thompson, 2007), which may contribute to the elucidation of different transmission pathways, including the role of animals as a reservoir for human giardiasis. In the present study, phylogenetic analyses revealed variation within assemblage B and particularly within B3 isolates. Only 14 of the 44 B3 isolates were identical to the B3 reference isolate BAH12. The remaining 31 B3 isolates had between one and four SNPs from BAH12. Alignment analysis of the polymorphisms within the B3 and B4 gdh sequences revealed that all nucleotide substitutions observed among the assemblage B3/B4 sub-groups were positioned at the third nucleotide of the codons and were transition mutations, i.e., they were purine–purine or pyrimidine–pyrimidine substitutions. Assemblage B was thought to be largely restricted to humans, however more recently assemblage B has been reported in beavers, cattle, dogs, horses, monkeys, muskrats, rabbits and sheep (cf. Caccio and Ryan, 2008). Several of the assemblage B subtypes identified in the present study have recently been identified in marsupials, indicating potential transmission between humans and marsupials (Thompson et al., 2008). For example, the B3 subtype identified in isolate GH172 (and eight other human isolates) has also been identified in a western grey kangaroo (Macropus fuliginosus) and a red kangaroo (Macropus rufus). The B4 subtype identified in 23 isolates (including GH20) (Fig. 1), has recently been identified in a quokka (Setonix brachyurus), a yellow-footed rock-wallaby (Petrogale xanthopus), a tammar wallaby (Macropus eugenii), a swamp wallaby (Wallabia bicolor) and red kangaroos (Thompson et al., 2008). The
identification of a B3 variant isolate (GH78) that was identical to a sequence from a Norwegian red fox (Hamnes et al., 2007) also suggests potential zoonotic transmission.

In conclusion, assemblage B dominated in *Giardia* isolates from humans in sporadic cases of giardiasis in Western Australia. The assemblage A isolates were completely homogenous genetically at the *gdh* locus, while assemblage B showed variability at the nucleotide but not at the amino acid level. Some of the assemblage B3 and B4 subtypes identified in humans have been identified in marsupials in Australia and in a fox, indicating potential zoonotic transmission.

**Acknowledgements**

We are most grateful to Dr. Brian McKenzie, PathWest, W.A. for provision of samples and clinical data.
References


Fig. 1. Phylogenetic relationships of *Giardia* isolates inferred by Neighbor Joining analysis of Kinura’s distances calculated from pair-wise comparisons of glutamate dehydrogenase (*gdh*) sequences. Percentage bootstrap support (>70%) from 1,000 replicate samples is indicated at the left of the supported node.
Table 1. Distribution of *Giardia* assemblages in Western Australian humans by age at the 18S and glutamate dehydrogenase (*gdh*) loci.

<table>
<thead>
<tr>
<th>Age range (years)</th>
<th>No. of cases</th>
<th>%</th>
<th>18S locus</th>
<th>gdh locus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>≤ 5</td>
<td>70/124</td>
<td>56.5</td>
<td>16</td>
<td>54</td>
</tr>
<tr>
<td>6-15</td>
<td>17/124</td>
<td>13.7</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>16-39</td>
<td>19/124</td>
<td>15.3</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>40-70</td>
<td>18/124</td>
<td>14.5</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>93</td>
<td>27</td>
<td>44</td>
</tr>
</tbody>
</table>

* Mixed A and B infection.
Table 2. Distribution of *Giardia* assemblages in Western Australian humans in urban and rural areas at the 18S and glutamate dehydrogenase (*gdh*) loci.

<table>
<thead>
<tr>
<th>Location</th>
<th>18S locus</th>
<th>gdh locus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Urban</td>
<td>8</td>
<td>25</td>
</tr>
<tr>
<td>Rural</td>
<td>23(^a)</td>
<td>68(^a)</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>93</td>
</tr>
</tbody>
</table>

\(^a\)Two mixed A and B infections.
Table 3. Polymorphisms in 10 *Giardia* B3 sub-group isolates compared with B3 reference isolate BAH12 ([AF069059](#)) at the glutamate dehydrogenase (*gdh*) locus. Polymorphic sites are numbered with reference to the full-length gene.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BAH-12</td>
<td>C</td>
<td>C</td>
<td>T</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>GH15</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>T</td>
<td>T</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>GH25</td>
<td>C</td>
<td>C</td>
<td>T</td>
<td>G</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>GH27</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>GH31</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>GH78</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>G36</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>G</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>GH46</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>T</td>
<td>A</td>
</tr>
<tr>
<td>GH50</td>
<td>C</td>
<td>C</td>
<td>T</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>GH104</td>
<td>T</td>
<td>T</td>
<td>C</td>
<td>G</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>GH171</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>G</td>
</tr>
</tbody>
</table>
Table 4. Polymorphisms in *Giardia* B4 isolates compared with B4 reference isolate Ad45 (AY178739) at the glutamate dehydrogenase (*gdh*) locus. Polymorphic sites are numbered with reference to the full-length gene.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Position</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>357</td>
<td>597</td>
</tr>
<tr>
<td>Ad-45</td>
<td>T</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>GH18</td>
<td>C</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>GH16</td>
<td>C</td>
<td>T</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1

Assemblage B3 (BAH12, AF069059)

Assemblage B4 (Ad-45 AY178138)

Assemblage A1 - Livestock

Assemblage A2 - Ad2-L40510

G. ardeae_