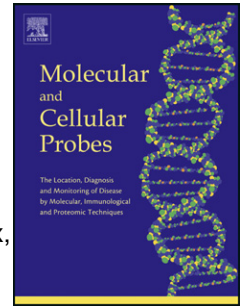


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First molecular characterization of *Giardia duodenalis* from goats in Malaysia*

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*Note: The nucleotide sequences determined in the present study are available from the GenBank database under accession numbers HQ283227 to HQ283232.

ABSTRACT

In the present study, 310 faecal samples from goats from eight different farms in Malaysia were tested for the presence of *Giardia* using a PCR-coupled approach. The nested PCR for SSU amplified products of the expected size (~200 bp) from 21 of 310 (6.8%) samples. Sixteen of these 21 products could be sequenced successfully and represented six distinct sequence types. Phylogenetic analysis of the SSU sequence data using Bayesian Inference (BI) identified *Giardia* assemblages A, B and E. The identification of the 'zoonotic' assemblages A and B suggests that *Giardia*-infected goats represent a possible reservoir for human giardiasis in Malaysia.

*Keywords:**Giardia duodenalis*

Small subunit of the nuclear ribosomal RNA (SSU) gene

Goats

Malaysia

1. Introduction

Giardia duodenalis (Protista) represents an important group of enteric pathogens of mammals, including humans and ruminants [1,2]. Giardiasis causes intestinal disease in cattle, particularly young calves [3]. Animals that are affected by giardiasis can exhibit clinical signs of diarrhoea, weight loss and a failure to thrive, often resulting in significant production losses and sometimes death [4]. The relatively high prevalence of *G. duodenalis* in young ruminants (often > 30%) [5] is a public health concern, because of the possibility of zoonotic transmission from infected animals to humans (e.g., farmers and their families) and, on a larger scale, through the contamination of the environmental or water with viable cysts, which have the potential to infect large human populations [6].

Based on molecular evidence, *G. duodenalis* is currently divided into eight genotypes or assemblages (A to H) [3,7], with assemblages A, B, and, occasionally E, being found to infect humans [8]. In ruminants, *G. duodenalis* assemblage E is most frequently being recorded in calves [9], sheep [10,11] and goats [12]. However, the “zoonotic” assemblages A and B are also common (with prevalences varying from 2.9% to 14%, depending on study) [13-15], suggesting that ruminant hosts could be significant reservoirs of *G. duodenalis* infection to humans.

Presently, there is considerable information on the epidemiology of *Giardia* of ruminants (e.g., [11,16,17]), particularly goats [5,12,18]. However, molecular studies have not been widely conducted on *Giardia* from animals in Malaysia. In this country, goat farming is a key agricultural sector, and goats represent a major food source. Goats constitute ~ 70% of the total small ruminant livestock industry in this country, being second after cattle and involving ~12,500 farms (Department of Veterinary Services, 2006; <http://agrolink.moa.my/jph/>). Except for a study of the prevalence of *Giardia* in cattle using a conventional microscopic detection method [6], there has been no study of *Giardia* of any ruminant species in Malaysia using molecular methods. The

present study genetically characterized, for the first time, *Giardia* from goats in this country to assess whether they harbour genotypes with the potential to infect humans.

2. Materials and methods

A total of 310 faecal samples were collected from goats (Jamnapuri breed; both sexes; 3 months to 7 years of age) from eight farms in four states of peninsular Malaysia (i.e., Selangor, Perak, Terengganu and Sarawak). The farms had similar farm management strategies, in accordance with guidelines for goat breeding (Department of Veterinary Services, Malaysia; <http://www.dvs.gov.my/web/guest/penerbitan>). Goats were kept in a semi-intensive system in raised wooden houses with slated flooring. The goats were fed concentrates and local grasses, and provided with water *ad libitum*.

Genomic DNA was extracted from each faecal sample using the PowerSoil DNA isolation kit (MOBIO Laboratories, USA) according to the manufacturer's protocol. To identify *G. duodenalis*, part (~ 200 bp) of the small subunit of nuclear ribosomal RNA (SSU) gene (cf. [19]) was amplified from individual genomic DNA samples by nested PCR assay and then sequenced. Primary PCR was carried out using primers RH11 (5'-CAT CCG GTC GAT CCT GCC-3') and RH4 (5'-AGT CGA ACC CTG ATT CTC CGC CAGG-3') [19], and the secondary PCR with primers Giar-F (5'-GAC GCT CTC CCC AAG GAC-3') and Giar-R (5'-CTG CGT CAC GCT GCTC-3') [20]. Both primary and secondary PCRs were performed in a 25 µl reaction volume containing 12.5 pmol of each primer, 2 U of ThermoPol polymerase (New England BioLabs, USA) in the buffer provided, 200 µM of each dNTP, 2.0 mM of MgCl₂, 5% of dimethyl sulfoxide (Sigma, USA), 0.2 mg/ml of gelatin and 400 mg/ml of bovine serum albumin (BSA). Two µl of genomic DNA and primary amplicon were added to the primary and secondary PCRs, respectively. Known *G. duodenalis* genomic DNA (positive control) samples were included in each PCR run, as were samples without

DNA template (negative control). PCR was carried out in a thermal cycler (MyCycler, Bio-Rad, Hercules, USA) at 95 °C for 2 min (initial denaturation), followed by 35 cycles of 95 °C for 20 s (denaturation), 59 °C for 20 s (annealing) and 72 °C for 20 s (extension), followed by 72 °C for 7 min (final extension). Secondary amplicons were purified over spin columns (Wizard PCR Preps, Promega), and then sequenced in both directions by direct, automated sequencing (BigDye® Terminator v.3.1 chemistry, Applied Biosystems, USA), using the same primers employed in the secondary PCR. The quality of each sequence was assessed based on the corresponding electropherogram, and sequences were compared with reference sequences using the Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov/BLAST>). Nucleotide sequence data reported here have been deposited in the GenBank database.

The six nucleotide sequences determined for SSU (GenBank accession nos. HQ283227 to HQ283232) were aligned with 16 published reference sequences (see Table 1). Phylogenetic analysis of each SSU sequence data were conducted using Bayesian Inference (BI), utilising MrBayes v.3.1.2 [21]. The General Time Reversible (GTR) evolutionary model was utilised, allowing for a 'site-specific rates model', with the rates varying according to a defined partition (codon position) (prset ratepr = variable). A 50 % majority rule consensus tree was constructed based on 75 % of final trees generated, and was viewed using the program TreeviewX v.0.5.0 [22]. SSU sequences for *G. ardeae* (accession number Z17210) and *G. muris* (accession number AF113895) were used as outgroups.

3. Results and discussion

All 310 genomic DNA samples derived from the goat faecal samples from eight farms in four different states in Malaysia were subjected to the molecular detection of *Giardia* DNA. The nested PCR for SSU amplified products of the expected size (~200 bp) from 21 of 310 (6.8 %) samples. Sixteen of these 21 amplicons could be sequenced successfully and represented six distinct

sequence types [GenBank accession numbers HQ283227 (isolate GS5), HQ283228 (isolate GT8), HQ283229 (isolate GT9), HQ283230 (isolate GX5), HQ283231 (isolate GX6), HQ283232 (isolate GX7)]. Phylogenetic analysis of these sequences, together with reference sequence data from the GenBank database (see Table 1) using BI, placed isolate GT8 in a cluster with assemblage B, isolates GX6 and GX7 in a cluster with assemblage A, and isolates GS5, GX5 and GT9 in a cluster with assemblage E (Fig. 1).

There is a paucity of information on the population genetics of *Giardia* in animals and humans in many countries, particularly in Southeast Asia [23]. Although *Giardia* has been detected in dogs [24], wild and domestic rats [25] and cattle [6] in Malaysia, there has been a scarcity of information on the genotypes in ruminants in this country. Nonetheless, some PCR-based epidemiological studies have detected *G. duodenalis* assemblage A in water samples from a recreational lake and a watershed near a zoological garden in Kuala Lumpur [26,27]. This 'zoonotic' assemblage has been detected also in HIV/AIDS patients [28], and both assemblages A and B have been recorded in Aboriginal communities in Malaysia [29].

In the present study, *G. duodenalis* was detected in 6.8% of 310 samples from goats on three of eight farms studied. For goats, this percentage is lower than those previously reported in studies conducted in Belgium [14], Brazil [30], Spain [12,31] and Turkey [32] among which prevalence for this species ranged from 14.3% to 42.2%. This difference could relate to the age of the goats tested (most goats tested here were ≥ 3 years of age), the diagnostic tests used or the fact that goats were tested once (rather than multiple times) in the present study.

G. duodenalis assemblages A, B and E were represented by three, one and 12 of 16 samples (for which sequences could be determined), with a preponderance of assemblage E (5.5% of 310). Studies in Spain [12,18], the Netherlands [33] and Uganda [34] have also reported exclusively assemblage E in goats. Although this assemblage is commonly found in ungulate hosts, particularly cattle [35], assemblages A and B, which are of significant human health concern, might also infect

these hosts in particular situations [36]. Both of these latter assemblages are commonly found in humans [3] and might be cross-transmissible from small ruminants (including goats and sheep) to humans.

For comparison, in a large, multi-institutional study of samples from 978 humans and 1,440 animals in Europe (i.e. Denmark, France, Germany, Italy, the Netherlands, Poland, Spain, Sweden and the UK) using four genetic loci (i.e., SSU, beta-giardin, glutamate dehydrogenase and *tpi*), *G. duodenalis* assemblages E, A and B were detected in 82 %, 17 % and 1 % of 207 faecal samples, respectively, from goats and sheep [37], with 78 % and 22 % of the assemblage A isolates determined to represent sub-assemblages A1 and AII, respectively. In humans, 25 %, 75 %, 56% and 44% represented sub-assemblages AI, AII, BIII and BIV, respectively [37]. In another investigation of 10 farms in Belgium, 25.5 % (35/137) of lambs and 35.8 % (53/148) of goat kids were test-positive for *Giardia* using molecular tools, targeting the beta-giardin and *tpi* genes. Although assemblage E was most commonly identified in both goat kids and lambs, assemblage A was also identified in 21.4 % of 28 goat kids, and 25 % of eight lambs. In addition, mixed infections of assemblages A and E were detected in some goat kids and lambs [14].

More recently, the genetic composition of *G. duodenalis* populations in 284 lambs was investigated in three regions in Victoria, Australia [16]. *Giardia* was detected in 43 (15.1%) of 284 faecal samples from individual sheep; the sequencing of all 43 *tpi* amplicons yielded 14 distinct sequence types, of which five of them (35.7 % of 14) corresponded to *G. duodenalis* assemblage A, and the rest (64.3 %) matched assemblage E. It was also noted that a particular sequence type representing assemblage A, which had not been reported previously in sheep, was detected in 10.6 % of the 284 sheep, and was the only type shared by all farms included in the study [16]. These studies reaffirmed the fact that the zoonotic assemblage A may be prevalent in small ruminants and may, thus, play a significant role in the transmission of giardiasis to humans.

In conclusion, although *G. duodenalis* assemblage E is reported to be dominant in goats in the present study, and in ruminants in general [3,7], this assemblage has also been detected in humans living in close contact with livestock [8], suggesting a potential for zoonotic transmission in particular epidemiological circumstances (i.e., close human-animal contact). However, more studies are needed to test this proposal. Importantly, the identification of the ‘zoonotic’ assemblages A and B in the present study suggests that *Giardia*-infected goats represent a possible source for human infections in Malaysia. Large-scale epidemiological studies of domesticated and native animals as well as humans are needed to gain better insights into the prevalence, host affiliations and geographical distributions of the different assemblages of *G. duodenalis* in Malaysia.

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Table 1

Sequences from this study (bold-type) and reference sequences from the GenBank database used for phylogenetic analysis of SSU sequence data.

Species	Origin	Country	Accession numbers	References
<i>Assemblage</i>				
<i>G. duodenalis</i>				
A	Goat	Malaysia	HQ283231, HQ283232	This study
	Ferret	Japan	AB469363, AB508812	[38]
	Water	Malaysia	FJ652976	[26]
B	“Aquatic bird”	Hungary	FJ984566	[41]
	Goat	Malaysia	HQ283228	This study
	Beaver	USA	DQ789112	[39]
	Human	Malaysia	FJ460009, FJ460012	[29]
C	“Aquatic bird”	Hungary	FJ984567	[41]
	Dog	Australia	AF113899	[40]
D	Dog	Australia	AF113900	[40]
E	Goat	Netherlands	AY826210	[33]
	Cattle	Canada	DQ448634, DQ448635	Wielinga et al. (unpublished)
	Cattle	Australia	DQ448641	Wielinga et al. (unpublished)
	Goat	Malaysia	HQ283227, HQ283229, HQ283230	This study
<i>G. ardeae</i>	Golden hamster		Z17210	[42]
<i>G. muris</i>	Mouse		AF113895	[40]

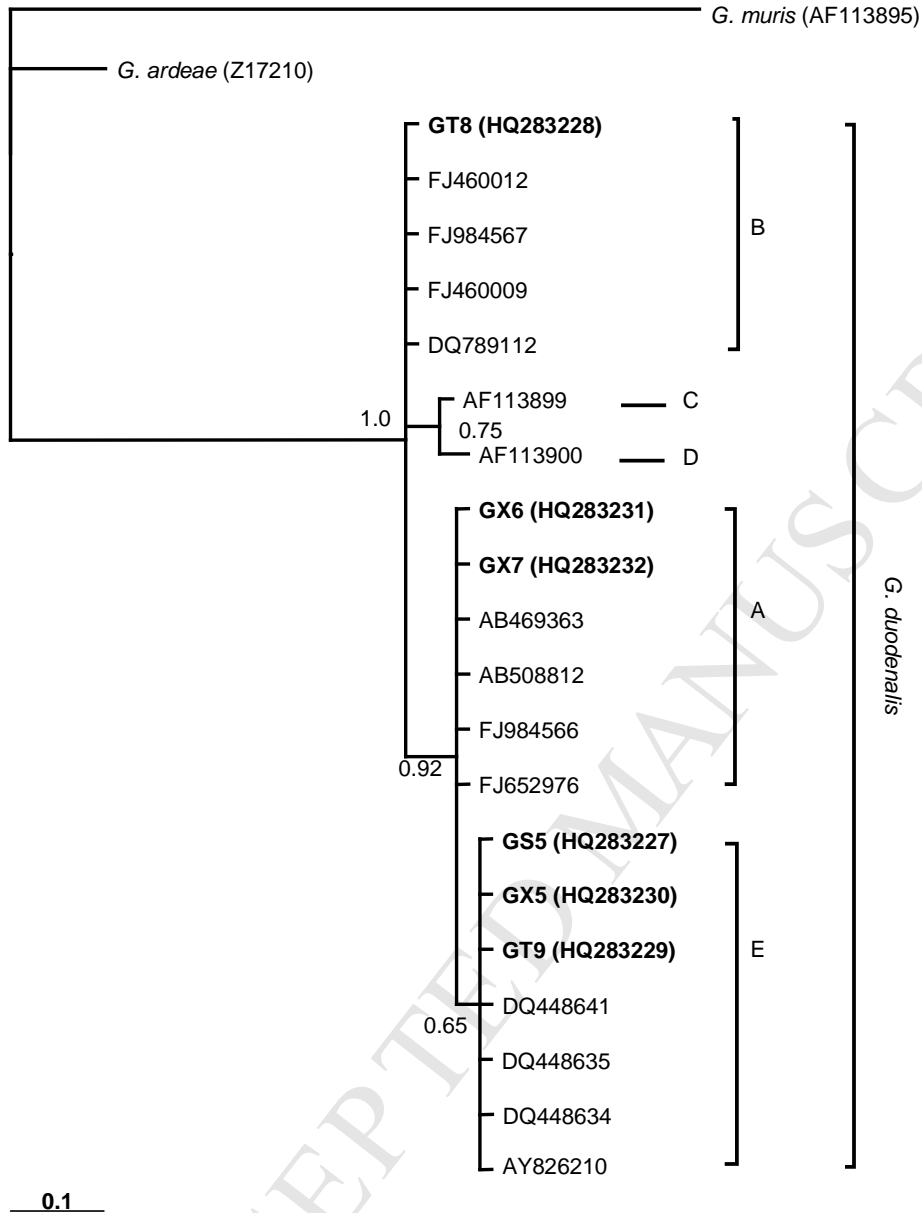


Fig. 1. The genetic relationships of *Giardia duodenalis* inferred from partial ribosomal RNA (SSU) sequence data following analysis using Bayesian Inference (BI). Sequences from the present study (bold type) as well as 16 reference sequences representing *G. duodenalis* assemblages, *G. ardeae* and *G. muris* (acquired from the GenBank database) (see Table 1) are indicated. Isolate code (accession number): GS5 (HQ283227), GT8 (HQ283228), GT9 (HQ283229), GX5 (HQ283230), GX6 (HQ283231) and GX7 (HQ283232) (see Table 1). Posterior probabilities (pp) are indicated at all major nodes.