SHORT REPORT
Mixed *Giardia duodenalis* assemblage infections in children and adults in South India

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SUMMARY

The assemblages of *Giardia duodenalis* in 25 children with and 25 children without diarrhoea and 24 adults with gastrointestinal symptoms in South India were determined. Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) targeting the glutamate dehydrogenase (*gdh*), *β-giardin* and triosephosphate isomerase (*tpi*) genes was used. The *tpi* PCR was the most sensitive and detected *G. duodenalis* in all 74 microscopy-positive samples, while *gdh* and *β-giardin* PCR were positive in 62·2% and 56·8% of the samples. Assemblage B was predominant in both children and adults (82·4%) followed by assemblage AII (9·4%); assemblage AI was not detected. Infections with both assemblages A and B (detected by *tpi* PCR–RFLP) were seen exclusively in children and the mixed assemblage BIII and BIV (detected by *gdh* PCR–RFLP) was more common in children than adults (*P* = 0·058).

Key words: Assemblage, *Giardia*, South India.

Giardia duodenalis, the causative agent of giardiasis in humans, is prevalent worldwide with a broad clinical spectrum ranging from asymptomatic infections to acute or chronic diarrhoea. Giardiasis in children in developing countries results in malnutrition, stunting and deficits in cognitive function [1]. Eight assemblages (A–H) have been described of which assemblages A and B are described as being human associated but have also been detected in a wide range of animal hosts. The other assemblages infect animals, although occasionally occur in human samples and are often linked with suppression in immunity [2, 3]. The distribution of the human-associated assemblages A and B varies geographically. There are conflicting reports on their association with symptomatic disease with some authors, including our group, finding an association with assemblage A [4, 5] and others with assemblage B [2]. Several loci have been described for determining assemblage of *Giardia* isolates including triose phosphate isomerase (*tpi*), glutamate dehydrogenase (*gdh*), *β-giardin*, small subunit ribosomal RNA (*SSU rRNA*) and elongation factor genes. Discrepancy between the loci for both human and animal isolates has been reported, possibly due to recombination or multiple infections. Some studies have suggested applying a multi-locus genotyping approach to assign assemblage type to a particular isolate (e.g. [6]). In this study, we characterized *Giardia* isolates from children and adults in South India to determine the predominant circulating assemblages using a multi-locus genotyping approach.

Faecal samples from 25 children with and without diarrhoea collected between January 2002 and July 2008 from an urban slum in Vellore district of

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Table 1. *Pairwise identity matrix of assemblage A and assemblage B sequences*

Assemblage A

	tpi					*gdh*					*β-giardin*				
			Reference sequences					Reference sequences					Reference sequences		
Samples	RFLP result	Genbank number (bp size)	EF688040 AI	U57897 AII	EU041754 AIII	RFLP result	Genbank number (bp size)	EF685701 AI	EF507677 AII	EU637582 AIII	RFLP result	Genbank number (bp size)	X14185 AI	DQ116610 AII	EU621373 AIII
AG23	AII	JN616258 (450 bp)	92·9	99·8	47·1	AII	JN616248 (414 bp)	99·5	100	51·5	A	JN616243 (511 bp)	99·6	99·8	97·9
CRI 25888	AII	JN616259(450 bp)	92·9	99·8	47·1	AII	JN616249 (419 bp)	99·3	99·8	49·2	A	JN616245 (501 bp)	98·8	99	97·4
GS9	AII	JN616260 (440 bp)	95	100	48·2	AII	JN616250 (441 bp)	98·4	98·9	49·2	A	JN616246 (453 bp)	99·6	99·6	98·5
KB193	AII	JN616261 (450 bp)	92·7	99·8	46·9	AII	JN616251 (426 bp)	99·5	100	50·2	A	JN616247 (463 bp)	99·4	99·4	98·3
CRI 8325	AII	JN616262 (436 bp)	95·6	99·8	48·4	AII	JN616252 (360 bp)	99·7	99·7	41·7	A	JN616244 (509 bp)	99·4	99·4	98·3

Assemblage B

	gdh				
			Reference sequences		
Samples	RFLP result	Genbank number (bp size)	AF069059 BIII	AY178738 BIV	L40508 BIV
AG26	BIII	JN616255 (415 bp)	99·3	97·8	97·8
AG28	BIII	JN616256 (426 bp)	97·4	97·9	97·9
GS33	BIII	JN616253 (424 bp)	98·6	97·6	97·6
AG3	BIV	JN616257 (426 bp)	98·6	99·1	99·1
CRI12635	BIV	JN616254 (426 bp)	98·4	98·8	98·8

AG, Adult with giardiasis; CRI, child with giardial diarrhoea; GS and KB, child with asymptomatic giardiasis; RFLP, restriction fragment length polymorphism.

Table 2. *Giardial assemblages determined by polymerase chain reaction–restriction fragment length polymorphism at the* tpi, gdh *and* β-giardin *loci*

Locus …	*tpi* (74)			*gdh* (46)*				*β-giardin* (42)*	
Assemblage …	AII	B	Mixed AII and B	AII	BIII	BIV	Mixed BIII and BIV	A	B
Children with diarrhoea (25)	3	18	4	3	1	3	8	3	11
Children without diarrhoea (25)	2	21	2	1	4	3	5	1	13
Adults with gastrointestinal symptoms (24)	2	22	0	1	8	6	3	1	13

* No mixed assemblage A and assemblage B infections detected.

Tamil Nadu, India with *Giardia* cysts or trophozoites identified by microscopic examination were included in this study. Samples from children with diarrhoea are referred to as 'CRI' as they were collected as part of a study on childhood rotavirus infections while samples from children without diarrhoea are referred to as *Giardia* study (GS) or Kaniyambadi area (KB). In addition, *Giardia*-positive samples from 24 adults (referred to as adult *Giardia* or 'AG') with gastrointestinal symptoms visiting Christian Medical College Hospital, Vellore collected between September and October 2008 were included. Diarrhoea in children was defined as at least three loose stools in a 24-h period and gastrointestinal symptoms for the adult subjects were defined as having any one or combination of the following symptoms or clinical diagnosis of dyspepsia or functional bowel disorder: diarrhoea, malaise, flatulence, foul smelling greasy stool, abdominal cramps, bloating, anorexia, nausea, weight loss, vomiting, fever and constipation. For samples from children enrolled in this study, informed consent was obtained from parents or guardians. Consent for samples from adults was waived as samples were taken from routine diagnostic work without patient identifiers. This study was approved by the Institutional Review Board of the Christian Medical College, Vellore.

DNA was extracted from the faecal samples using the Qiamp DNA stool minikit (Qiagen, USA) according to the manufacturers' protocol. The *tpi*, *gdh* and *β-giardin* PCR–RFLP was performed using previously published protocols with the minor modification of adding 1% DMSO to the PCR master mix [4, 7, 8]. The three markers were chosen to resolve sub-assemblages AI and AII (*tpi*), BII and BIV (*gdh*) and to differentiate human-associated assemblages (A and B) from the other, mostly zoonotic, assemblages (C–G, *β-giardin*). For confirmation of RFLP results, DNA extracted from representative samples of each assemblage determined by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) at the three loci were sequenced (MWG Biotech Pvt Ltd, India) and uploaded to Genbank (see Table 1 for accession numbers). Samples that did not have mixed infections/recombinations with more than one assemblage or sub-assemblage and for which PCR at all three loci had worked were selected. Raw sequences were then compared with reference sequences [3] in a pairwise identity matrix using Genetool version 1.0 [9]. The reference sequences used were EF688040 for *tpi* assemblage AI [10], U57897 for assemblage AII [11] and EU041754 for assemblage AIII [12]. For the *gdh* locus, the reference sequences used were EF685701 for assemblage AI [10], EF507677 for assemblage AII [13], EU637582 for assemblage AIII [6], AF069059 for BIII [14] and AY178738 and L40508 for assemblage BIV [15]. The *β-giardin* reference sequences used were X14185 for assemblage AI [16], DQ116610 for AII [17], and EU621373 for assemblage AIII.

Of the three typing methods, *tpi* PCR–RFLP was the most sensitive with amplification of DNA extracted from all 74 samples, while *gdh* and *β-giardin* PCRs amplified DNA extracted from 46 (62·2%) and 42 (56·8%) of the samples. In a previous study, Bertrand *et al*. also found *tpi* PCR to be more sensitive than *gdh* PCR [8]. The *gdh* PCR was positive in 15 children with diarrhoea (60%), 13 (52%) without diarrhoea and in 18 adults (75%) while the *β-giardin* PCR was positive in 14 children with and without diarrhoea (56%) and 14 (58%) adults. The assemblage and sub-assemblage distribution for each locus is given in Table 2. In both adults and children, assemblage B was predominant (by *tpi* PCR–RFLP). Previous studies by us as well as other authors in the Indian subcontinent have also demonstrated a

predominance of this assemblage [4, 5, 18]. The *tpi* PCR–RFLP detected a few sub-assemblage AII infections but no sub-assemblage AI. When the *gdh* PCR–RFLP was performed, both sub-assemblage BIII and BIV were detected. PCR–RFLP techniques (*gdh* and *β-giardin*) did not detect any other non-human assemblages in this population. This could indicate that zoonotic transmission is not common in this setting. However, since we did not use assemblage-specific primers, amplification of the more predominant assemblages could have occurred.

The *tpi* PCR–RFLP was better able to resolve mixed infections/recombinations with assemblages A and B since both the *gdh* and *β-giardin* PCR–RFLP typed these samples as either assemblage A or assemblage B infection alone. Interestingly, these mixed infections/recombinations with two assemblages were seen only in children (6/50), four of whom had diarrhoea indicating that they are probably mixed infections occurring in younger and possibly more susceptible individuals. Among these samples, *β-giardin* PCR identified three samples as assemblage B and one as assemblage A while *gdh* PCR identified one as an assemblage BIII and BIV co-infection, one as assemblage BIII and one as assemblage AII. Mixed-infections/recombinations with sub-assemblages BIII and BIV identified by *gdh* PCR–RFLP were also seen more frequently in children (8/15 in children with diarrhoea and 5/13 in children without diarrhoea) than adults (3/18) (Fisher's exact test, $P = 0.058$).

When a pairwise identity matrix of a few isolates identified as assemblage AII by RFLP was constructed (Table 1), *tpi* sequences were better able to resolve the sub-assemblages ($>98\%$ identity with sub-assemblage AII and $92–95\%$ with sub-assemblage AI) while *gdh* and *β-giardin* loci showed a poorer resolution. For assemblage B, *gdh* sequences of a few isolates identified as sub-assemblages BIII and BIV were analysed but were of limited use in resolving sub-assemblages. Limitations in the current sub-assemblage classification based on sequencing due to heterogeneity at these loci and the need for refinement of these techniques has been described elsewhere [19].

In our study, the *tpi* PCR was more sensitive in detecting *Giardia* infection and mixed assemblage infections/recombinations than the *gdh* or *β-giardin* PCRs. Comparison of the assemblage A sequences at the *tpi* locus in the pairwise identity matrix was more discriminatory in identifying sub-assemblages than the other loci. The predominant circulating *Giardia* assemblage in adults and children in this geographical location was found to be assemblage B and no non-human assemblages were detected suggesting a mostly anthroponotic transmission. However, more detailed studies using assemblage-specific primers and sequencing are required to corroborate findings of the absence of assemblage C–H as well as sub-assemblage AI and AIII in human samples. Studies on animal samples are also required to determine the true risk of zoonotic transmission. We also found that mixed assemblage and mixed sub-assemblage infections occur more commonly in children and the significance of this novel finding needs to be evaluated in future longitudinal studies.

DECLARATION OF INTEREST

None.

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