Molecular typing of *Giardia duodenalis* in humans in Queensland – first report of Assemblage E

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SUMMARY

Little is known about the genetic diversity of the protozoan parasite, *Giardia duodenalis*, infecting humans in Queensland, Australia. The present study typed 88 microscopically *Giardia*-positive isolates using assemblage-specific primers at the triose phosphate isomerase (*tpi*) gene and sequenced a subset of isolates at the glutamate dehydrogenase (*gdh*) gene ($n = 30$) and *tpi* locus ($n = 27$). Using the *tpi*-assemblage specific primers, *G. duodenalis* assemblage A and assemblage B were detected in 50% (44/88) and 38·6% (34/88) of samples, respectively. Mixed infections with assemblages A and B were identified in 4·5% (4/88) and assemblage E was identified in 6·8% (6/88) of samples. Sequence analysis at the *gdh* and *tpi* loci also confirmed the presence of assemblage E in these isolates. Cyst numbers per gram of feces (g^{-1}) were determined using quantitative polymerase chain reaction and of the isolates that were typed as assemblage E, cyst numbers ranged 13·8–$68{\cdot}3 \times 10^6$ cysts g^{-1}. This is the first report of assemblage E in humans in Australia, indicating that in certain settings, this assemblage may be zoonotic.

Key words: *Giardia duodenalis*, assemblage E, humans, zoonotic.

INTRODUCTION

Giardia duodenalis (syn. *Giardia intestinalis* and *Giardia lamblia*), is a protozoan enteric parasite that causes acute, watery diarrhoea or giardiasis in 280 million people annually and is a common cause of waterborne outbreaks (Lane and Lloyd, 2002; Baldursson and Karanis, 2011; Painter *et al*. 2015; Einarsson *et al*. 2016). Most infections are self-limiting but chronic infections can lead to weight loss and malabsorption (Ryan and Cacciò, 2013) and infections are associated with stunting (low height for age), wasting (low weight for height) and cognitive impairment in children in developing countries (Berkman *et al*. 2002; Feng and Xiao, 2011). Furthermore, acute giardiasis may disable patients for extended periods and can elicit protracted post-infectious syndromes, including irritable bowel syndrome and chronic fatigue (Hanevik *et al*. 2014). In Australia, the overall prevalence is ~2 to 7%, with a steady increase of reports each year (Asher *et al*. 2016). *Giardia* is responsible for ~614, 740 sporadic cases of acute gastroenteritis per year in Australia and a disease burden of 4·3 disability-adjusted life years (Gibney *et al*. 2014).

Giardia duodenalis consists of eight genetic assemblages (A–H) with different host specificities; assemblage A in humans, livestock and other mammals; B in humans, primates and some other mammals, C and D in dogs and other canids; E mainly in hoofed animals including cattle, sheep and goats, and more recently in rabbits, non-human primates and humans; F in cats and humans, and more recently in cattle; G in rats and mice; and H in marine mammals (Andrews *et al*. 1989; Mayrhofer *et al*. 1995; Ey *et al*. 1997; Monis *et al*. 1998; Ryan and Cacciò, 2013; Cardona *et al*. 2015; Qi *et al*. 2015). Of these, assemblages A and B are the predominant assemblages in humans and exhibit a broad host range including cattle, sheep, pigs, horses, non-human primates, dogs, cats and fish (Yang *et al*. 2010*a*; Feng and Xiao, 2011; Ghoneim *et al*. 2012; Ryan and Cacciò, 2013; Durigan *et al*. 2014). The remaining assemblages are considered to be largely host-specific. However recent studies have reported the occurrence of animal assemblages in humans including assemblage F in Ethiopia (Gelanew *et al*. 2007), assemblage E in Egypt and Brazil (Foronda *et al*. 2008; Helmy *et al*. 2014; Abdel-Moein and Saeed, 2016; Fantinatti *et al*. 2016; Scalia *et al*. 2016), assemblage C in China and Slovakia (Liu *et al*. 2014; Štrkolcová *et al*. 2015) and assemblage D in German travellers (Broglia *et al*. 2013). Very little is known about the prevalence and genetic diversity of *Giardia* assemblages infecting humans in Queensland, Australia. To date, only two studies have investigated the molecular characteristics of *Giardia* species in human patients from Queensland (Nolan *et al*. 2011; Ebner *et al*. 2015), with the latter reporting a prevalence of 1·9% for Northern Queensland and Darwin, while a prevalence of up to 12% in Queensland patients has been reported using

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microscopy (Boreham and Phillips, 1986). The aim of the present study therefore was to examine *G. duodenalis* assemblages in human patients suffering from sporadic cases of giardiasis in order to better understand the epidemiology and transmission dynamics of this ubiquitous parasite in Queensland.

MATERIALS AND METHODS

Samples and DNA extraction

A total of 88 faecal samples diagnosed as *Giardia*-positive by microscopy (collected from patients from rural and urban areas in South-East Queensland) were shipped to Murdoch University by Department of Health (Queensland) staff under Murdoch University Human Ethics permit 2014/159. All samples were stored at 4 °C until analysed. Genomic DNA was extracted from 250 mg of each faecal sample using a Power Soil DNA Kit (MO BIO, Carlsbad, California). A negative control (no faecal sample) was used in each extraction group.

Quantitative polymerase chain reaction (qPCR) determination of cysts per gram of feces

All samples were initially amplified by qPCR analysis of the glutamate dehydrogenase (*gdh*) locus as previously described (Yang *et al*. 2014). Copy numbers detected were converted to cyst numbers on the basis that the *gdh* gene in *Giardia* is a single copy gene (Yee and Denis, 1992) and the fact that there are 4 haploid nuclei per cyst. Therefore, every 4 copies of *gdh* detected by qPCR were equivalent to 1 cyst.

Molecular typing

All samples were typed at the triose phosphate isomerase (*tpi*) locus using assemblage A, B and E specific primers as previously described (Sulaiman *et al*. 2003; Geurden *et al*. 2008; Levecke *et al*. 2009). A representative subset of samples were also amplified using a nested PCR and sequenced at the glutamate dehydrogenase (*gdh*) locus ($n = 30$) as described by Read *et al*. (2004) and at the *tpi* ($n = 27$) locus using the assemblage-specific primers described above. Briefly, the amplified DNA from secondary *tpi* and *gdh* PCR products were separated by gel electrophoresis and purified for sequencing using an in-house filter tip method (Yang *et al*. 2013). Purified PCR products were sequenced independently using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions at 60 °C annealing temperature for PCR products at the *gdh* locus and 64, 62 and 67 °C annealing temperature for assemblages A, B and E *tpi* PCR products, respectively (Read *et al*. 2004; Geurden *et al*. 2008; Levecke *et al*. 2009). Sanger sequencing chromatogram files were imported into Geneious Pro 8·1·6 (Kearse *et al*. 2012), where they were analysed, edited and aligned with reference sequences from GenBank using MUSCLE (Edgar, 2004) and trimmed.

Phylogenetic analysis

Phylogenetic analysis for both loci was conducted using Distance and Maximum Likelihood (ML) analysis in MEGA6 (after selection of the best nucleotide substitution models) (Tamura *et al*. 2013). Distance estimation was conducted based on evolutionary distances calculated with the Tamura-Nei model and grouped using Neighbour-Joining (NJ). ML was conducted using the Tamura 3-parameter model. The confidence of groupings was assessed by bootstrapping, using 1000 replicates.

RESULTS

Giardia duodenalis assemblage prevalences

Assemblage A and B were detected in 50% (44/88) and 38·6% (34/88) of samples respectively. Mixed A and B assemblages were identified in 4·5% (4/88) and assemblage E in 6·8% (6/88) of samples (Table 1). Information on age at the time of collection was only available for a subset of samples ($n = 25$) and of these, ages ranged from 3 months to 79 years. Information for three of the samples typed as assemblage E was available (Qld86, Qld81 and Qld82) and their ages were 11, 19 and 67 years, respectively and were from urban (Qld86, Qld81) and rural locations. Information on contact with animals was not available.

Molecular typing at the gdh and tpi loci

Phylogenetic analysis at the *gdh* and *tpi* loci using both Distance and ML analysis produced trees of similar topology (data not shown) and therefore only NJ trees are shown. Assignment to assemblage based on sequence analysis of the *gdh* and *tpi* loci was 100% concordant at the assemblage level between the two loci (i.e. all isolates typed as assemblage A by assemblage-specific PCR and sequence analysis of the *tpi* locus were also assemblage A at the *gdh* locus (Table 1). There were however differences at the sub-assemblage level. For example, at the *gdh* locus, all the assemblage A isolates that were typed ($n = 11$) were AII and exhibited 100% similarity to a Queensland human AII subtype from a Brisbane patient (AY178737) and an AII subtype from an Australian sheep (KY083429) (Fig. 1 – NJ tree and Table 1). At the *tpi* locus, two isolates (Qld 44 and Qld71) were typed as AI, while the remaining assemblage A isolates ($n = 8$) were typed as AII

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Table 1. *Giardia duodenalis* assemblages and sub-assemblages (where available) and cysts per gram of feces detected in *Giardia*-positive human faecal samples from Queensland, Australia

Sample ID	Cysts $g^{-1} \times 10^6$	Assemblage-specific primers at *tpi* locus	*tpi* sequence data	*gdh* sequence data
QLD 2	2·3	B	–	–
QLD 3	63·1	B	–	–
QLD 4	3·8	A	–	–
QLD 5	21·2	B	–	–
QLD 6	8·9	A	–	–
QLD 8	3·8	A	AII	AII
QLD 9	3·2	A	–	–
QLD 10	9·6	A	–	–
QLD 11	14·5	A	–	–
QLD 12	32	B	–	–
QLD 13	11·4	A	AII	AII
QLD 14	24·3	B	–	–
QLD 15	67·2	B	BIV	BIV
QLD 16	58·7	B	–	BIV
QLD 17	19·2	A	–	–
QLD 18	4·5	B	BIII	BIV
QLD 19	39·8	B	BIV	BIV
QLD 20	12·4	A	–	–
QLD 21	11·6	A	AII	AII
QLD 22	0·8	A	AII	AII
QLD 24	6·6	B	–	–
QLD 25	28·7	B	–	–
QLD 26	20·4	B	–	–
QLD 27	72·2	B	BIV	BIV
QLD 28	15·6	B	–	–
QLD 29	1·2	A	–	–
QLD 30	45·9	A + B	–	–
QLD 31	11·8	A	–	–
QLD 32	6·3	B	–	–
QLD 33	9·7	B	–	–
QLD 34	26·5	B	BIV	BIV
QLD 36	16·1	A + B	–	–
QLD 37	18·6	B	–	–
QLD 39	56·1	B	–	–
QLD 41	12·6	B	–	–
QLD 42	38·2	B	–	BIV
QLD 43	23·8	B	–	–
QLD 44	11·6	A	AI	AII
QLD 45	0·4	A	–	–
QLD 46	1·9	A	–	–
QLD 47	8·8	A	AII	AII
QLD 48	6·2	B	–	–
QLD 49	2·2	A	–	–
QLD 50	18·4	A	–	–
QLD 51	11·9	A	AII	AII
QLD 52	9·6	A	AII	AII
QLD 53	0·9	A	–	–
QLD 54	24·9	E	E	E
QLD 55	122	B	–	–
QLD 56	13·8	E	E	E
QLD 57	42·5	E	E	E
QLD 58	88·1	B	–	–
QLD 59	19	B	BIV	BIV
QLD 60	26·6	A	–	–
QLD 61	48·3	B	–	–
QLD 62	22·6	A	–	–
QLD 63	3·7	A	AII	AII
QLD 64	5·6	A	–	–
QLD 65	14·1	B	–	–
QLD 66	6·2	A	–	–
QLD 67	14·4	B	–	–
QLD 68	53·8	B	–	–
QLD 69	44·8	A + B	–	–
QLD 70	52·2	A + B	–	–
QLD 71	33·8	A	AI	AII

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Table 1. (*Cont.*)

Sample ID	Cysts $g^{-1} \times 10^6$	Assemblage-specific primers at *tpi* locus	*tpi* sequence data	*gdh* sequence data
QLD 72	18·9	A	–	AII
QLD 73	144	B	–	–
QLD 74	84·1	B	–	–
QLD 75	92·6	B	BIV	BIV
QLD 76	37·2	B	BIV	BIV
QLD 77	19·2	B	–	–
QLD 78	58·8	B	–	–
QLD 79	64·7	B	–	–
QLD 80	22	A	–	–
QLD 81	68·3	E	E	E
QLD 82	55·8	E	E	E
QLD 83	12·2	A	–	–
QLD 84	82·8	B	–	–
QLD 85	13·5	B	–	–
QLD 86	39·8	E	E	E
QLD 87	58·8	B	–	–
QLD 88	18·2	B	BIV	BIV
QLD 89	66·8	A	–	–
QLD 90	56·9	A	–	–
QLD 91	92·4	B	–	–
QLD 92	106	B	BIV	BIV
QLD 93	98·4	B	–	–
QLD 94	132	B	BIII	BIV

N/A, not available; "–", not sequenced.

(Fig. 2 – NJ tree and Table 1). At the *gdh* locus, four of the assemblage B isolates (Qld16, Qld18, Qld42 and Qld94) grouped with an Australian reference BIV subtype (L40508), but exhibited 5 single nucleotide polymorphisms (SNP's) from the reference subtype across 666 bp of sequence. The remaining assemblage B isolates also grouped in the BIV clade but exhibited 7 SNP's with isolate VANC/94/UBC/125 (KP687771), across 699 bp of sequence. At the *tpi* locus, two isolates (Qld18 and Qld94) grouped with sub-assemblage BIII (100% similarity across 355 bp of sequence), while the remaining assemblage B isolates ($n = 9$) were 100% identical to a BIV sub-assemblage from a foal (KM926543) across 319 bp of sequence. At both the *tpi* and *gdh* locus, six isolates (Qld54, Qld56, Qld57, Qld81, Qld82, and Qld86) grouped in a clade with assemblage E. At the *gdh* locus, all six were 100% identical to a Western Australian (WA) cattle-derived assemblage E isolate (HQ398327), while at the *tpi* locus, all six were identical to a sheep-derived assemblage E isolate from Victoria (Vic) (GQ444454). Representative sequences have been submitted to GenBank under accession numbers KY655475- KY655485.

Cysts per gram of feces

Cyst numbers per gram of feces (g^{-1}) were determined using qPCR and ranged from $0{\cdot}8 \times 10^6$ to 106×10^6 cysts g^{-1} (Table 1). Of the isolates that were typed as assemblage E, cyst numbers ranged from 13·8 to $68{\cdot}3 \times 10^6$ cysts g^{-1}.

DISCUSSION

In the present study, assemblage A and B were detected in 50% (44/88) and 38·6% (34/88) of samples, respectively. Mixed A and B assemblages were identified in 4·5% (4/88) and assemblage E in 6·8% (6/88) of samples. The predominance of assemblage A over B is in contrast with the findings from other studies of *Giardia* in humans in Australia. Previous studies have reported ~70–75% assemblage B and 25–30% assemblage A ($n = 23$–124) in Western Australia (Read *et al*. 2002; Yang *et al*. 2010*b*), 69% B ($n = 9$) and 31% A ($n = 4$) in patients from parts of the tropical North of Australia (Ebner *et al*. 2015) and 86·1% B and 12·7% A ($n = 165$) in New South Wales (Asher *et al*. 2016). Current evidence indicates that, on a global level, assemblage B is slightly more prevalent than assemblage A in both developed and developing countries (Feng and Xiao, 2011). The only two previous studies, which analysed a small number of *Giardia* isolates from Queensland, identified both assemblage A and B (Nolan *et al*. 2011; Ebner *et al*. 2015).

Analysis of genetic variability within assemblages has shown that isolates of assemblage A can be divided into four sub-assemblages (AI, AII, AIII and AIV) by protein polymorphisms of 23 loci (Monis *et al*. 1996, 2003), with human isolates

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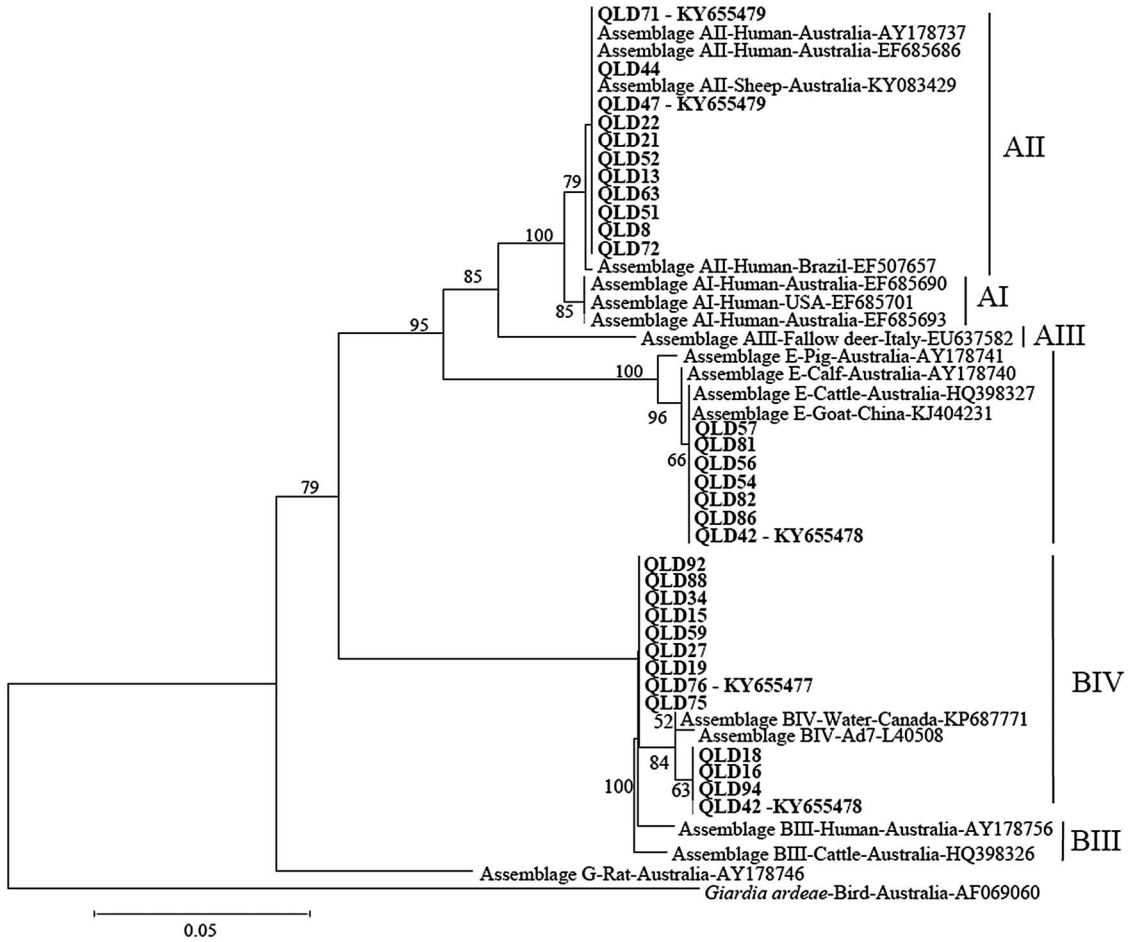

Fig. 1. Phylogenetic analysis of partial *Giardia* glutamate dehydrogenase (*gdh*) sequences amplified from human faecal samples inferred from Neighbour-Joining (NJ) analysis of Kimura's distances calculated from pair-wise comparisons. Percentage support (>50%) from 1000 pseudoreplicates from NJ analyses is indicated at the left of the supported node. Isolates from this study are indicated in bold font.

belonging to AI and AII and animal isolates belonged to AI, AIII and AIV (Monis *et al*. 2003; Cacciò *et al*. 2008; Sprong *et al*. 2009; Ryan and Cacciò, 2013). In the present study, although there was 100% concordance between loci in assignment to assemblage, there were some differences in assignment to sub-assemblage level. Of the subset of assemblage A isolates that were sequenced at the *gdh* locus ($n = 30$), all were AII, while at the *tpi* locus, two of the 10 assemblage A isolates were typed as AI. Sub-assemblages AI and AII are found in both humans and animals, with sub-assemblage AI preferentially found in livestock and pets, whereas sub-assemblage AII is predominantly found in humans (Sprong *et al*. 2009). Previous studies in Australia have also reported that AII is the predominant sub-assemblage in humans (Yang *et al*. 2010*b*; Asher *et al*. 2016). It is also the dominant sub-assemblage in sheep across Australia (Yang *et al*. 2014) and therefore zoonotic transmission of AII is possible.

Within assemblage B, sub-assemblages BI, BII, BIII and BIV have been described by enzyme electrophoresis, with human isolates forming two clusters (BIII and BIV) and animal isolates (monkeys and a dog) belonging to sub-assemblages BI and BII (Monis *et al*. 2003). However, BIII and BIV sub-assemblages identified by allozyme electrophoresis are not always supported by DNA sequence analysis as subtyping analyses of field isolates produced inconsistent sub-assemblages among different loci (Feng and Xiao, 2011). In the present study, all the assemblage B isolates typed at the *gdh* locus ($n = 13$) are considered BIV variants, whereas at the *tpi* locus, two of the 11 assemblage B isolates were typed as BII, with the remainder BIV. Lack of concordance in the assignment to assemblages and sub-assemblages has been reported in many recent studies (Cacciò *et al*. 2008; Ryan and Cacciò, 2013) and is explained by either mixed infections or recombination (Cacciò and Sprong, 2010). In the first case, two different assemblages/sub-assemblages are responsible for the infection of a single host and their detection depends on the relative proportion (with the majority population being favoured by PCR) and on the lack of bias during amplification. In the second case, genetic exchanges within or among assemblages can create

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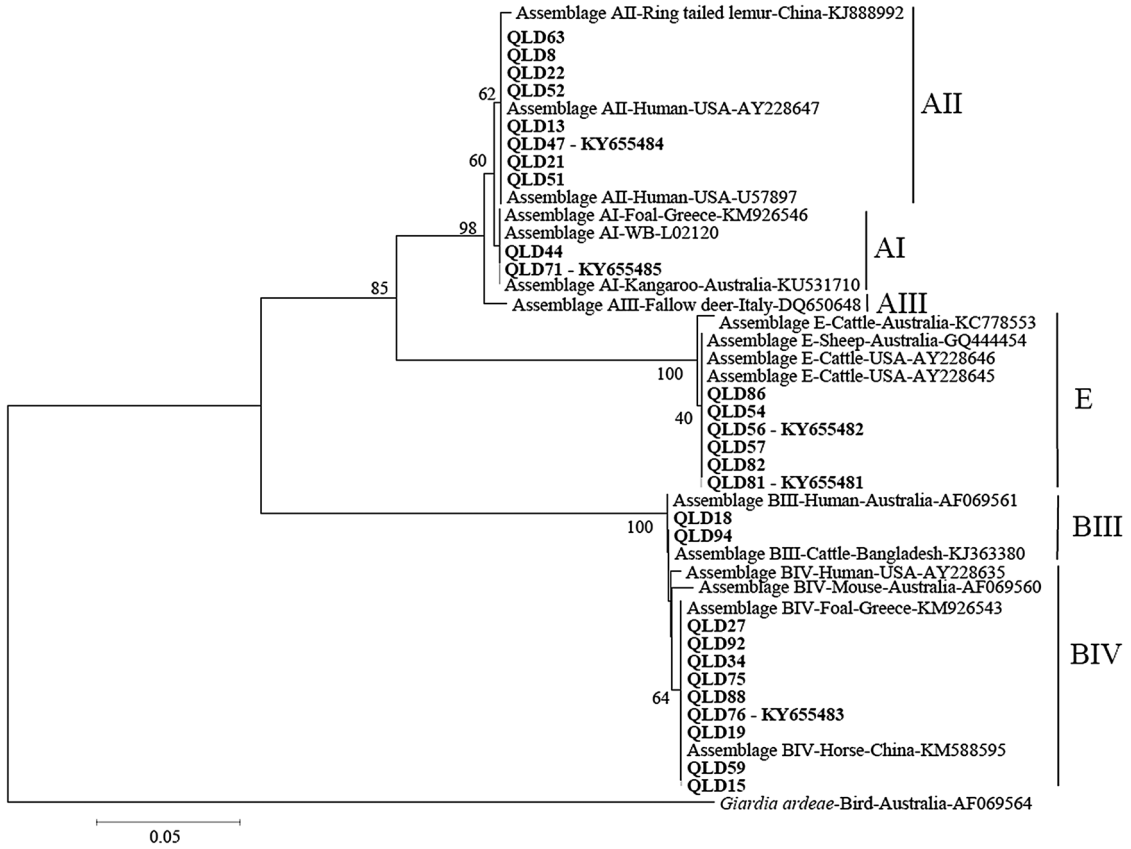

Fig. 2. Phylogenetic analysis of partial *Giardia* triose phosphate isomerase (*tpi*) sequences amplified from human faecal samples inferred from Neighbour-Joining (NJ) analysis of Kimura's distances calculated from pair-wise comparisons. Percentage support (>50%) from 1000 pseudoreplicates from NJ analyses is indicated at the left of the supported node. Isolates from this study are indicated in bold font.

recombinants that may contain specific sequences of assemblage A in a genome of assemblage B (or recombinants within sub-assemblages).

At both the *gdh* and *tpi* loci, six isolates were typed as assemblage E. At the *gdh* locus, all six were 100% identical to a WA cattle-derived assemblage E isolate (HQ398327) and at the *tpi* locus, were 100% identical to a Victorian sheep isolate (GQ444454). This is the first report of assemblage E in humans in Australia. A previous study in Egypt reported a high prevalence of assemblage E – 62·5% (25/40) in children living in agricultural areas in Egypt (Abdel-Moein and Saeed, 2016). In that study, assemblage E was detected in 42·1% of *Giardia*-positive diarrheic and 81% of non-diarrheic children suggesting that assemblage E may cause clinical giardiasis (Abdel-Moein and Saeed, 2016). The high prevalence of assemblage E was attributed to the fact that the children lived in rural villages with large cattle populations (Abdel-Moein and Saeed, 2016). Other studies in Egypt have reported assemblage E in 15 and 11·1%, respectively, of *Giardia* positive human samples (Foronda *et al*. 2008; Helmy *et al*. 2014). In Brazil, assemblage E was identified in 34% (15/44) of *Giardia*-positive samples amongst preschoolers (aged between 10 months and 4 years) in a community of Rio de Janeiro (Fantinatti *et al*. 2016). In that study, all samples were collected from children attending a day-care unit located in the slum with no sewerage network coverage, and stray animals including pigs and cattle moving throughout the location (Fantinatti *et al*. 2016).

Assemblages A and E are common amongst sheep and cattle in Australia with assemblage E the most dominant assemblage in hoofed animals (Nolan *et al*. 2010; Ng *et al*. 2011; Abeywardena *et al*. 2013; Yang *et al*. 2014; Asher *et al*. 2016). In the present study, the individuals that were positive for Assemblage E were experiencing diarrhoea, came from both rural and urban areas and shed variable levels of cysts in their feces. All were identical to assemblage E from Australian cattle and sheep, suggesting possible zoonotic transmission. The data generated demonstrates that zoonotic transmission from cattle and sheep may be occurring and warrants further investigation.

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