Prevalence and clinical correlations of genetic subtypes of *Giardia lamblia* in an urban setting

A. S. BREATHNACH, T. D. McHUGH and P. D. BUTCHER

Prevalence and clinical correlations of genetic subtypes of *Giardia lamblia* in an urban setting

A. S. BREATHNACH[1]*, T. D. McHUGH[2] AND P. D. BUTCHER[1]

[1] *Centre for Infection, Division of Cellular and Molecular Medicine, St George's University of London, London, UK*
[2] *Department of Infection, Royal Free Campus, University College London, London, UK*

SUMMARY

The clinical significance of different genetic subtypes or assemblages of *Giardia lamblia* is uncertain. Cases of giardiasis in south-west London between 1999 and 2005 were studied, comparing molecular-typing results with clinical and epidemiological findings from routine surveillance. We identified 819 cases, of whom 389 returned surveillance questionnaires. A subset of 267 faecal samples was submitted for typing by sequencing of the triose phosphate isomerase (*tpi*) and ribosomal RNA genes, and/or a separate duplex PCR of the *tpi* gene. Typing was successful in 199 (75%) samples by at least one of the molecular methods. Assemblage A accounted for 48 (24%) samples and Assemblage B for 145 (73%); six (3%) were mixed. Both assemblages had similar seasonality, age distribution and association with travel. Clinical features were available for 59 successfully typed cases: both assemblages caused similar illness, but Assemblage A was significantly more frequently associated with fever than Assemblage B.

Key words: Gastrointestinal infections, genetics, *Giardia lamblia*, molecular epidemiology, typing.

INTRODUCTION

Giardia lamblia (syn. *G. duodenalis* or *G. intestinalis*) is a human and animal intestinal protozoan parasite and is a frequent cause of diarrhoea. Repeated or chronic infection is common in areas of poverty and overcrowding [1]. In affluent countries, rates are lower, but when hygiene breaks down (e.g. in children's nurseries, or in association with travel abroad, camping and recreational water contact, or as a result of contaminated drinking water systems) rates can be similar to those seen in developing countries [2–4].

Molecular techniques developed in the 1980s and 1990s have revealed a rich diversity of genetic subtypes within the species *G. lamblia* [5–7]. As currently understood, there are two main genogroups or genetic assemblages of *G. lamblia* which infect humans – Assemblage A and Assemblage B [8]. These genogroups are also found in animals, and have been divided into subassemblages: AI, AII, BIII and BIV. A further subassemblage (AIII) has recently been described, but only in animals [9]. Other genogroups also affect animals, but are rarely or never found in humans: genogroups C and D in dogs [6], genogroup E in ruminants [5], and genogroups F and G in cats and rats, respectively [7].

Assemblages A and B express different surface proteins [10], and differ in their ability to be cultivated *in vitro* [11]; recent genomic studies suggest that the

* Author for correspondence: Dr A. S. Breathnach, Centre for Infection, Division of Cellular and Molecular Medicine, St George's University of London, London SW17 0RE, UK.
(Email: aodhan.breathnach@stgeorges.nhs.uk)

genetic differences are so great that they should be regarded as different species [12]. These differences could lead to differences in the organisms' ability to spread and cause disease, although the degree to which this occurs is uncertain. The purpose of this study was to examine cases of giardiasis in an urban cosmopolitan setting in the UK, to determine the prevalence of different genetic subtypes and whether these subtypes differed in terms of clinical or epidemiological features.

MATERIALS AND METHODS

Clinical samples and surveillance data

Cases of microscopically confirmed giardiasis occurring in south-west London between 1999 and 2005 were studied. Most cases were symptomatic; a small proportion (<2%) were detected when asymptomatic contacts of symptomatic cases were screened.

The three hospital diagnostic microbiology laboratories in the area (St George's, St Helier's, Kingston hospitals) were asked to provide faecal samples that had been found to contain *Giardia* cysts. Two laboratories used direct microscopy, and one laboratory used a commercial concentration procedure (Parasep™, DiaSys, UK) before microscopy. Samples were stored (not formalinized) at 4 °C until analysis.

The five environmental health departments in the area (Kingston-upon-Thames, Richmond-upon-Thames, Merton, Sutton, Wandsworth) were asked to provide surveillance data on cases of giardiasis that had been notified to them. These departments investigate giardiasis using a standard postal questionnaire. These surveillance data were combined with demographic information included on the laboratory request form.

Descriptive statistical methods were employed, using an electronic spreadsheet program (Microsoft Excel version 9.0). Tests of significance included Fisher's exact test, the Mann–Whitney U test, and where appropriate, 95% confidence intervals.

DNA extraction and purification

Whole faecal DNA extraction and purification was performed using the Boom method [13] as modified by McLauchlin *et al.* [14]. In brief, 200 mg faecal samples were homogenized with 0·5 mm zirconia/silica beads, to disrupt cysts, with isoamyl alcohol to reduce foaming. DNA was extracted with guanidine isothiocyanate (GuSCN), with binding to silica beads. Following removal of denatured proteins, fats and other faecal material by repeated washing with GuSCN buffer, alcohol and acetone, the DNA was eluted with 150 μl water. Samples which were negative by initial PCR were further purified with polyvinyl pyrrolidone (PVP) to remove residual PCR inhibitors [15]. Control DNA was from two axenic strains of *G. lamblia*: PO-1 [16] and NESS, a strain isolated from a human case in Scotland (H. V. Smith, Scottish Parasite Diagnostic Laboratory, personal communication).

Sequenced-based typing

Sequence-based typing at the ribosomal RNA gene (*rRNA*) and triose phosphate isomerase gene (*tpi*) loci was performed by the following method. Candidate gene sequences were retrieved from the Genbank sequence database [17]. Multiple sequences for the same genes were aligned using the ClustalW program, available from the website of the European Bioinformatics Institute (EBI) [18]. Where possible, gene sequences representing divergent species of *Giardia* were included, including *G. lamblia*, *G. ardeae*, *G. muris* and *G. microti*, in order that genus-specific primers could be selected. Candidate primers were selected from conserved areas, and checked for compatibility and stability using the Netprimer program [19].

Primer oligonucleotides (Table 1) were synthesized by Invitrogen Corp. (USA). These were suspended in distilled water to a concentration of 50 pmol/μl. PCR reaction mixtures were prepared with 10 μl faecal DNA extract and 40 μl mastermix, with a final concentration of 1× PCR buffer (Invitrogen), 0·2 mM dGTP, 0·2 mM dCTP, 0·2 mM dATP, 0·2 mM dTTP, 1·5 mM MgCl$_2$, 1 μM of each primer, 2·5 U *Taq* polymerase (Platinum™, Invitrogen Corp.) per reaction, 0·2× Q-solution (Qiagen GmbH, Germany). The mastermix was prepared on ice, and nested or heminested PCRs were performed with a hot-start protocol: 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min, finishing with 7 min at 72 °C. For the second-round reaction, 10 μl of a 1/20 dilution of the first-round product was processed using the same conditions. PCR products were detected by gel electrophoresis.

Sequencing was performed by Qiagen GmbH using the Sanger chain termination method [20]. Sequencing

Table 1. *Primers used in the rRNA and* tpi *PCRs*

PCR target	Primer name (function)	Sequence (5′–3′)*	T_m	GC%
rRNA gene	r37fw (outer forward)	caagg acrma agcca tgc	59 °C	56%
	r883rev (outer reverse)	ccttc aagtt ycagc cttgc	60 °C	50%
	r275fw (inner forward)	ggagr atcag ggtty gactc	59 °C	55%
	r780rev (inner reverse)	gttta cgsyc gggaa tacg	62 °C	58%
tpi gene	tpi1f† (outer & inner forward)	ccctt catcg gyggt aac	58 °C	61%
	tpi479r (outer reverse)	cccgt gccra trgac cacac	61 °C	60%
	tpi300r (inner reverse)	agrgc acgct tmgcc ttc	60 °C	66%

T_m, Melting temperature; GC, guanine-cytosine content.

* Key to degenerate nucleotides in primer sequences: m=a/c; r=a/g; s=g/c; y=c/t.

† Primer tpi1f was used as the forward primer in both rounds of a hemi-nested reaction.

Predicted *rRNA* gene PCR product sizes, in base pairs: 847 (outer), 506 (inner).

Predicted *tpi* gene PCR product sizes: 518 (outer), 339 (inner).

was done in both the forward and reverse directions. ClustalW was used to align sequences with comparator *Giardia* sequences of known assemblage. Phylogenetic trees of these alignments were generated using two methods: the neighbour-joining method [21], using the ClustalX program, available from the Clustal website [22] and a maximum parsimony algorithm within the PHYLIP set of programs [23]. The trees were visualized using NJPlot, a tree-drawing program available from Pôle Bio-Informatique Lyonnais [24], and bootstrap analysis was performed using PHYLIP. Sequences were assigned to Assemblages A or B according to which Genbank sequence they most closely aligned with.

Duplex PCR-based typing

This was a hemi-nested PCR for *tpi*, as described previously [25]: the first round was duplex, with separate primers for Assemblages A and B; the second round consisted of separate PCR reactions for each assemblage. As each reaction yielded different sized fragments (Assemblage A product 452 bp, Assemblage B product 141 bp), gel visualization of the PCR product could distinguish the two assemblages. This method also enabled mixed infections of Assemblages A and B to be detected.

Sequence data accession numbers

DNA sequences from this project are available in Genbank [17]: *tpi* accession numbers FM242108–FM242136; *rRNA* gene accession numbers FN252252–FN252290.

RESULTS

Demographics of patients

Between 1999 and 2005, 819 cases of giardiasis were diagnosed in the three laboratories in south-west London. Environmental health questionnaires were returned by 389/819 cases.

The age distribution was bimodal, with peaks in infancy and in adults in their 30s (Fig. 1). The sex distribution was 47·7% male, 52·3% female. Outbreaks (as defined by two or more linked cases) were common, with 101 separate outbreaks reported involving 170 (21%) of the cases; but 95 of these outbreaks were small, single household clusters: the mean number of study cases in each outbreak was 1·6.

Recent travel was recorded in 208/389 (53%) questionnaire responses; 104 (27%) patients indicated they had not travelled and the remainder left the question unanswered.

Stool sample selection for molecular analysis

Of the 819 identified cases, 574 had samples submitted for this study. Only 144 cases had both samples submitted and questionnaires returned. Samples that had clinical and/or epidemiological information available were favoured for molecular analysis. On this basis, 267/574 available stool samples were selected for DNA extraction and PCR analysis (Fig. 2).

Sequence-based typing

PCR for the *rRNA* and *tpi* genes was performed on 62 DNA extracts. Products of the predicted size were
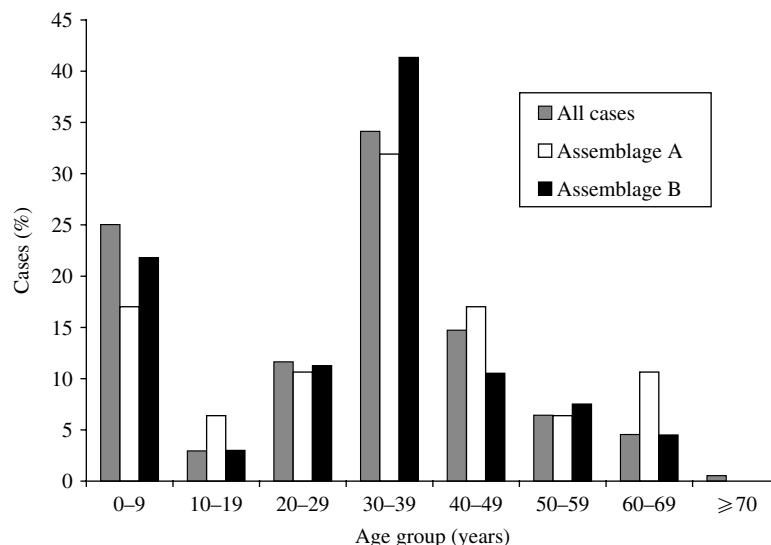
Fig. 1. Age distribution of cases of giardiasis, for all cases where age was recorded ($n = 747$), and Assemblage A ($n = 47$) and Assemblage B ($n = 133$). Mixed infections excluded.
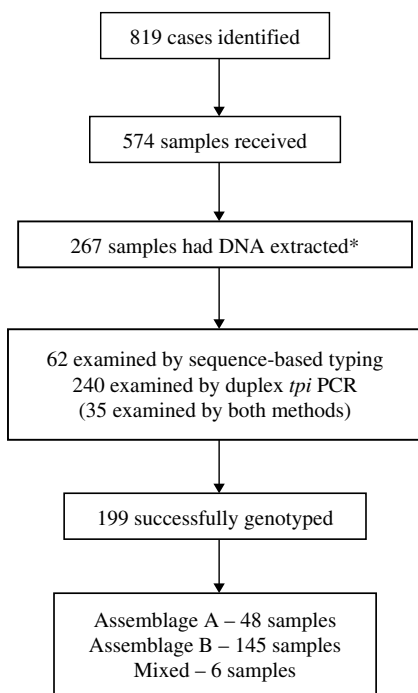

Fig. 2. Flow chart showing numbers of cases of giardiasis identified and samples received, and numbers subsequently analysed and successfully genotyped. (* Samples with epidemiological or clinical information were preferred for molecular analysis.)

obtained from 41/62 *rRNA* and 48/62 *tpi* PCR reactions. From these PCR products 39 *rRNA* gene sequences and 28 *tpi* sequences were obtained. A positive sequence for one or both genes was obtained from 45 samples.

Phylogenetic trees generated from the sequence data showed that all clinical samples clustered with either Assemblage A or Assemblage B; there were no intermediate clusters (Fig. 3). Different tree-generating algorithms, as well as bootstrapping, were used in order to assess the reliability of any inferences being made. Clustering was concordant between the two genes for all samples, and the trees generated by both neighbour-joining and maximum parsimony methods were similar. It was possible to assign 14 (31%) isolates to Assemblage A, and 31 (69%) to Assemblage B, by one or both sequencing reactions.

The *rRNA* gene sequences did not reveal any sub-assemblages, but the *tpi* sequences had subgroups which aligned with known Assemblage BIII and BIV sequences (Fig. 3). The *tpi* sequences of all Assemblage A clinical samples clustered with Assemblage AII – none aligned with AI. The *tpi* sequences showed most variation between the two assemblages, at 57/246 bp aligned (23%), compared to only four differences out of 431 aligned base pairs for the *rRNA* gene sequences (0·9%).

Duplex PCR-based typing

Duplex PCR-based typing was performed on 240 DNA extracts. Of these, 35 had already been typed by sequencing, to ensure concordance between the two methods. Molecular typing was successful for 164 (70%) samples. Assemblage A accounted for 36 (22%) samples and Assemblage B for 125 (75%); six (4%) samples were mixed.
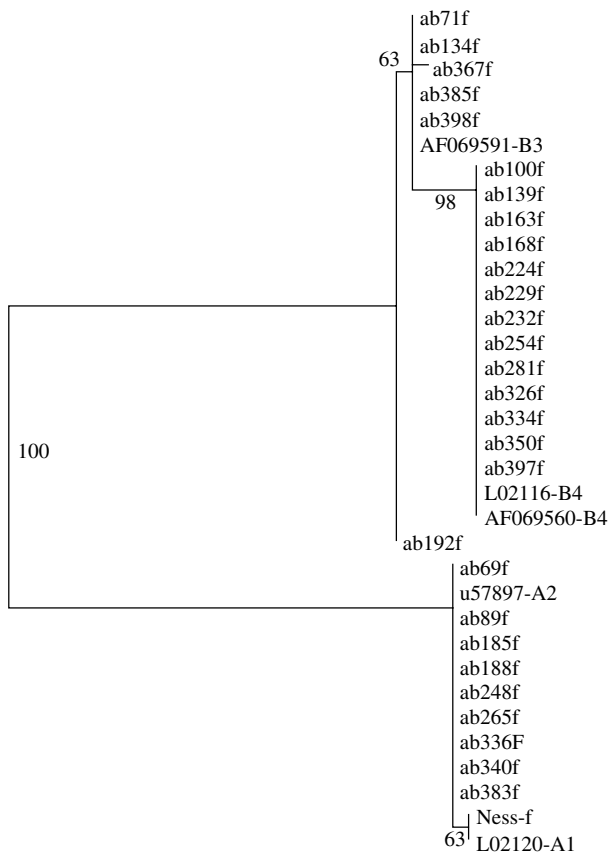
Fig. 3. Phylogram derived from forward *tpi* sequences, using a maximum parsimony algorithm. Genbank reference sequences of known assemblages are included. Bootstrap values are shown, from 100 iterations. Ab192f differed from Assemblage B3 by just 1 base pair.

Combined typing results

In total, 267 samples were examined by at least one of the PCR methods, including 35 that were tested by both methods. Genetic typing was successful by at least one method for 199 (75%) samples. Assemblage A accounted for 48 (24%) samples; Assemblage B for 145 (73%), and six (3%) were mixed. Where samples were successfully typed by both methods, the results were concordant in all cases.

Clinical and epidemiological correlations with Assemblages A and B

Assemblages A and B had similar patient demographics. Both assemblages had a bimodal age distribution. Males accounted for 44% of Assemblage A cases, and 49% of Assemblage B cases ($P = 0.6159$, Fisher's exact test). Both assemblages had similar

proportions of cases associated with household outbreaks: 6/49 (18%) Assemblage A cases, and 33/148 (22%) Assemblage B cases ($P = 0.358$). In nine outbreaks (all family clusters), two or more samples were successfully genotyped: in seven of these, the assemblages were concordant, but in two outbreaks Assemblages A and B were detected.

Travel histories were available for 98 of the cases where molecular typing was successfully performed. The proportions of Assemblage A in recent travellers ($n = 73$) and non-travellers ($n = 25$) were similar (32% and 33%, respectively; $P = 0.56$). The travel-associated cases had visited a wide number of destinations (Table 2); the numbers for each destination were too low for meaningful statistical analysis.

Both assemblages peaked in incidence at the end of the year (October–December) (Fig. 4). The duration of illness before diagnosis was known for 91 cases, and ranged widely for both assemblages, from 4 or 5 to 180 days. Assemblage A had a shorter mean duration of illness (25·8 days, compared to 26·6 days for Assemblage B), and a greater proportion of Assemblage B cases (44%, compared to 21% of Assemblage A) had a prolonged illness ($\geqslant 20$ days). However, the differences between the two assemblages did not reach statistical significance ($P = 0.17$, Mann–Whitney *U* test).

Fifty-nine of the patients whose isolates had been successfully genotyped provided details of symptoms (Table 3). Fever was significantly more common in Assemblage A than Assemblage B [odds ratio (OR) 5·091, 95% confidence interval (CI) 1·39–18·81; $P = 0.018$ by Fisher's exact test]. For all other symptoms, there were no significant differences.

Patients with Assemblage A organisms reported more symptoms (mean 2·88) than those with Assemblage B (mean 2·29), although this is not statistically significant ($P = 0.1738$, Mann–Whitney *U* test). Of note, three patients with no symptoms, who were picked up on screening of contacts of cases, were all infected with Assemblage B.

DISCUSSION

This study shows the proportions of *G. lamblia* Assemblages A and B in a cosmopolitan urban UK population. Both assemblages have similar epidemiological and clinical features, but there are differences between assemblages in terms of presence of fever.

Table 2. *Destinations of travel-associated giardiasis cases by assemblage (n = 71). Two infections with mixed Assemblages A and B are excluded (note that some cases had visited more than one destination)*

Destination	Assemblage A (n = 23)	Assemblage B (n = 48)	Total
North & South America	3	5	8
Europe	9	15	24
Far East	6	2	8
Indian subcontinent	3	9	12
Middle East & North Africa	4	6	10
Sub-Saharan Africa	1	7	8
UK & Ireland	1	2	3
Australasia	1	1	2
Not stated	0	1	1

Table 3. *Symptoms in cases of Assemblages A and B*

Symptom	Assemblage A (n = 19) Yes	No/not stated	Assemblage B (n = 40) Yes	No/not stated	OR (95 % CI)
Diarrhoea	17	2	37	3	0·69 (0·11–4·51)
Nausea	8	11	22	18	0·55 (0·20–1·79)
Aching muscles	1	18	8	32	0·22 (0·03–1·92)
Vomiting	8	11	13	27	1·51 (0·49–4·66)
Blood in faeces	0	19	3	37	0
Fever	8	11	5	35	**5·09 (1·38–18·81)**
Abdominal cramps/bloating	10	9	22	18	0·91 (0·30–2·72)
Headache	4	15	10	30	0·80 (0·44–2·98)
Malaise/fatigue	7	12	19	21	0·65 (0·21–1·98)
Weight loss	2	17	3	37	1·45 (0·22–9·50)

OR, Odds ratio; CI, confidence interval.
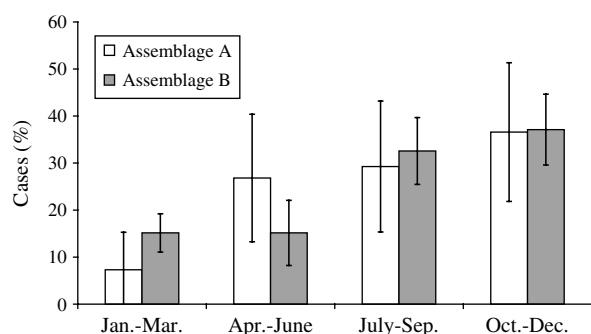Bold values indicate statistically significant values.

Fig. 4. Seasonal distribution of Assemblages A and B cases.

Methodological approach

PCR applied directly to clinical samples was used. Two housekeeping genes were chosen for PCR analysis, in order to enhance robustness of typing. A quarter of samples failed to amplify by either PCR assay, despite PVP purification. Probable explanations are persisting PCR inhibitors in the samples, and/or low numbers of cysts (cyst numbers were not quantified before PCR). The *tpi* gene PCR products did not yield a sequence in 20/48 cases, compared to only 2/41 *rRNA* gene PCR products which failed to sequence. The reason for this difference is uncertain. The *tpi* PCR reactions were noted to have a higher proportion of non-specific bands, and the gene is common to most faecal bacteria, so it is possible that some of the positive PCR results included products of non-specific amplification, which could have interfered with the sequencing process.

Sequence-based typing for the *tpi* gene allowed differentiation of strains within the two major assemblages, into the four recognized subassemblages already described (AI, AII, BIII, BIV – although A1 was absent from the clinical isolates). Sequencing may miss mixed infections with different assemblages in a single host (subsequently shown to be <5% by duplex PCR). Sequencing may also miss heterozygosity between the nuclei in a binucleate organism. The Assemblage A genome sequencing project has estimated heterozygosity in the genome of the strain studied (the WB clone C6) to be <0·01% [26], but the Assemblage B genome has an estimated heterozygosity of 0·53%, which could explain some single-nucleotide differences between strains within Assemblage B [12].

The duplex PCR-based typing method was adopted when the initial sequence results showed the limited genetic diversity within Assemblages A and B, and the absence of infections due to other genogroups. This method had the advantage of relative ease and faster throughput, and also avoided the loss of data that occurred with the extra sequencing step.

Preponderance of Assemblage B

The typing results show that all *Giardia* infections in this population are due to *G. lamblia* Assemblages A and B. This confirms the results of a number of studies performed elsewhere [8, 11, 27].

The preponderance of Assemblage B strains in this London population (73%) is similar to the results of a smaller British study which showed that 21 (64%) of 33 *Giardia* isolates were Assemblage B, with 9% mixed Assemblages A and B [25]. The worldwide distribution is less consistent, with a reported proportion of Assemblage A ranging from 0% in a small number of Indian isolates [28] to 100% in studies from Central America and Portugal [29, 30]. However, molecular studies of *Giardia* may not be directly comparable: some studies are limited to particular outbreaks [31], whereas other studies use diagnostic samples from symptomatic patients [30], case-control studies [32], and surveillance studies of particular cohorts of patients. These studies also use many different primers and molecular-typing methods, and it is possible that different methodologies may favour one assemblage over another [33].

Although outbreaks were common in our study, these were mostly small family clusters of cases; there were no large outbreaks which could potentially have skewed the overall results. Outbreak- and non-outbreak-associated cases had similar proportions of each assemblage. Of note, 2/9 outbreaks had mixed assemblages detected, which serves as a reminder that, while some outbreaks may represent cross-infection from one linked case to another, others may represent shared exposure to unhygienic conditions, where mixed assemblages may be more likely.

Differences between Assemblages A and B

Given the genetic and phenotypic differences between assemblages, it might be expected that there would be correlating clinical/epidemiological differences. In fact, in most of the parameters examined Assemblages A and B caused broadly similar illness, although the number of patients with both genotype and symptom data was low, and only one statistically significant difference was noted: fever was significantly more common in Assemblage A. Other differences which did not reach statistical significance included a trend towards a higher number of symptoms in Assemblage A, and a longer illness or asymptomatic infection in Assemblage B. Several other studies which examined clinical differences between assemblages support the hypothesis that Assemblage B is associated with persistence or asymptomatic carriage [32, 34–37]. Such a tendency may explain discrepancies between the prevalence of Assemblages A and B in returned travellers, compared to local genetic typing studies carried out in the destination of travel; for example, our study found 3/4 (unlinked) cases in travellers returned from Portugal to be due to Assemblage B, whereas a study carried out in Portugal found all of 25 symptomatic cases to be due to Assemblage A [30].

However, other work has found greater proportions of asymptomatic infection in Assemblage A [38]. A small Dutch study ($n = 18$) looked only at symptomatic cases in some detail [39]: they found that the nine Assemblage B cases all had active diarrhoea at the time of diagnosis, whereas the nine Assemblage A cases all had intermittent and short-lived diarrhoea. Contrary to the present study, they also found that Assemblage B cases were more likely to present with fever, and had more severe illness than Assemblage A cases. On the basis of their findings, they suggested that Assemblage A infection is more likely to lead to asymptomatic carriage – an hypothesis not supported by the present study or other studies [32, 34–37].

This study confirms the predominance of Assemblage B in *Giardia* cases in an urban British setting, in both travel-associated and non-travel-associated cases. Both assemblages had similar clinical and epidemiological features, although Assemblage A infections more commonly presented with fever, and had non-significant trends towards a greater number of reported symptoms and a shorter duration of illness. Further work is necessary to definitively characterize clinical differences between the assemblages.

ACKNOWLEDGEMENTS

DECLARATION OF INTEREST

None.

REFERENCES

1. **Gilman RH, et al.** Rapid reinfection by *Giardia lamblia* after treatment in a hyperendemic Third World community. *Lancet* 1988; **1**: 343–345.
2. **Pickering LK, et al.** Occurrence of *Giardia lamblia* in children in day care centers. *Journal of Pediatrics* 1984; **104**: 522–526.
3. **Dennis DT, et al.** Endemic giardiasis in New Hampshire: a case-control study of environmental risks. *Journal of Infectious Diseases* 1993; **167**: 1391–1395.
4. **Gray SF, Rouse AR.** Giardiasis – a cause of travellers' diarrhoea. *Communicable Disease Report CDR Review* 1992; **2**: R45–R47.
5. **Ey PL, et al.** Genetic analysis of giardia from hoofed farm animals reveals artiodactyl-specific and potentially zoonotic genotypes. *Journal of Eukaryotic Microbiology* 1997; **44**: 626–635.
6. **Hopkins RM, et al.** Ribosomal RNA sequencing reveals differences between the genotypes of giardia isolates recovered from humans and dogs living in the same locality. *Journal of Parasitology* 1997; **83**: 44–51.
7. **Monis PT, et al.** Molecular systematics of the parasitic protozoan *Giardia intestinalis*. *Molecular Biology and Evolution* 1999; **16**: 1135–1144.
8. **Caccio SM, Ryan U.** Molecular epidemiology of giardiasis. *Molecular and Biochemical Parasitology* 2008; **160**: 75–80.
9. **Caccio SM, et al.** Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages A and B. *International Journal for Parasitology* 2008; **38**: 1523–1531.
10. **Ey PL, Darby JM.** VSP417-6, a variant-specific surface protein encoded at a sixth locus within the vsp417 gene subfamily of *Giardia intestinalis*. *International Journal for Parasitology* 2002; **32**: 425–436.
11. **Karanis P, Ey PL.** Characterization of axenic isolates of *Giardia intestinalis* established from humans and animals in Germany. *Parasitology Research* 1998; **84**: 442–449.
12. **Franzen O, et al.** Draft genome sequencing of *Giardia intestinalis* assemblage B isolate GS: is human giardiasis caused by two different species? *PLoS Pathogens* 2009; **5**: e1000560.
13. **Boom R, et al.** Rapid and simple method for purification of nucleic acids. *Journal of Clinical Microbiology* 1990; **28**: 495–503.
14. **McLauchlin J, et al.** Genetic characterization of Cryptosporidium strains from 218 patients with diarrhea diagnosed as having sporadic cryptosporidiosis. *Journal of Clinical Microbiology* 1999; **37**: 3153–3158.
15. **Lawson AJ, et al.** Polymerase chain reaction detection and speciation of Campylobacter upsaliensis and C. helveticus in human faeces and comparison with culture techniques. *Journal of Applied Microbiology* 1997; **83**: 375–380.
16. **Meyer EA.** *Giardia lamblia*: isolation and axenic cultivation. *Experimental Parasitology* 1976; **39**: 101–105.
17. **Genbank.** Genbank nucleotide sequence database (http://www.ncbi.nlm.nih.gov). Accessed 9 March 2009.
18. **European Bioinformatics Institute.** (http://www.ebi.ac.uk/). Accessed 9 January 2008.
19. **Netprimer analysis program.** (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html). Accessed 2 July 2003.
20. **Sanger F, Nicklen S, Coulson AR.** DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA* 1977; **74**: 5463–5467.
21. **Saitou N, Nei M.** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 1987; **4**: 406–425.
22. **Clustal.** (http://www.clustal.org/). Accessed 10 January 2008.
23. **Felsenstein J.** PHYLIP (Phylogeny Inference Package) Version 3.5c (http://bioweb2.pasteur.fr/phylogeny/intro-en.html#parsalgo). Accessed 10 January 2008.
24. **Guoy M.** NJplot (http://pbil.univ-lyon1.fr/software/). Accessed 9 January 2008.
25. **Amar CF, et al.** Sensitive PCR-restriction fragment length polymorphism assay for detection and

genotyping of *Giardia duodenalis* in human feces. *Journal of Clinical Microbiology* 2002; **40**: 446–452.

26. **Morrison HG, *et al.*** Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia*. *Science* 2007; **317**: 1921–1926.

27. **Lalle M, *et al.*** Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. *International Journal for Parasitology* 2005; **35**: 207–213.

28. **Sulaiman IM, *et al.*** Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. *Emerging Infectious Diseases* 2003; **9**: 1444–1452.

29. **Eligio-Garcia L, Cortes-Campos A, Jimenez-Cardoso E.** Genotype of *Giardia intestinalis* isolates from children and dogs and its relationship to host origin. *Parasitology Research* 2005; **97**: 1–6.

30. **Sousa MC, *et al.*** Genotyping of *Giardia lamblia* human isolates from Portugal by PCR-RFLP and sequencing. *Journal of Eukaryotic Microbiology* 2006; **53** (Suppl. 1): S174–S176.

31. **Robertson LJ, *et al.*** Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004. *Applied and Environmental Microbiology* 2006; **72**: 2212–2217.

32. **Haque R, *et al.*** Giardia assemblage A infection and diarrhea in Bangladesh. *Journal of Infectious Diseases* 2005; **192**: 2171–2173.

33. **Lu S, *et al.*** The intraspecific difference of the triose phosphate isomerase (tim) gene from *Giardia lamblia*. *Chinese Medical Journal* 2002; **115**: 763–766.

34. **Aydin AF, *et al.*** Classification of *Giardia duodenalis* parasites in Turkey into groups A and B using restriction fragment length polymorphism. *Diagnostic Microbiology and Infectious Disease* 2004; **50**: 147–151.

35. **Paintlia AS *et al.*** *Giardia lamblia* groups A and B among young adults in India. *Clinical Infectious Diseases* 1998; **26**: 190–191.

36. **Read C, *et al.*** Correlation between genotype of *Giardia duodenalis* and diarrhoea. *International Journal for Parasitology* 2002; **32**: 229–231.

37. **Sahagun J, *et al.*** Correlation between the presence of symptoms and the *Giardia duodenalis* genotype. *European Journal of Clinical Microbiology and Infectious Diseases* 2008; **27**: 81–83.

38. **Gelanew T, *et al.*** Molecular characterization of human isolates of *Giardia duodenalis* from Ethiopia. *Acta Tropica* 2007; **102**: 92–99.

39. **Homan WL, Mank TG.** Human giardiasis: genotype linked differences in clinical symptomatology. *International Journal for Parasitology* 2001; **31**: 822–826.