Parasitol Res (2013) 112:3449–3456
DOI 10.1007/s00436-013-3524-y

ORIGINAL PAPER

Molecular typing of *Giardia duodenalis* isolates from German travellers

A. Broglia · T. Weitzel · G. Harms · S. M. Cacció · K. Nöckler

Received: 23 May 2013 / Accepted: 25 June 2013 / Published online: 28 July 2013
© Springer-Verlag Berlin Heidelberg 2013

Abstract *Giardia duodenalis* isolates from German travellers returning from tropical areas were characterised by PCR amplification and sequencing of fragments of the beta-giardin (*bg*), glutamate dehydrogenase (*gdh*) and triose phosphate isomerase (*tpi*) genes. Assignment of isolates to specific *G. duodenalis* assemblages was found to differ according to the marker used. Indeed, at the *bg* locus, assemblages A and B were identified, with a higher prevalence of the former over the latter, whereas at the *tpi* and *gdh* loci, most samples were classified as assemblage B. In agreement with previous studies, sequence analysis showed that assemblage B isolates have a higher genetic polymorphism than assemblage A isolates, and novel variants were described. The degree of polymorphism was shown in a graphical representation of the polymorphic sites generating a novel sequence, the heterogeneous positions common to assemblages A and B (double peaks), that may represent mixed assemblage infection and the heterogeneous positions detected at random sites. Notably, assemblage D, which is considered to be adapted to dogs, was found at the *gdh* locus in two samples originating from southern Asia, as novel genotypes. By comparing the geographical origin of the infected cases and the number of German travellers visiting the areas considered, India and west Africa appeared to be the areas associated to the highest risk of acquiring *Giardia* infection. The analysis of the geographical distribution of the genotypes did not suggest any particular geographical clustering pattern, but it may be useful to evaluate these results with a higher number of isolates. Most of the samples typed at the three markers could not be assigned unequivocally to either assemblage A or B, and this was confirmed also by a real-time PCR assay, using a set of assemblage-specific primers. The results of this study reinforce the notion that genetic exchanges and allelic sequence heterogeneity represent major obstacles towards understanding the epidemiology of giardiasis and that exposure to *Giardia* parasites in endemic areas often results in mixed infections in returning travellers.

Electronic supplementary material The online version of this article (doi:10.1007/s00436-013-3524-y) contains supplementary material, which is available to authorized users.

A. Broglia (✉)
European Food Safety Authority, via C.Magno 1, 43126 Parma, Italy
e-mail: alessandro.broglia@efsa.europa.eu

T. Weitzel · G. Harms
Institute of Tropical Medicine and International Health, Charité, University Medicine, Berlin, Germany

T. Weitzel
Clínica Alemana, Universidad del Desarrollo, Santiago, Chile

S. M. Cacció
Istituto Superiore di Sanitá, Rome, Italy

K. Nöckler
Federal Institute for Risk Assessment, Berlin, Germany

Introduction

Giardiasis is one of the most common gastrointestinal protozoan diseases of humans and also affects many other mammalian species. It occurs more frequently in developing countries where approximately 200 million people suffer symptomatic giardiasis every year (WHO 1992). Clinical symptoms, when present, include diarrhoea, flatulence, stomach cramps and nausea, which sometimes can lead to weight loss and dehydration (Gardner and Hill 2001).

Currently, six species are recognized in the *Giardia* genus, *G. duodenalis* is the only species that infects humans, albeit it is found in many other mammalian hosts (Thompson and Monis 2011). In order to understand the epidemiology of giardiasis, particularly the role of zoonotic transmission, and the correlation between the genetic variation of the parasite and its pathogenicity in the host, a large number of isolates

have been characterised genetically (Amar et al. 2002; Caccio et al. 2005; Sprong et al. 2009; Vanni et al. 2012). These data have shown that isolates of *G. duodenalis*, which cannot be distinguished morphologically, can be assigned to at least eight distinct genetic groups (assemblages). Among these, assemblages A and B have a broad host range and infect humans and many other mammals (Alyousefi et al. 2013; Caccio and Sprong 2010; Eligio-Garcia et al. 2005; Feng and Xiao 2011; Geurden et al. 2009; Yang et al. 2010). The other assemblages are thought to be more host specific (assemblages C and D for canids, assemblage E for cloven-hoofed domestic mammals, assemblage F for felids, assemblage G for rats and assemblage H for marine mammals), although recent studies have detected some of these assemblages in humans from developing countries (Foronda et al. 2008; Gelanew et al. 2007).

Further genetic variation exists within each assemblage: subgroups within assemblages A and B were originally defined by isoenzyme analysis and classified as AI and AII, BIII and BIV (Monis et al. 1999). This technique, however, is not suitable for routine genotyping studies because it requires large amount of biological material that can only be obtained by in vitro growth of *Giardia* isolates. Currently, the genetic characterization of *Giardia* is based on PCR amplification and sequencing of fragments of the small subunit rRNA, beta-giardin (*bg*), triose phosphate isomerase (*tpi*) and glutamate dehydrogenase (*gdh*) genes, which differ in terms of sensitivity and degree of genetic polymorphism (Wielinga and Thompson 2007). Genotyping data on human isolates have been generated from many parts of the world, albeit most of the data are from Europe, Australia and the USA (Caccio et al. 2002; Read et al. 2004; Sulaiman et al. 2004; van der Giessen et al. 2006). Recent genotyping studies have also been performed in tropical, developing or newly industrialized countries such as India, Philippines, Mexico, Argentina, Brazil, Peru, Nicaragua, Egypt, Guinea Bissau and Ethiopia (Eligio-Garcia et al. 2008; Ferreira et al. 2012; Foronda et al. 2008; Gelanew et al. 2007; Kohli et al. 2008; Lebbad and Svard 2008; Minvielle et al. 2008; Traub et al. 2004; Yason and Rivera 2007). These studies reported high genetic variability, mixed assemblage infections and assemblages not typically associated with human infections, thus confirming the complexity of *Giardia* molecular epidemiology.

To obtain an informative picture of the epidemiology of giardiasis, the analysis of single markers is not sufficient. Indeed, recent studies based on a multilocus typing approach have revealed an unexpected complexity due to mixed infections, allelic sequence heterogeneity and lack of concordance in the assignment of isolates to a specific assemblage (Almeida et al. 2010; Caccio et al. 2002; Geurden et al. 2009; Laishram et al. 2012; Lebbad et al. 2008, 2010, 2011; Soliman et al. 2011; Sprong et al. 2009; Wielinga et al. 2011).

In Germany, approximately 4,700 cases of giardiasis are officially registered yearly (RKI 2008), but, due to underreporting and under diagnosing, the actual number might be much higher. Most cases of human giardiasis in Germany are related to travel, particularly to tropical and subtropical areas.

The present study was aimed to perform a molecular analysis through multilocus genotyping on isolates collected from German patients with a recent history of travel to endemic regions, which may provide further insights on the distribution of *Giardia* molecular patterns in different geographical areas.

Materials and methods

Sources of isolates

Faecal samples from travel returnees were collected at the Institute of Tropical Medicine and International Health in Berlin (Germany). The detection of *Giardia* cysts and/or trophozoites in those samples was based on light microscopy using conventional MIF concentration as well as direct immunofluorescence (Merifluor Crypto & Giardia, Meridian Bioscience, Cincinnati, OH, USA). In 1 year, a total of 66 patients mostly suffering gastrointestinal disturbances were found positive for *Giardia*. For 57 patients, data about previous stays abroad were available: recent travels to Latin America (Mexico, Guatemala, Honduras, Argentina, Bolivia, Brazil), India, Southeast Asia (Thailand, Cambodia, Laos), western Africa (Ghana, Burkina Faso, Benin), eastern Africa (Sudan, Tanzania, Kenya), southern Africa (Namibia, Malawi) and northern Africa (Egypt) were reported.

Sample treatment and DNA extraction

About 3 g of faecal material were dissolved in phosphate-buffered saline (PBS), vortexed, filtered through a mesh and centrifuged for 5 min at 4,700 rpm. The supernatant was discarded, and the pellet was dissolved in 3 ml of PBS. The faecal suspension was laid over 10 ml of a 1-M sucrose solution and centrifuged at 2,000 rpm for 10 min without brake. The intermediate layer containing the *Giardia* cysts was recovered, brought to 50 ml with PBS and concentrated by centrifugation at 4,700 rpm for 5 min. The pelleted cysts were used for DNA extraction using the QIAamp Stool kit (Qiagen, Hildesheim, Germany), with an initial incubation of 10 min at 95 °C and a final elution in 100 μl of elution buffer instead of 200 μl. The eluted DNA samples were stored at −20 °C before use.

Molecular and phylogenetic analysis

Nested PCR amplification of fragments of the *bg* (511 bp), *tpi* (530 bp) and *gdh* (530 bp) genes was performed using published protocols from Lalle et al. (2005), Sulaiman et al.

(2003) and Caccio et al. (2008), respectively. A published quantitative TaqMan real-time PCR assay (qPCR) targeting the *bg* locus (Guy et al. 2004) was performed on samples for which assignment to assemblages was non-concordant.

PCR products were purified using spin columns (GE Healthcare, Germany) and sequenced from both strands. Sequences were edited using SeqMan 7.0 software (DNASTAR, WI, USA) and aligned with reference sequences retrieved from the GenBank using Clustal W (Aiyar 2000).

Representative sequences of subgroups AI (accession numbers EU014394, L40509, AF069556), AII (accession numbers AY072723, L40510, AF069557), BIII (accession numbers AY072726, AF069059, AF069561) and BIV (accession numbers AY072725, L40508, AF069560) were retrieved from GenBank and used as reference. As reference for sub-assemblages A3–A6 at the *bg* locus, the sequences submitted by Cacciò et al. (2008) were used.

Results

PCR amplicons were obtained from 61 of the 66 samples at the *bg* locus, from 29 samples at the *tpi* locus and from 17 samples at the *gdh* locus. All positive isolates were sequenced and the sequences aligned with those from reference strains representing sub-assemblages AI, AII, BIII and BIV and D (Baruch et al. 1996; Homan et al. 1998; Monis et al. 1999). The degree of polymorphisms detected at the three loci in the different assemblages is shown in Fig. 1 and in the Supplementary Tables S2, S3, S4. The heterogeneity index reported in Fig. 1 is a graphical representation of the degree of polymorphism expressed as the mean number of (1) polymorphic sites generating a novel sequence (single-nucleotide polymorphism (SNP)); (2) the heterogeneous positions common to assemblages A and B, representing mixed assemblage infection (*mixAssem*) and (3) the heterogeneous positions (double peaks) at random sites not common to assemblage A or B (*Heter*), where the denominator is the number of isolates of a given assemblage.

The novel sequences without heterogeneous positions detected in the present study are available in the GenBank database under accession numbers FJ007840–FJ007859.

Multilocus typing analysis

The assignment of the samples to different assemblages through standard PCR and sequencing and qPCR by using assemblage-specific primers is reported in Table 1.

Sequence data for all three loci were available for 14 samples. By comparing the assignment to assemblages at the *bg*, *tpi* and *gdh* loci, nine isolates were consistently classified as assemblage B (seven samples) or assemblage A (two samples), whereas the remaining five isolates were not. In two cases, assemblage A was detected at the *bg* and *gdh* loci and

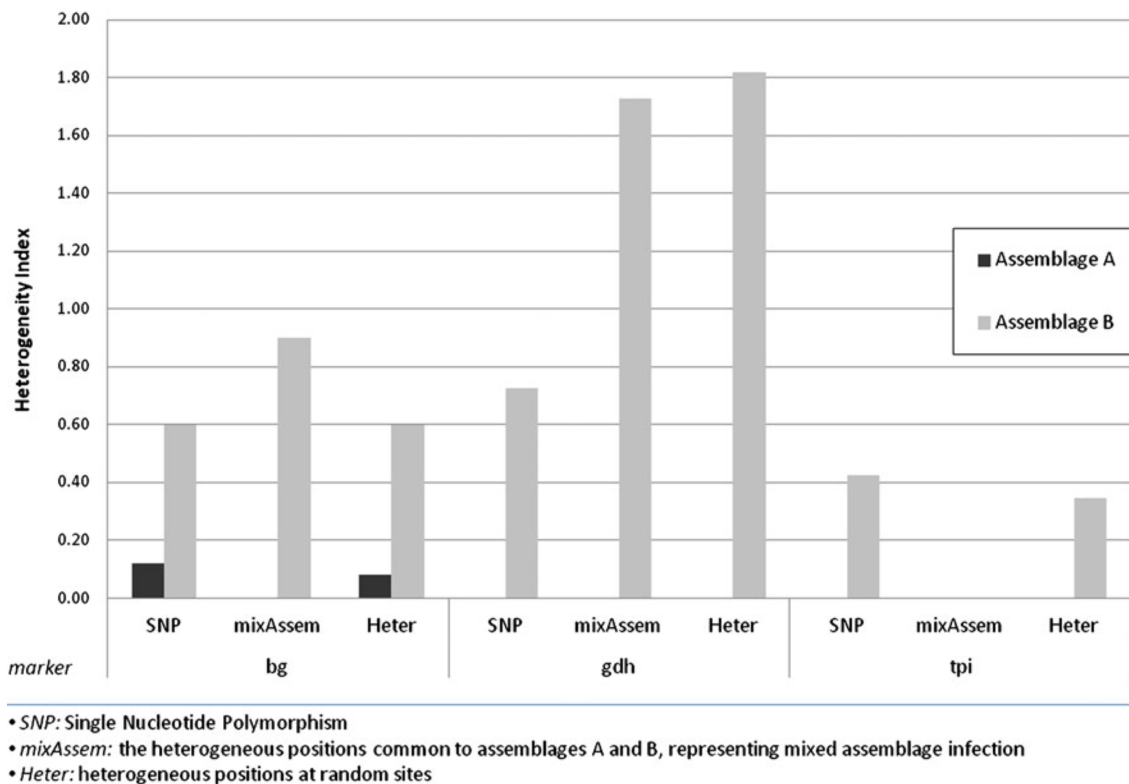


Fig. 1 Heterogeneity index (number of SNP/number isolates of a given assemblage) of *Giardia* isolates at the three different loci considered

assemblage B was detected at the *tpi* locus, whereas three isolates were typed as assemblage A at *bg*, but as assemblage B at the *tpi* and *gdh* loci.

Furthermore, the two samples typed at the *gdh* locus as novel genotypes of assemblage D were classified as novel genotypes of assemblage A at *bg* locus. Among the 32 samples for which genotyping data were available for at least two markers, the majority of them showed incongruent assignment to assemblages (21 out of 32 samples), in particular assemblage A at *bg* and assemblage B at *tpi* (19 out of 32 samples).

In order to further analyse cases of inconsistent assignment to assemblage, ten samples with available multilocus typing data were tested by qPCR assays with assemblage-specific primers (A and B) at the beta-giardin. The samples BfR163, BfR164, BfR167, BfR169 and BfR172, typed as assemblage A at *bg*, showed also an amplification signal for assemblage B, while in samples BfR162 and BfR168, unambiguously typed as B at *bg*, *gdh*, and *tpi*, the assemblage A was also detected by qPCR.

Polymorphisms at single loci

At the *bg* locus, 51 out of 61 samples were classified as assemblage A, 43 being identical to genotype A3 (accession number AY072724), whereas two were identical to genotype A2 (accession number AY072723). Four samples of these (8 % isolates) were new variants with unique SNP patterns, distinct from the six subtypes (A1–A6) previously described by Cacciò et al. (2008).

Table 1 Genotyping results (assemblage, sub-assemblage) of human isolates of *Giardia duodenalis* obtained at the *bg*, *gdh* and *tpi* loci by standard PCR and by qPCR at the *bg* locus and geographical origin of infection (recent travel)

Isolate	*bg*	*gdh*	*tpi*	qPCR *bg*	Geographical origin of infection
BfR152	B	B	B	–	Central America
BfR168	B	B	B	A and B	Central America
BfR235	A3	B	B	–	Central America
BfR156	A3	A2	A2	–	Eastern Africa
BfR164	A3	B	neg	B	Eastern Africa
BfR269	A3	neg	B	–	Eastern Africa
BfR275	A3	neg	B	–	Eastern Africa
BfR153	B	B	B	B	India
BfR157	B	B	B	–	India
BfR161	B	B	B	–	India
BfR162	B	B	B	A and B	India
BfR163	A	D	neg	A and B	India
BfR166	A3	A2	A2	neg	India
BfR169	A3	B	B	A and B	India
BfR170	B	B	B		India
BfR238	A3	neg	B	–	India
BfR239	A2	A2	B	–	India
BfR266	A3	neg	B	–	India
BfR268	A3	neg	B	–	India
BfR270	A3	neg	B	–	India
BfR281	A3	neg	A2	–	India
BfR282	A3	neg	B	–	India
BfR160	B		B	–	na
BfR167	A3	B	B	A and B	na
BfR240	A2	A2	B	–	na
BfR236	A3	neg	B	–	South Africa
BfR237	A3	neg	B	–	South America
BfR267	A3	neg	B	–	South America
BfR277	A3	neg	B	–	South America
BfR172	A3	D	neg	A and B	Southeast Asia
BfR265	A3	neg	B	–	Southeast Asia
BfR165	A3	neg	B	neg	Western Africa

Among the ten isolates classified as assemblage B, the percentage of new variants was much higher (six out of ten) than among assemblage A isolates.

At *gdh* locus, the majority of isolates were typed as assemblage B (11 out of 17) and were characterised by heterogeneous positions at random sites (Supplementary Table S3). In six of the isolates, a sequence corresponded to a mixture of sub-assemblages BIII and BIV, and all of the isolates also contained mixed positions. Only four out of 17 isolates were classified as assemblage A, where no heterogeneous positions were observed. Interestingly, two samples (BfR163 and BfR172) were classified as assemblage D, which is considered to be adapted to canid hosts. These isolates were from two patients with symptomatic giardiasis who spent holidays both in Southeastern Asia. This result was confirmed by repeating PCR and sequencing.

Sequence analysis at the *tpi* locus showed 26 isolates genotyped as assemblage B and only three typed as assemblage A, with novel polymorphisms of assemblage B in six samples with unique SNPs (Supplementary Table S4). Eight isolates of assemblage B were characterised by heterogeneous positions at random sites not to be ascribed to mixed assemblages. No evidence of heterogeneous template was detected among the isolates grouped in assemblage A.

Geographical origin of the infection

Information about travel destinations (where most likely the *Giardia* infection was acquired) was available for 57 patients. The information concerning the areas visited by the infected patients was compared with figures about German travellers visiting yearly each area considered in this study (DESTATIS 2006). Although the data were insufficient for statistical comparison, India and west Africa seemed to be the areas associated to the highest risk of acquiring *Giardia* infection (Fig. 2). Further, in Table 1, the geographical origin of the isolates next to the genotyping results of at least two loci is reported. The most common genotype combination typed as assemblage A at *bg* and B at *tpi* (18 isolates) locus was detected in all the geographical areas considered in the present study. The seven isolates consistently assigned to assemblage B at all three markers (a relatively uncommon multilocus assemblage typed in this study), originated from India (five samples) and Central America (two samples). The two isolated typed as assemblage D at *gdh* marker were both originating from southern Asian continent (India and Southeast Asia).

Discussion

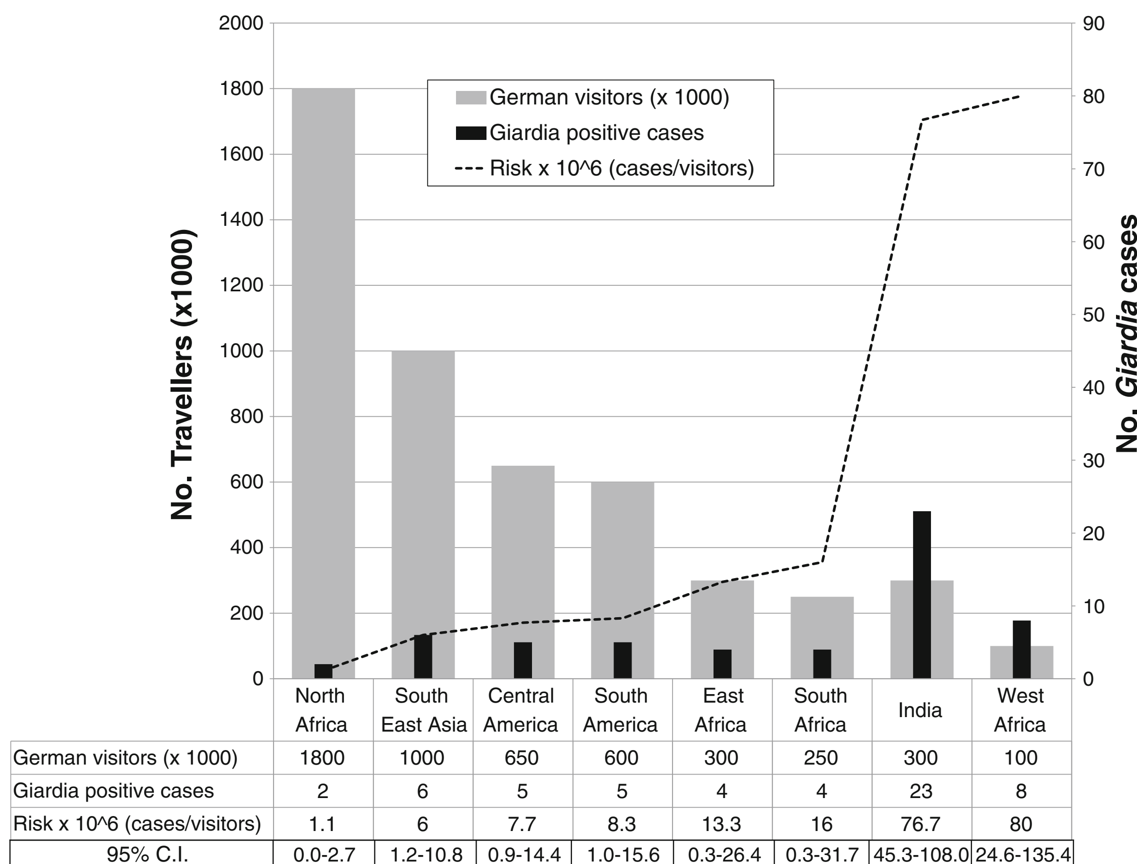
The main objective of the present study was to investigate the genetic variability of *Giardia duodenalis* isolates collected from returning travellers by a multilocus analysis at the *bg*, *gdh* and *tpi* loci. This analysis, however, was negatively impacted by the low rate of amplification observed at the *gdh* and *tpi* loci (26 and 48 % of samples, respectively), compared with that obtained at the *bg* locus (92 %). These markers are all single-copy genes, thus the different rate of amplification cannot be explained by difference in target copy number. The presence of mismatches between the genomic sequences and the primers used for PCR, that may cause a strong reduction or even a lack of amplification, cannot be excluded. Interestingly, similar findings were reported by other researchers (Amar et al. 2002; Covacin et al. 2011; Robertson et al. 2006; Soliman et al. 2011). Soliman et al. (2011) reported a rate of amplification close to 50 % at the *tpi* and *gdh* loci, whereas 100 % of the samples could be amplified at the *bg* locus; Covacin et al. (2011) reported even only 7 % successful amplification at *gdh* locus.

Despite this limitation, our study provided several interesting results, which are discussed in turn below.

The most intriguing observation was the difficulty in assigning isolates to specific *G. duodenalis* assemblages. Indeed, only ten of the 31 samples for which sequence information was available at more than one locus were unequivocally assigned to a specific assemblage. On the contrary, most of the samples classified as assemblage A at the *bg* locus were classified as assemblage B at the *tpi* or the *gdh* locus. This lack of concordance in the assignment to assemblages has been reported in many recent studies (e.g. Cacciò et al. 2008, references) and can be explained by two very different mechanisms, namely mixed infections or recombination (Caccio and Sprong 2010). In the first case, two different 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 recombinants that may contain specific sequences of assemblage A in a genome of assemblage B (or vice versa).

High frequency of multiple/mixed infections has been increasingly reported from multilocus studies in other countries in both humans and dogs and indeed hypothesis of meiotic recombination or preferential amplification of one assemblage over another in mixed infections have been suggested (Caccio et al. 2005; Covacin et al. 2011; Sprong et al. 2009; Wielinga and Thompson 2007).

To detect mixed infections, both conventional and real-time PCR assays, based on the use of assemblage-specific primers have been developed (Almeida et al. 2010; Geurden et al. 2009) and tested on DNA extracted from stools or purified cysts. In this study, a real-time PCR assay detected DNA of assemblages A and B in six of eight samples tested (two isolates were negative); four of these six isolates gave an inconsistent assignment when tested by conventional PCR (A or B in two cases, A or D in the other two cases), whereas the other two isolates were classified as assemblage B. Therefore, mixed infections can potentially explain only a fraction of cases where different



	North Africa	South East Asia	Central America	South America	East Africa	South Africa	India	West Africa
German visitors (x 1000)	1800	1000	650	600	300	250	300	100
Giardia positive cases	2	6	5	5	4	4	23	8
Risk x 10^6 (cases/visitors)	1.1	6	7.7	8.3	13.3	16	76.7	80
95% C.I.	0.0-2.7	1.2-10.8	0.9-14.4	1.0-15.6	0.3-26.4	0.3-31.7	45.3-108.0	24.6-135.4

Fig. 2 Prevalence of giardiasis in patients returning from the areas considered compared to the destinations of German travellers

assemblages were detected using different PCR assays. The occurrence of recombinants could not be investigated.

A second relevant finding was the identification of assemblage D (at the *gdh* locus) in two patients returning from India and Southeast Asia, *G. duodenalis*. The occurrence of the dog-adapted assemblage D has been already reported in human isolates from India (Traub et al. 2004) when genotyping was performed at the 18S rDNA locus, but this finding could not be supported by genetic characterization at other loci (*ef1*-α, *tpi*), as was the case in our study (see Table 1). It should be noted, however, that assemblage E was found in 15 % of the human isolates from Egypt by genotyping at the *tpi* locus (Foronda et al. 2008), and more recently, also assemblage C was found at *bg* locus in human patients from the same country (Soliman et al. 2011). Further, a study conducted in Ethiopia revealed the presence of assemblage F in three human isolates (Gelanew et al. 2007). The epidemiology of giardiasis in developing countries is greatly influenced by poor hygienic conditions and the closeness between humans and animals, which could favour the transmission of unusual parasitic strains. However, passive carriage of cysts from those assemblages cannot be excluded.

Our results reinforced previous data concerning the amount of polymorphism and the occurrence of allelic sequence heterogeneity (ASH) in isolates from assemblages A and B. Among the isolates classified as assemblage A (at the *bg* locus), the majority were identical to an already described genotype (A3), albeit three of them represented novel variants. In more than 30 kb of sequenced DNA, only four heterogeneous positions were found in three isolates, which do not represent mixed infections with other known assemblage A genotypes. At the *gdh* and *tpi* loci, the few samples ascribed to assemblage A were identical to the reference strain AII (Monis et al. 1996; Souza et al. 2007), and novel variants or heterogeneous sites were not detected. This is illustrated in Fig.1, where the heterogeneity of assemblage A isolates was at the lowest level at *bg* locus, and even absent at *gdh* and *tpi* locus. This result largely agrees with previous studies in which ASH in assemblage A isolates was very low or absent (Caccio et al. 2008; Morrison et al. 2007). On the contrary, isolates classified as assemblage B showed a larger amount of polymorphism and of ASH at all the three loci investigated, in agreement with previous studies (Caccio et al. 2008; Lebbad et al. 2008, 2011;Robertson et al. 2006; Sulaiman et al. 2004; Teodorovic et al. 2007; Winkworth et al. 2008). Novel genotypes were observed at all three markers, and the heterogeneity index for SNPs and heterogeneous positions was high, in particular at *gdh* and *bg* loci. The reasons for the different ASH levels in assemblage A and B are not understood, but a recent study has demonstrated, by typing single cysts isolated

by micromanipulation, that ASH occurs between the two nuclei of single cysts but also between different cysts taken from the same patient (Ankarklev et al. 2012). Therefore, the genetics of *Giardia* is complex, and the occurrence of ASH complicates the assignment of isolates to specific genotypes, especially for assemblage B.

Finally, interesting observations were made with respect to the geographical origin of the infection. Indeed, by comparing the number of *Giardia*-positive travellers to the total number of tourists visiting these areas yearly, a higher risk of acquiring the infection was found to be associated with travel to India and west Africa. Although statistically weak, this finding agrees with previous studies that showed a higher prevalence of giardiasis among travellers returning from the Indian subcontinent and from west Africa compared to other tropical areas (Ekdahl and Andersson 2005; Jelinek and Loscher 2000). This increased risk could be associated to the poor management of water supply and wastewater treatment in areas with high-population density. The comparison of the genotyping results with the geographical origin of the isolates allows only limited conclusion, mainly due to the limited number of isolates. The majority of the isolates originated from India and were typed as assemblage A at *bg* and assemblage B at *tpi*, the most common multilocus assemblage registered in this study. Similarly, in other studies conducted in India, *Giardia* isolates in human samples were typed as assemblage A1 at *bg* and as assemblage B at *tpi* (Khan et al. 2011; Sulaiman et al. 2003; Traub et al. 2004). The samples originating from west Africa were typed mostly (five out of six samples) as assemblage A at *bg*, which is in contrast with another study conducted in west Africa (Guinea Bissau), where the predominance of assemblage B was detected at *bg* locus in 82 % of samples analysed (Ferreira et al. 2012). No particular geographical clustering pattern of multilocus assemblage was detected, but these results may provide a preliminary contribution to further spatial analysis of multilocus assemblage distribution, if assessed together with more genotyping data.

In conclusion, the results of this study have confirmed that the epidemiology of giardiasis still presents several unclear aspects. Further studies are needed to clarify the host specificity of this parasite, given the finding of unusual assemblages in human and animal isolates. Moreover, the molecular typing of isolates from travellers returning from endemic areas may provide a useful framework for comparing the genotype variability from different geographic regions and may help in investigating the pathogenic potential of different strains and in identifying risk-associated areas.

Acknowledgments This work was supported by the European MED-VET-NET project—contract FOOD-CT-2004-506122.

Disclaimer This paper is published under the sole responsibility of the authors and is not considered as an EFSA output. The positions and opinions presented are those of the authors alone and are not intended to represent the views of EFSA.

References

Aiyar A (2000) The use of CLUSTAL W and CLUSTAL X for multiple sequence alignment. Methods Mol Biol 132:221–41

Almeida A, Pozio E, Caccio SM (2010) Genotyping of *Giardia duodenalis* cysts by new real-time PCR assays for detection of mixed infections in human samples. Appl Environ Microbiol 76(6):1895–901

Alyousefi NA, Mahdy MAK, Xiao L, Mahmud R, Lim YAL (2013) Molecular characterization of *Giardia duodenalis* in Yemen. Exp Parasitol 134(2):141–147. doi:10.1016/j.exppara.2013.03.001

Amar CF, Dear PH, Pedraza-Diaz S, Looker N, Linnane E, McLauchlin J (2002) Sensitive PCR-restriction fragment length polymorphism assay for detection and genotyping of *Giardia duodenalis* in human feces. J Clin Microbiol 40(2):446–52

Ankarklev J, Svard SG, Lebbad M (2012) Allelic sequence heterozygosity in single *Giardia* parasites. BMC Microbiol 12(1):65. doi:10.1186/1471-2180-12-65

Baruch AC, Isaac-Renton J, Adam RD (1996) The molecular epidemiology of *Giardia lamblia*: a sequence-based approach. J Infect Dis 174(1):233–6

Caccio SM, Beck R, Lalle M, Marinculic A, Pozio E (2008) Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages A and B. Int J Parasitol 38(13):1523–31. doi:10.1016/j.ijpara.2008.04.008

Caccio SM, De Giacomo M, Pozio E (2002) Sequence analysis of the beta-giardin gene and development of a polymerase chain reaction-restriction fragment length polymorphism assay to genotype *Giardia duodenalis* cysts from human faecal samples. Int J Parasitol 32(8):1023–30

Caccio SM, Sprong H (2010) *Giardia duodenalis*: genetic recombination and its implications for taxonomy and molecular epidemiology. Exp Parasitol 124(1):107–12. doi:10.1016/j.exppara.2009.02.007

Caccio SM, Thompson RC, McLauchlin J, Smith HV (2005) Unravelling *Cryptosporidium* and *Giardia* epidemiology. Trends Parasitol 21(9):430–7. doi:10.1016/j.pt.2005.06.013

Covacin C, Aucoin DP, Elliot A, Thompson RC (2011) Genotypic characterisation of *Giardia*from domestic dogs in the USA. Vet Parasitol 177(1–2):28–32. doi:10.1016/j.vetpar.2010.11.029

DESTATIS (2006) Tourism in numbers (German). German Federal Statistical Office, Wiesbaden. https://www.destatis.de/DE/Publikationen/Thematisch/BinnenhandelGastgewerbeTourismus/Tourismus/TourismusinZahlen1021500067004.pdf?__blob=publicationFile

Ekdahl K, Andersson Y (2005) Imported giardiasis: impact of international travel, immigration, and adoption. AmJTrop Med Hyg 72(6):825–30

Eligio-Garcia L, Cortes-Campos A, Jimenez-Cardoso E (2005) Genotype of Giardia intestinalis isolates from children and dogs and its relationship to host origin. Parasitol Res 97(1):1–6

Eligio-Garcia L, Cortes-Campos A, Cota-Guajardo S, Gaxiola S, Jimenez-Cardoso E (2008) Frequency of *Giardia intestinalis* assemblages isolated from dogs and humans in a community from Culiacan, Sinaloa, Mexico using beta-giardin restriction gene. Vet Parasitol 156(3–4):205–9. doi:10.1016/j.vetpar.2008.04.029

Feng Y, Xiao L (2011) Zoonotic potential and molecular epidemiology of *Giardia* species and giardiasis. Clin Microbiol Rev 24(1):110–40. doi:10.1128/CMR.00033-10

Ferreira FS et al (2012) Molecular characterization of Giardia duodenalis in children from the Cufada Lagoon Natural Park, Guinea-Bissau. Parasitol Res 111(5):2173–7. doi:10.1007/s00436-012-3068-6

Foronda P et al (2008) Identification of genotypes of *Giardia intestinalis* of human isolates in Egypt. Parasitol Res 103(5):1177–81. doi:10.1007/s00436-008-1113-2

Gardner TB, Hill DR (2001) Treatment of giardiasis. Clin Microbiol Rev 14(1):114–28. doi:10.1128/CMR.14.1.114-128.2001

Gelanew T, Lalle M, Hailu A, Pozio E, Caccio SM (2007) Molecular characterization of human isolates of *Giardia duodenalis* from Ethiopia. Acta Trop 102(2):92–9. doi:10.1016/j.actatropica.2007.04.003

Geurden T et al (2009) Multilocus genotyping of *Cryptosporidium* and *Giardia* in non-outbreak related cases of diarrhoea in human patients in Belgium. Parasitology 136(10):1161–8. doi:10.1017/S0031182009990436

Guy RA, Xiao C, Horgen PA (2004) Real-time PCR assay for detection and genotype differentiation of *Giardia lamblia* in stool specimens. J Clin Microbiol 42(7):3317–20. doi:10.1128/JCM.42.7.3317-3320.2004

Homan WL, Gilsing M, Bentala H, Limper L, van Knapen F (1998) Characterization of *Giardia duodenalis* by polymerase-chain-reaction fingerprinting. Parasitol Res 84(9):707–14

Jelinek T, Loscher T (2000) Epidemiology of giardiasis in German travelers. J Travel Med 7(2):70–3

Khan SM, Debnath C, Pramanik AK, Xiao L, Nozaki T, Ganguly S (2011) Molecular evidence for zoonotic transmission of Giardia duodenalis among dairy farm workers in West Bengal, India. Vet Parasitol 178(3–4):342–345. doi:10.1016/j.vetpar.2011.01.029

Kohli A et al (2008) *Giardia duodenalis* assemblage, clinical presentation and markers of intestinal inflammation in Brazilian children. Trans R Soc Trop Med Hyg 102(7):718–25. doi:10.1016/j.trstmh.2008.03.002

Laishram S, Kannan A, Rajendran P, Kang G, Ajjampur SS (2012) Mixed *Giardia duodenalis* assemblage infections in children and adults in South India. Epidemiol Infect 140(11):2023–2027. doi:10.1017/S0950268811002767

Lalle M, Pozio E, Capelli G, Bruschi F, Crotti D, Caccio SM (2005) Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. Int J Parasitol 35(2):207–13. doi:10.1016/j.ijpara.2004.10.022

Lebbad M, Ankarklev J, Tellez A, Leiva B, Andersson JO, Svard S (2008) Dominance of *Giardia* assemblage B in Leon, Nicaragua. Acta Trop 106(1):44–53. doi:10.1016/j.actatropica.2008.01.004

Lebbad M et al (2010) From mouse to moose: multilocus genotyping of *Giardia* isolates from various animal species. Vet Parasitol 168(3–4):231–9. doi:10.1016/j.vetpar.2009.11.003

Lebbad M et al (2011) Multilocus genotyping of human *Giardia* isolates suggests limited zoonotic transmission and association between assemblage B and flatulence in children. PLoS Negl Trop Dis 5(8):e1262. doi:10.1371/journal.pntd.0001262

Lebbad M, Svard S (2008) Comment on article by Eligio-Garcia et al. entitled "Frequency of *Giardia intestinalis* assemblages isolated from dogs and humans in a community from Culiacan, Sinaloa, Mexico using beta-giardin restriction gene". Vet Parasitol 158(1–2):159–60. doi:10.1016/j.vetpar.2008.08.011

Minvielle MC, Molina NB, Polverino D, Basualdo JA (2008) First genotyping of *Giardia lamblia* from human and animal feces in Argentina, South America. Mem Inst Oswaldo Cruz 103(1):98–103

Monis PT, Andrews RH, Mayrhofer G, Ey PL (1999) Molecular systematics of the parasitic protozoan *Giardia intestinalis*. Mol Biol Evol 16(9):1135–44

Monis PT, Mayrhofer G, Andrews RH, Homan WL, Limper L, Ey PL (1996) Molecular genetic analysis of *Giardia intestinalis* isolates at the glutamate dehydrogenase locus. Parasitology 112(Pt 1):1–12

Morrison HG et al (2007) Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia*. Science 317(5846):1921–6. doi:10.1126/science.1143837

Read C, Monis P, Thompson R (2004) Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. Infect Genet Evol 4:125–130

RKI (2008) Epidemiological bullettin of notifiable disease in 2008 Robert Koch Institute. Robert Koch Institute, Berlin

Robertson LJ, Hermansen L, Gjerde BK, Strand E, Alvsvag JO, Langeland N (2006) Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004. Appl Environ Microbiol 72(3):2212–7. doi:10.1128/AEM.72.3.2212-2217.2006

Soliman RH, Fuentes I, Rubio JM (2011) Identification of a novel Assemblage B subgenotype and a zoonotic Assemblage C in human isolates of *Giardia intestinalis* in Egypt. Parasitol Int 60(4):507–11. doi:10.1016/j.parint.2011.09.006

Souza SL et al (2007) Molecular identification of *Giardia duodenalis* isolates from humans, dogs, cats and cattle from the state of Sao Paulo, Brazil, by sequence analysis of fragments of glutamate dehydrogenase (gdh) coding gene. Vet Parasitol 149(3–4):258–64. doi:10.1016/j.vetpar.2007.08.019

Sprong H, Caccio SM, van der Giessen JW, ZOOPNET network and partners (2009) Identification of zoonotic genotypes of Giardia duodenalis. PLoS Negl Trop Dis 3(12):e558. doi:10.1371/journal.pntd.0000558

Sulaiman IM et al (2003) Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. Emerg Infect Dis 9(11):1444–52

Sulaiman IM, Jiang J, Singh A, Xiao L (2004) Distribution of *Giardia duodenalis* genotypes and subgenotypes in raw urban wastewater in Milwaukee, Wisconsin. Appl Environ Microbiol 70(6):3776–80. doi:10.1128/AEM.70.6.3776-3780.2004

Teodorovic S, Braverman JM, Elmendorf HG (2007) Unusually low levels of genetic variation among *Giardia lamblia* isolates. Eukaryot Cell 6(8):1421–30. doi:10.1128/EC.00138-07

Thompson RCA, Monis P (2011) Taxonomy of *Giardia* species. In: Luján H, Svärd S (eds) *Giardia*. Springer, Vienna, pp 3–15

Traub RJ, Monis PT, Robertson I, Irwin P, Mencke N, Thompson RC (2004) Epidemiological and molecular evidence supports the zoonotic transmission of *Giardia* among humans and dogs living in the same community. Parasitology 128(3):253–62

van der Giessen JW, de Vries A, Roos M, Wielinga P, Kortbeek LM, Mank TG (2006) Genotyping of *Giardia* in Dutch patients and animals: a phylogenetic analysis of human and animal isolates. Int J Parasitol 36(7):849–58. doi:10.1016/j.ijpara.2006.03.001

Vanni I et al (2012) Detection of *Giardia duodenalis* assemblages A and B in human feces by simple, assemblage-specific PCR assays. PLoS Negl Trop Dis 6(8):e1776. doi:10.1371/journal.pntd.0001776

WHO (1992) Global Health Situation and Projections: Estimates. WHO/HST/921 World Health Organization. World Health Organisation, Geneva

Wielinga C, Ryan U, Andrew Thompson RC, Monis P (2011) Multilocus analysis of *Giardia duodenalis* intra-Assemblage B substitution patterns in cloned culture isolates suggests sub-Assemblage B analyses will require multi-locus genotyping with conserved and variable genes. Int J Parasitol 41(5):495–503. doi:10.1016/j.ijpara.2010.11.007

Wielinga CM, Thompson RC (2007) Comparative evaluation of *Giardia duodenalis* sequence data. Parasitology 134(Pt 12):1795–821. doi:10.1017/S0031182007003071

Winkworth CL, Learmonth JJ, Matthaei CD, Townsend CR (2008) Molecular characterization of *Giardia* isolates from calves and humans in a region in which dairy farming has recently intensified. Appl Environ Microbiol 74(16):5100–5. doi:10.1128/AEM.00232-08

Yang R, Lee J, Ng J, Ryan U (2010) High prevalence *Giardia duodenalis* assemblage B and potentially zoonotic subtypes in sporadic human cases in Western Australia. Int J Parasitol 40(3):293–7. doi:10.1016/j.ijpara.2009.08.003

Yason JA, Rivera WL (2007) Genotyping of *Giardia duodenalis* isolates among residents of slum area in Manila, Philippines. Parasitol Res 101(3):681–7. doi:10.1007/s00436-007-0533-8