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ORIGINAL PAPER

Multilocus sequence analysis of *Giardia* spp. isolated from patients with diarrhea in Austria

Mellesia F. Lee[1,2] · **Herbert Auer**[1] · **John F. Lindo**[2] · **Julia Walochnik**[1]

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Abstract *Giardia duodenalis* is a protozoan parasite causing intestinal infections in a wide range of mammals. Two distinct assemblages, A and B, infect humans predominantly; however, both are believed to be generally zoonotic. *Giardia* strains associated with infections in Austria have not been investigated at the molecular level. In this study, 65 human stool samples microscopically positive for *Giardia* spp. were subjected to DNA isolation and nested PCR targeting fragments of the glutamate dehydrogenase (*gdh*), triose phosphate isomerase (*tpi*), and beta-gardin (*bg*) genes. A total of 52 samples were successfully analyzed using PCR and DNA sequencing. Assemblage B was detected most frequently and accounted for 65.4% (34/52) of infections, while Assemblage A accounted for 34.6% (18/52). There was a high level of genetic diversity among the isolates with 46.2% designated as sub-assemblage BIV (24/52), 25% sub-assemblage AII (13/52), 19.2% sub-assemblage BIII (10/52), and 9.6% sub-assemblage AI (5/52). No mixed infections were detected. The results suggest that the majority of infections were imported and that endemic anthroponotic transmission plays a minor role in Austria.

Keywords Austria · *Giardia* · Assemblages · Molecular epidemiology · Multilocus sequencing

✉ John F. Lindo
john.lindo@uwimona.edu.jm

[1] Institute of Specific Prophylaxis and Tropical Medicine, Center of Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

[2] Department of Microbiology, The University of the West Indies, Kingston 7, Mona, Jamaica

Introduction

Giardia spp. are flagellated protozoan parasites that colonize the intestinal tract of a wide range of, mammals reptiles, and birds (Adam 2001; Thompson and Monis 2004). They have a worldwide distribution, approximately 2% of adults and 6–8% of children in developed countries, and up to 33% in developing countries are infected (CDC 2012). The life cycle involves the fragile reproductive and feeding trophozoite and a highly resistant cyst which can survive harsh environmental conditions for prolonged periods of time (Adam 2001; Feng and Xiao 2011). Infection is initiated when cysts are ingested with contaminated water, food, or direct fecal-oral contact. Excystation results in the release of motile trophozoites which multiply in the small intestine causing asymptomatic or mild self-limiting infections in most cases or acute to severe gastroenteritis, occasionally resulting in nutrient malabsorption and long-term growth retardation in children. Children in day care and school settings and immunocompromised persons are most at risk of acquiring symptomatic infections (Fraser et al. 2000; Thompson et al. 2008). Giardiasis is often underreported due to the self-limiting nature of infections.

Giardia duodenalis (synonyms *G. intestinalis* and *G. lamblia*) consists of eight assemblages (A–H), which today are regarded as separate species each with varying host specificities (Thompson and Monis 2012). Assemblage A, corresponding to *G. duodenalis*, and assemblage B, corresponding to *Giardia enterica*, are known to infect humans and are responsible for 37 and 58% of human infections, respectively, (Ryan and Caccio 2013). While assemblage B is usually associated with prolonged and severe cases of diarrhea and is considered more pathogenic, both, A and B, are zoonotic and infect a wide host range including dogs, cats, livestock, and wildlife (Feng and Xiao 2011). The assemblages are further divided into subtypes/sub-assemblages I to IV. Sub-

assemblage AI and AIII are responsible for zoonotic infections in humans, while sub-assemblage BIII and BIV have been reported to infect humans predominantly (Plutzer et al. 2010; Wang et al. 2014). However, further division of assemblage B into BIII and BIV previously established by allozyme electrophoresis is no longer supported by sequence analysis due to inconsistencies at the subtype level (Feng and Xiao 2011). Assemblages C–H are found in different groups of animals such as dogs and other canids (C/D), cattle and other livestock (E), cats (F), and rats (G) and seals (H) (Feng and Xiao 2011). Although less often, sporadic cases of human infection with assemblages C, D, E, and F have been reported providing evidence of zoonotic transmission (Ryan and Caccio 2013).

Advances in molecular techniques have led to an improvement in the classification of *Giardia* isolates allowing identification of strains at the subtype level. Such analyses revealed that using a single genetic locus for characterization often produces conflicting results when the same isolates are compared using another genetic locus (Feng and Xiao 2011; Traub et al. 2004). Thus, a multilocus sequence analysis targeting more than one locus is crucial for identifying *Giardia* at the sub-assemblage level.

Giardia infections are not well studied in Austria, although giardiasis was reported as the most common parasitic infection among travelers returning from endemic areas (Reinthaler et al. 1998). Furthermore, the parasite was recorded at low prevalence (2.7%) among patients with acute gastroenteritis in three sentinel clinics (Reinthaler et al. 1998) and small outbreaks and occasionally complicated cases of giardiasis have been reported from rural areas and from the young and elderly population (Fortunat et al. 2004). However, there are no molecular data on isolates from humans. Uncharacterized *Giardia* infections have been reported from calves, and infections with *G. duodenalis* assemblage A have been reported from cats (Haschek et al. 2006; Hinney et al. 2015). Pet ownership is high in many European countries with an average of 14.9% (range 7.2 to 35.0%) cat ownership and 12.0% (range 5.4 to 35.0%) dog ownership in 12 birth cohorts (Anyo et al. 2002). In Germany, almost 50% of cases of giardiasis were suspected to be acquired autochthonously (Espelage et al. 2010).

The aim of this study was to identify assemblage and subtype diversity of *Giardia* strains isolated from Austrian patients using a multilocus approach.

Materials and methods

Safety

Safety regulations using biological and chemically hazardous material by international standards, the Austrian Gene Technology Law, and the ISO 15189 standard for medical laboratories were strictly followed.

Sample collection

A total of 65 microscopically *Giardia*-positive stool samples submitted to the parasitology laboratory at the Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna from patients with diarrhea were included in the study. Stool samples were concentrated using a modified SAF procedure whereby ethanol was used instead of formalin to allow subsequent DNA isolation. Samples were stored at 4 °C.

Molecular investigation

DNA extraction was performed using the DNA stool Kit (QIAamp Fast DNA Stool Kit) following the manufacturer's instructions. Initial preparation included adding 20 mg/200 μl of concentrated stool sample to a microfuge tube followed by washing using distilled water then centrifugation at 16,000×*g* for 10 min. Extracted DNA was stored at −20 °C until PCR targeting segments of three housekeeping genes was performed. This included nested PCR using the *tpi*, *gdh*, and *bg* genes as markers for assemblage and subtype analysis and conventional PCR using species-specific primers for identification of mixed infection with assemblage A and B at the *tpi* locus (Table 1). Primers targeting the *tpi*, *gdh*, and *bg* loci were selected from previously published studies (Feng and Xiao 2011; Gelanew et al. 2007; Zheng et al. 2014). Primers specific for assemblage A and B targeting the *tpi* gene were used as described (Anuar et al. 2014). Conditions for primary and nested amplifications were identical: 35 cycles (94 °C for 1 min, 45 °C for 30 s, and 72 °C for 45 s) in an Eppendorf® thermocycler with an initial preheating at 94 °C for 10 min and a final extension at 72 °C for 7 min. The protocol for conventional PCR was similar to that used in nested PCR with the exception of 40 instead of 35 cycles. Products were visualized using 2% agarose gel electrophoresis and a GelRed™ stain (BioTrend, Cologne, Germany). Amplicons were purified using a PCR and Gel Band Purification kit (illustra GFX, GE Healthcare, Austria) and sequenced using an ABI automated sequencer and the respective dye (BigDye Terminator V3.1, Applied Biosystems, Austria). Multiple alignments of nucleotide sequences were conducted using the CLUSTAL X® alignment program and analyzed using GeneDoc® (version 2.7.000). Sequences were obtained from both strands, assembled to a consensus sequence, and compared to reference sequence from GenBank. Assemblages and

Table 1 Primers used in identification of *Giardia* at the *tpi*, *gdh*, and *bg* loci

Primer	Oligonucleotide 5′-3′	Amplicon size	Assay
TPI1 TPI2	Forward (5′-AAC GCA ATC ACT GTA TCT-3′) Reverse (5′-CAA TGA CAA CCT CCT TCC-3′)	650 bp	Nested
TPI3 TPI4	Forward (5′-CTT CAT CGG CGG TAA CTT-3′) Reverse (5′-GGC ACG CTT AGC CTT CTT-3′)	334 bp	
GDH1 GDH2	Forward (5′-TTC CGT GTT CAG TAC AAC TC-3′) Reverse (5′-ACC TCG TCC TGA GTG GCG CA-3′)	754 bp	Nested
GDH3 GDH4	Forward (5′-ATG ACT GAG CTT CAG AGG CAC GT-3′) Reverse (5′-GTG GCG CAA GGC ATG ATG CA-3′)	530 bp	
GiarF bg GiarR bg	Forward (5′-GAA CGA ACG AGA TCG AGG TCC G-3′) Reverse (5′-CTC GAC GAG CTT CGT GTT-3′)	511 bp	Nested
G7 G759	Forward (5′-AGG CCC GAC CTC ACC CGC AGT GC-3′) Reverse (5′-GAG GCC GCC CTG GAT CTT CGA GAC GAC-3′)	753 bp	
AssAF AssAR	(5′-CGC CGT ACA CCT GTC-3′) (5′-AGC AAT GAC AAC CTC CTT CC-3′)	332 bp	Conventional
AssBF AssBR	Forward (5′-GTT GTT GTT GCT CCC TCC TTT-3′) Reverse (5′-CCG GCT CAT AGG CAA TTA CA-3′)	500 bp	Conventional

sub-assemblages were identified according to Feng and Xiao (2011) and Thompson et al. (2000).

Results

Distribution of *Giardia* assemblages

Of all 65 samples positive for *Giardia* by microscopy, DNA extraction and PCR were successful from 52 samples. Failure to extract sufficient *Giardia* DNA of good quality from all samples may have been due to low concentration of cysts in some samples and/or long-term storage of samples. Molecular analysis revealed that assemblage A was responsible for 34.6% (18/52) while assemblage B was responsible for 65.4% (34/53) of the infections. There was no evidence of mixed infections among the patients included in the study.

Sequence analysis of the *tpi*, *gdh*, and *bg* genes

On sequence analysis, sub-assemblage BIV was the most common subtype identified and was seen in 46.2% (24/52) of the samples. This was followed by sub-assemblage AII which was identified in 25% (13/52), sub-assemblage BIII in 19.2% (10/52), and sub-assemblage AI in 9.6% (5/52) of patients. Amplification of the *tpi* locus was successful in 100% of all 52 samples, while amplification of the *bg* and *gdh* loci was successful only in 86.5% (45) and 55.8% (29), respectively. There were inconsistencies in subtyping between the different loci. Seven samples revealed sequences conforming to sub-assemblage BIV at the *bg* and *gdh* loci but correlated to BIII at the *tpi* locus. Another four samples were also of the sub-assemblage BIV type at the *bg* locus but were of the BIII type at the *gdh* locus. Similarly, sub-assemblage AI was identified at the *gdh* locus in three samples, which revealed higher identity to sub-assemblage AII at the *tpi* locus. The observed inconsistencies were highest at the *bg* locus, in which 44.1% (15/34) of all samples produced subtypes which were non-concordant with the other loci.

Discussion

Molecular characterization of *Giardia* isolates from patients in Austria revealed that assemblage B accounted for 65.4% of all cases while assemblage A accounted for 34.6%. These results are similar to results from other European countries, e.g., in Sweden, assemblage B was identified in 61.1% (127 of 208) of patient samples (Lebbad et al. 2011) and analysis from sporadic cases in England and Wales identified assemblage B from 60% (21 of 35) of cases (Amar et al. 2002). The pattern of assemblage distribution across the neighboring countries of Austria is more diverse and also difficult to compare. While there seems to be a higher prevalence of assemblage A over assemblage B in German travelers (Broglia et al. 2013), in children living in a disprivileged setting in Rome, Italy (Marangi et al. 2010), and also in a small sample of asymptomatic persons in Hungary (Plutzer et al. 2014), there was a predominance of assemblage B in samples from Slovakia; however, based only on *tpi* sequences and with only nine samples investigated (Strkolcova et al. 2016). Generally, assemblage B seems to be the most common cause of giardiasis in both, developing and developed countries, and has also been responsible for food and water-borne outbreaks in, England, Wales, Norway, and the USA (Amar et al. 2002; Daly et al. 2010; Feng and Xiao 2011; Karon et al. 2011;

Robertson et al. 2006; Sulaiman et al. 2003). This dominance of assemblage B in clinical cases was suspected to be the result of increased virulence which may result in enhanced transmission cycles and higher detection rates, as infections with assemblage A may more often remain asymptomatic and thus not be detected (Lebbad et al. 2008; Lebbad et al. 2011). It was also shown that children under 15 years of age are more likely to acquire infections with assemblage B (Anuar et al. 2014).

Assemblage A is dominant in certain geographical settings (Feng and Xiao 2011; Thompson and Monis 2004), e.g., it was isolated from 75% of 41 patients investigated in Egypt, from all 26 samples tested in a study conducted in Mexico (Anuar et al. 2014; Helmy et al. 2009), and it was the only assemblage found in symptomatic patients in Jamaica (Lee et al. 2016).

Even though mixed infections with different *Giardia* assemblages have been frequently reported in humans (Carvalho-Costa et al. 2007; Epe et al. 2010; Feng and Xiao 2011), there were no cases of mixed infection in this study. Similarly, no case of mixed infection was found in a study conducted on patients in France where all the isolates were assemblage A (Bertrand et al. 2005). Mixed infections might be more likely detected with a larger sample size; however, they could also simply be infrequent in areas of low endemicity (Anuar et al. 2014).

At the sub-assemblage level, sub-assemblage AII was isolated from 25% (13/52) of patients while AI was isolated from 9.6%. Likewise, sub-assemblage BIV (46.2%) accounted for far more cases than BIII (19.2%). However, the validity of subtypes within assemblage B is generally controversial (Feng and Xiao 2011). Analysis of the *bg* gene fragment from residents in Bangkok also showed a higher occurrence of sub-assemblage BIV over BIII (54.5 and 45.5%, respectively) while sub-assemblages AI and AII were identified at rates of 12 and 88%, respectively (Tungtrongchitr et al. 2010). Most studies showed that sub-assemblage AII was generally more frequently isolated from humans while AI is commonly identified in animals (Feng and Xiao 2011). Sub-assemblages BIV and AII are the most often isolated subtypes from human giardiasis patients (Feng and Xiao 2011). As such, this high prevalence of sub-assemblage BIV and AII suggests that infections were likely acquired through anthroponotic transmission. Human to human transmission is highly unlikely to occur in Austria as tap water is mostly mountain spring water and is not produced from surface water. Furthermore, all Austrian households are connected to sewage treatment plants, and the use of human feces as fertilizers has been prohibited decades ago. However, sub-assemblages AI, AII, BIII, and BIV have all been isolated from other hosts including cattle, monkey, horses, dogs, and cats which corroborates that zoonotic transmission cannot be totally excluded (Ballweber et al. 2010; Caccio and Ryan 2008; Sprong et al. 2009).

Interestingly, amplification of the *gdh* and *bg* loci was not always successful, although all samples revealed good amplicons with the primers targeting the *tpi* gene. This may be attributable to the shorter length of the *tpi* amplicon. Previous reports have also shown this high amplification yield and have concluded that this gene is particularly suited for analyses of evolutionary relationships within *Giardia* (Feng and Xiao 2011). A recent study reported amplification rates of 70, 45, and 33% for the *tpi*, *gdh*, and *bg* locus, respectively, (Huey et al. 2013).

Inconsistent subtyping results were seen when comparing the three genes. For example, a single sample showed highest similarity to assemblage AI at the *tpi* locus but highest similarity to AII at the *bg* locus. This was initially thought to be due to mixed infections, but this could be excluded. It is known that up to 15% of all *Giardia* isolates analyzed so far showed inconsistencies between different markers, including the SSUrRNA, *tpi*, *bg*, and *gdh* genes (Sprong et al. 2009). Assemblage B showed a particularly high level of genetic diversity at the *bg* locus.

In summary, the high diversity suggests that most human *Giardia* infections in Austria are imported, and this is also corroborated by the high prevalence of anthroponotic subtypes.

Compliance with ethical standards Ethical approval for the study was obtained from the Ethics Committee of the Medical University of Vienna (Ethics permit number: 1070/2015), and all patient identifiers were removed from the samples.

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