

Research Article

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## Multilocus genotyping of *Giardia duodenalis* (Lambl, 1859) from symptomatic human infections in Slovenia

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**Abstract:** Giardiasis is a common gastrointestinal infection of humans and animals with a worldwide distribution. Eight genetic groups (known as assemblages A to H) are currently recognised within the species complex of *Giardia duodenalis* (Lambl, 1859), of which assemblages A and B are responsible for infection of humans and other mammalian hosts. Genotyping data on giardiasis are not available from Slovenia. In this work, we have characterised isolates of *G. duodenalis* from 85 human symptomatic cases collected during 2002–2013. Genomic DNAs were first tested by a real-time (rt) PCR assay and then by conventional PCR at three loci (beta-giardin, *bg*; triose phosphate isomerase, *tpi*; and glutamate dehydrogenase, *gdh*). We found that the threshold cycle (Ct) values in rt-PCR testing were higher for samples collected during 2002–2005 and that this was paralleled by a low amplification rate in conventional PCR (6 of 32, i.e. 19%). In contrast, lower Ct values and higher amplification rate (45 of 53; 85%) were observed for samples collected during 2006–2013, suggesting an adverse effect of prolonged freezing of stools. Assemblages A and B were found with an almost identical frequency in the 51 genotyped samples. In agreement with previous studies, sequences from assemblage B isolates were characterised by larger genetic variability and by the presence of heterogeneous positions, which made assignment to specific genotypes difficult. Less variability was observed in sequences from assemblage A isolates, which belonged to the human-specific subassemblage AII. These data showed that the genotypes of *G. duodenalis* that circulate in humans in Slovenia are similar to those previously identified in Europe.

**Keywords:** giardiasis, molecular typing, assemblages, genotypes, epidemiology

The flagellated protist *Giardia duodenalis* (Lambl, 1859) (syn. *G. intestinalis*, *G. lamblia*) is a common gastrointestinal parasite of humans and other mammals, including pets and livestock (Feng and Xiao 2011). A considerable amount of data have shown that *G. duodenalis* is a species complex, whose members show little morphological variation, yet at least eight distinct genetic groups or assemblages (A to H) have been recognised based on protein or DNA sequence polymorphisms (Andrews et al. 1989, Monis et al. 2003, Ryan and Cacciò 2013).

The genetic distances separating the assemblages are very big and recent comparisons at the whole genome level (Frantzen et al. 2009, Jerlstrom-Hultqvist et al. 2013) strongly supported that assemblages A, B and E (the three assemblages for which genome sequences are currently available) represent distinct species. The same concept was raised before based on other biological and phenotypic features and the names *G. duodenalis* (assemblage A) and *G. enterica* (assemblage B) were proposed but not widely accepted (Monis et al. 2009).

The epidemiology of giardiasis is complex and multiple transmission routes contribute to human infection, with an

important role played by water (Feng and Xiao 2011). Despite many investigations, several important epidemiological aspects remain unclear, including the role of animals in zoonotic transmission (Ryan and Cacciò 2013) and the clinical significance of single and mixed infections with assemblages A and B (Kohli et al. 2008).

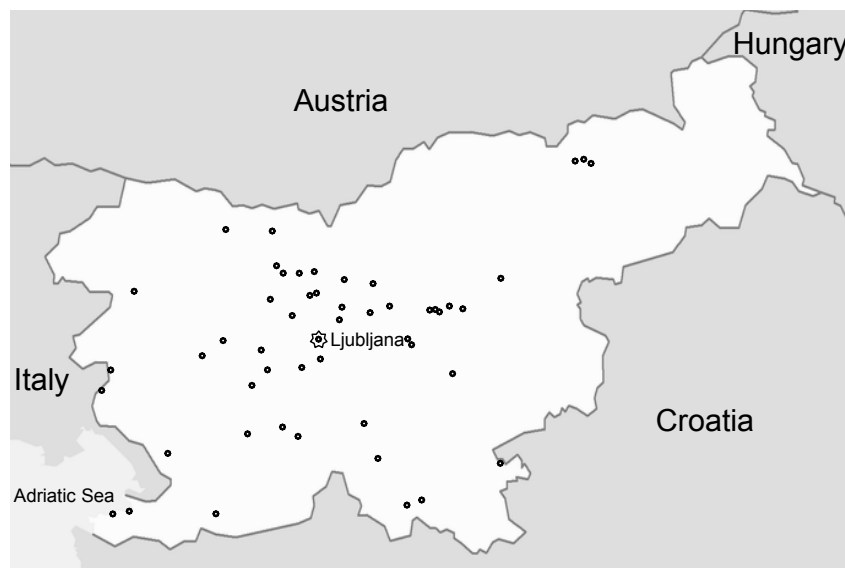
To date, no genetic information is available on human giardiasis in Slovenia. Therefore, the aim of the present work was to characterise isolates collected in this country using established molecular methods.

### MATERIALS AND METHODS

**Sources of isolates.** Stool samples from 85 sporadic, symptomatic human cases of giardiasis throughout Slovenia were referred to the Institute of Microbiology and Immunology (IMI) in Ljubljana between 2002 and 2013. The geographical distribution of cases included in the study is shown in Fig. 1. The mean age of the patients was 31.5 years (range 2–63 years, SD = 13), with 51 being males and 34 females.

Cysts of *Giardia* spp. were identified microscopically in stool smears using a direct immunofluorescence test (MeriFluor, Meridian Bioscience, Cincinnati, Ohio, USA), following the manu-

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**Fig. 1.** Map of Slovenia. Dots indicate the geographic origin of the human cases of giardiasis included in the study.

facturer's instructions. Cyst-containing stool samples were stored at  $-20^{\circ}\text{C}$  until used for molecular analysis. Positive stool samples were archived as a part of the laboratory collection. Forty-seven stools (55%) were also examined for other potential etiological agents of gastroenteritis, including bacteria (54% of stools) and viruses (12% of stools). Five cases of mixed infection of *Giardia* and bacteria (*Providencia alcalifaciens*, *Salmonella* sp., *Campylobacter coli*, *Shigella boydii* and *Shigella sonnei*), as well as one case of mixed infection of *Giardia* with rotavirus, were observed.

**Molecular analysis.** Genomic DNA was extracted from stool samples using either the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), with an initial incubation of 10 min at  $99^{\circ}\text{C}$ , or the FastDNA SPIN Kit for faeces (MP Biomedicals, Solon, Ohio, USA). When extracting DNA by the FastDNA SPIN Kit for faeces, the TissueLyser LT (Qiagen), set at 50 Hz for 5 min, was used instead of the FastPrep-24 instrument for sample homogenisation. The DNA extracts were stored at  $-20^{\circ}\text{C}$  until analysis. DNA from axenic cultures of strains of *Giardia duodenalis* of assemblage A (WB, genotype AI) and assemblage B (GS, genotype BIV) were used as controls in both real-time (rt) and conventional PCR assays.

All genomic DNAs were first tested using a TaqMan rt-PCR assay (Verweij et al. 2004), which targets a 62-base pair fragment of the small subunit ribosomal (SSU rRNA) gene of *G. duodenalis*. Next, conventional, nested PCR assays were used to amplify fragments of the *bg* (511 bp), *tpi* (530 bp) and *gdh* (530 bp) genes of human isolates of *G. duodenalis* according to published protocols (Sulaiman et al. 2003, Lalle et al. 2005, Cacciò et al. 2008). PCR products were purified using spin columns (QiaQuick PCR Purification kit – Qiagen) and sequenced from both strands. The sequences were edited using CLC Main Workbench 6.0 (CLC bio – Qiagen) and compared to all available sequences using BLAST. Novel sequences without heterogeneous positions have been deposited in the European Molecular Biology Laboratory (EMBL) database under the accession numbers LN626343–LN626354.

**Phylogenetic analysis.** Multiple alignments were generated using Clustal X (Larkin et al. 2007). For phylogenetic analyses, unambiguous sequences (i.e. sequences not containing heteroge-

neous positions) from the current investigation, from a previous study (Cacciò et al. 2008) and from representative sequences retrieved from GenBank were used. The sequences from the three loci were concatenated and phylogenetic trees were inferred by Neighbour-Joining (NJ) or Maximum Likelihood (ML) methods using the Kimura 2 parameter algorithm, as implemented in the software MEGA version 5.4 (Tamura et al. 2011). The robustness of the nodes was tested by a bootstrap analysis of 1 000 iterations.

## RESULTS

Using a SSU rRNA rt-PCR assay, all isolates of *G. duodenalis* ( $n = 85$ ) yielded positive results. It was noticed, however, that the average threshold cycle (Ct) value of DNAs from samples collected between 2002 and 2005, and stored at  $-20^{\circ}\text{C}$  for 8–11 years before extraction, was 31.1 (range 25.6–36.0), compared to an average Ct value of 26.6 (range 18.6–38.4) for samples collected between 2006 and 2013, which were stored for no longer than 3 years. The difference in Ct values between the two groups of samples was statistically significant ( $p < 0.01$ ).

Using conventional nested PCR assays, 51 of the 85 human isolates (60%) were amplified at least at one of the three loci tested (Table 1). In agreement with the rt-PCR results, amplification was largely more successful in the 2006–2013 human samples (85%) than in the 2002–2005 samples (19%).

BLAST comparison with homologous sequences from the GenBank database identified assemblage A in 26/51 (51%) isolates and assemblage B in 25/51 (49%). Assemblage A infections were observed in 9 females and 17 males, whereas assemblage B infections were found in 12 females and 13 males. The distribution of assemblage A and B in females versus males did not differ significantly. No discordant assignment to assemblages was observed for the 45 isolates that could be genotyped at more than one locus (Table 1).

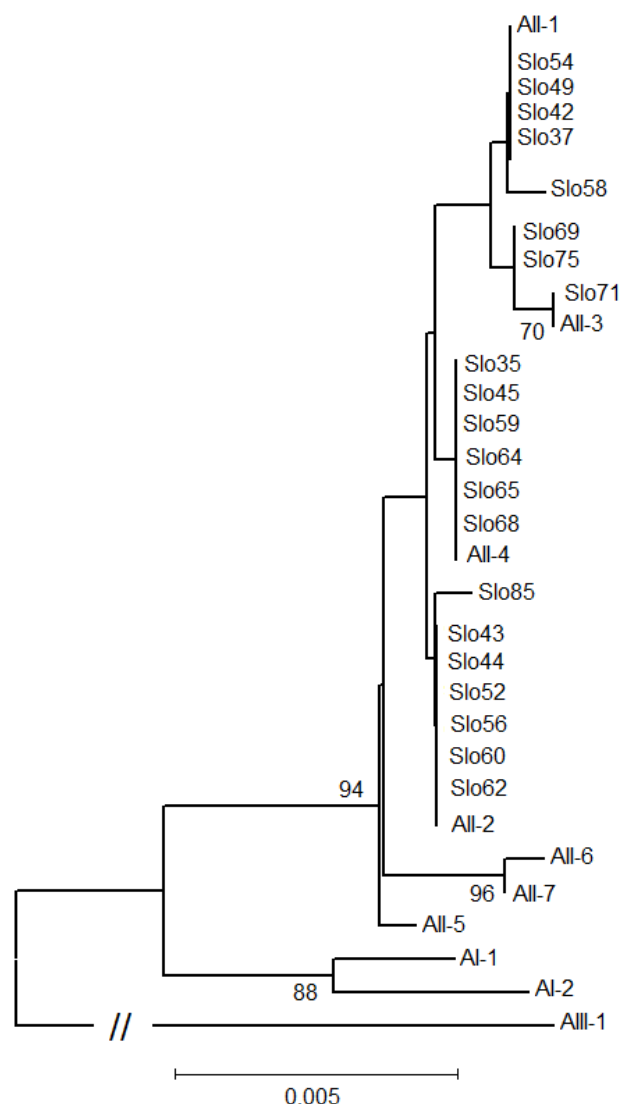
At the *bg* locus, 49 of 85 (58%) isolates were PCR positive and sequence analysis showed Assemblage A in 25

**Table 1.** Genotypes of human isolates of *Giardia duodenalis* (Lambl, 1859) identified at the *bg*, *tpi* and *gdh* loci.

Isolate	Age	Gender	<i>bg</i>	<i>tpi</i>	<i>gdh</i>	Assemblage/ MLG
Slo1	37	M	neg	A2	neg	A
Slo6	38	M	A3	neg	neg	A
Slo9	31	M	A3	neg	neg	A
Slo15	23	F	B <sup>b</sup>	B	neg	B
Slo16	36	M	B <sup>a</sup>	B	neg	B
Slo19	23	M	B <sup>b</sup>	neg	neg	B
Slo33	10	M	B <sup>b</sup>	B <sup>b</sup>	neg	B
Slo34	39	M	A2	A2	neg	A
Slo35	15	F	A3	A2	A4	AII-4
Slo37	24	M	A2	A2	A2	AII-1
Slo38	8	F	B	B	B	BIV
Slo39	36	M	A <sup>a</sup>	A2	A4	AII
Slo40	34	F	B	B	B	BIV
Slo41	33	F	B	neg	neg	B
Slo42	49	M	A2	A2	A2	AII-1
Slo43	37	M	A3	A2	A2	AII-2
Slo44	30	F	A3	A2	A2	AII-2
Slo45	63	M	A3	A2	A4	AII-4
Slo47	47	F	B <sup>b</sup>	B <sup>b</sup>	neg	B
Slo48	36	M	B	B	B	BIV
Slo49	29	M	A2	A2	A2	AII-1
Slo50	28	M	neg	B	neg	B
Slo52	25	F	A3	A2	A2	AII-2
Slo53	35	M	B <sup>a</sup>	B <sup>a</sup>	B <sup>b</sup>	B
Slo54	29	M	A2	A2	A2	AII-1
Slo55	26	M	B <sup>b</sup>	B <sup>b</sup>	B <sup>a</sup>	B
Slo56	33	F	A3	A2	A2	AII-2
Slo58	33	F	A2	A <sup>c</sup>	A2	AII
Slo59	39	M	A3	A2	A4	AII-4
Slo60	38	M	A3	A2	A2	AII-2
Slo61	21	M	B	B <sup>a</sup>	B <sup>a</sup>	B
Slo62	52	F	A3	A2	A2	AII-2
Slo63	42	M	B	B	B	BIV
Slo64	11	F	A3	A2	A4	AII-4
Slo65	2	M	A3	A2	A4	AII-4
Slo67	31	F	B <sup>a</sup>	B <sup>a</sup>	B <sup>a</sup>	B
Slo68	31	F	A3	A2	A4	AII-4
Slo69	45	F	A2	A2	A4	AII
Slo70	23	M	B	B	B <sup>a</sup>	B
Slo71	31	M	A2	A2	A3	AII-3
Slo72	58	F	B <sup>a</sup>	B <sup>b</sup>	B <sup>a</sup>	B
Slo73	26	M	B <sup>a</sup>	B <sup>a</sup>	B <sup>a</sup>	B
Slo75	58	M	A2	A2	A4	AII
Slo76	9	F	B <sup>b</sup>	B	B	BIV
Slo77	33	M	B	B	B <sup>a</sup>	B
Slo79	47	F	B <sup>a</sup>	B <sup>a</sup>	B <sup>a</sup>	B
Slo80	47	F	B <sup>a</sup>	B <sup>a</sup>	B <sup>a</sup>	B
Slo81	35	F	B <sup>a</sup>	B <sup>b</sup>	B <sup>b</sup>	B
Slo82	31	F	B <sup>a</sup>	B <sup>a</sup>	B <sup>a</sup>	B
Slo83	29	M	B	B	B	BIV
Slo85	27	M	A3	A <sup>c</sup>	A2	AII

MLG – Multi-locus genotypes; <sup>a</sup> sequences showing heterogeneous positions; <sup>b</sup> new variants without heterogeneous positions; <sup>c</sup> single SNP difference from genotype A2

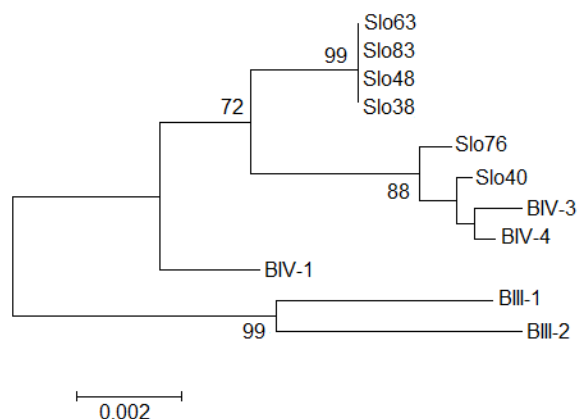
(51%) isolates and assemblage B in 24 (49%) isolates. Among assemblage A isolates, 9 were identical to genotype A2 (AY072723) and 15 were identical to genotype A3 (AY072724). A single isolate had a sequence identical to genotype A2 except for a single heterogeneous position (C261Y). Among assemblage B isolates, sequences from 9 of 24 (38%) isolates showed heterogeneous positions and

**Fig. 2.** Neighbour Joining phylogenetic tree of concatenated *bg*, *tpi* and *gdh* genes from assemblage A isolates. Multi-locus genotypes from a previous study (Cacciò et al. 2008) are indicated. Only bootstrap support values > 50% are shown.

could not be unequivocally assigned to a specific genotype, whereas no heterogeneous positions were observed in the remaining 15 isolates. Among the latter, six new variants were detected (Table 1).

At the *tpi* locus, 47 of 85 (55%) isolates were PCR positive and sequence analysis show assemblages A and B in 24 (51%) and 23 (49%) isolates, respectively. Among assemblage A, genotype A2 (U57897) was found in 22 isolates, whereas two isolates differed from genotype A2 by a single nucleotide polymorphism (SNP) at position 445 (G445A). There was no evidence of heterogeneous positions among assemblage A isolates. Among assemblage B isolates, sequences of 7 of 23 (30%) isolates showed heterogeneous positions, whereas no heterogeneous positions were observed in the remaining 16 isolates. Among the latter, five new variants were detected (Table 1).

At the *gdh* locus, 40 of 85 (47%) samples were PCR positive and sequence analysis showed assemblage A in 55% (22/40) and assemblage B in 45% (18/40) of the



**Fig. 3.** Neighbour Joining phylogenetic tree of concatenated *bg*, *tpi* and *gdh* genes from assemblage B isolates. Multi-locus genotypes from a previous study (Cacciò et al. 2008) are indicated. Only bootstrap support values > 50% are shown.

isolates. Among assemblage A isolates, 12 were identical to genotype A2 (AY178736), 9 were identical to genotype A4 (EF507657), and one was identical to genotype A3 (EU278608). Among assemblage B isolates, 10 of 18 (56%) sequences showed heterogeneous positions, whereas no heterogeneous positions were observed in the remaining eight isolates. Among the latter, two new variants were detected (Table 1).

Sequence data were generated from the three loci for 40 of 85 (47%) human isolates (Table 1). A phylogenetic analysis of the concatenated *bg*, *tpi* and *gdh* sequences from assemblage A (Fig. 2) showed that the so-defined Multi-locus genotypes (MLGs) from Slovenian isolates corresponded, or were very closely related, to previously described in human isolates from Europe, namely to MLG AII-1, MLG AII-2, MLG AII-3 and MLG AII-4. Among assemblage B isolates, phylogenetic analysis of MLGs was performed only for the six isolates that had sequences without heterogeneous positions; this showed that MLGs from Slovenia grouped with isolates from subassemblage BIV (Fig. 3).

## DISCUSSION

The main goal of this study was to characterise at the molecular level human isolates of *Giardia duodenalis* collected in Slovenia, a European country for which no such data were previously available. Giardiasis is included in the list of notifiable diseases in independent Slovenia since 1991, and cases are reported to the European Centre for Disease Control. In 2011, the crude incidence rate of giardiasis in Slovenia was estimated at 1.51 per 100 000 population, which is lower than the average European value of 5.49 (European Centre for Disease Prevention and Control 2013). These data, however, should be interpreted with caution, as giardiasis is likely to be underdiagnosed and under-reported in most European countries, including Slovenia. According to the information from the National Institute of Public Health (NIJZ) of the Republic of Slovenia, no epidemics, foodborne or waterborne outbreaks of giardiasis have been reported during the course of this study. However, cysts of *Giardia* have been detected in

several water supplies, though a link to human disease has not been established so far (Gorazd Pretnar and Matjaž Retelj, National Laboratory of Health, Environment and Food, Slovenia – pers. comm.). Since rural areas represent a significant part of the Slovenian territory and livestock farming is one of the most important agricultural activities, a role of livestock in the environmental contamination and transmission of zoonotic diseases is to be considered.

In this study, stool samples from 85 sporadic human cases of giardiasis collected between 2002 and 2013 were analysed. It is noteworthy that most cases are from adults (mean age, 31.5 years), and that children are underrepresented (only five cases were 0–10 years old). This seems to contrast with the fact that giardiasis is mostly a paediatric infection worldwide (Cacciò and Sprong 2011). In Europe, the highest notification rate was in 0–4 year old male children, with 11.2 per 100 000 population, followed by 0–4 year old female children, with 9.8 per 100 000 population (European Centre for Disease Prevention and Control 2013). However, according to the information from NIJZ, in Slovenia only 5.1% and 13.4% of all cases reported in the last 9 years were from children 0–4 and 0–14 years old, respectively.

Genotyping of 51 human samples showed assemblages A and B with almost identical frequencies. For the 45 isolates typed at more than one locus, assignment to assemblages was always concordant, indicating an apparent absence of inter-assemblage recombination or of mixed infections in the parasite circulating in Slovenia. Phylogenetic analysis of MLGs, performed on concatenated sequences with no detectable heterogeneity, showed that 21 assemblage A isolates from Slovenia clustered with other human-derived MLGs, all belonging to subassemblage AII (Fig. 2). The same analysis on assemblage B isolates showed clustering of six Slovenian isolates with reference sequences from subassemblage BIV (Fig. 3).

The fact that sequencing profiles from assemblage A isolates rarely showed heterogeneity, whereas the opposite characterise sequences from assemblage B isolates, largely agrees with previous studies (Sprong et al. 2009, Feng and Xiao 2011, Ryan and Cacciò 2013). As discussed extensively in the literature, this heterogeneity can be attributed to the presence of different alleles in the nuclei of single cysts or to the presence of genetically different cysts in a mixed infection. As demonstrated by the elegant study of Ankarklev et al. (2012), who genotyped single cysts from clinical samples, both situations occur, even in the parasite population that infects a single individual.

Apart from the underlining mechanism(s), the practical implication of allelic sequence heterozygosity (ASH) is that isolates cannot be assigned to subtypes, making meaningful comparisons with other available data impossible (Cacciò et al. 2008, Lebbad et al. 2010). It has been suggested that sequence analyses of both conserved and variable genes are required to characterise assemblage B isolates (Wielinga et al. 2011), but this is difficult to apply to large number of isolates. In contrast, since ASH is not uniformly distributed in the genome, at least in the GS strain of assemblage B that has been sequenced (Franzen



et al. 2009), it should be possible to develop new markers by targeting regions of the genome less affected by ASH (Wielinga et al 2015).

The clinical manifestations of giardiasis range from mild intestinal problems that resolve spontaneously in a week to complex symptoms that last up to several weeks, such as chronic diarrhoea, abdominal pain, nausea, weight loss, extra-intestinal manifestations and post-infectious fatigue (Halliez and Buret 2013). A number of investigations have tried to establish a correlation between symptomatology and the presence of either assemblage A or B (or both) in humans. Results from these studies have been controversial, with some claiming a correlation of assemblage A with symptoms, others a correlation of assemblage B with symptoms, and others no such correlation (Cacciò and Sprong 2011). In this work, assemblages A and B were found with similar frequency in the 51 symptomatic patients genotyped, thus no correlation appears to exist between parasite strains and symptoms in the host.

Another debated aspect is the occurrence of zoonotic transmission of giardiasis (Ryan and Cacciò 2013). As stated above, Slovenia is predominantly rural country and this has prompted a number of studies on zoonotic infections. Among intestinal parasites, a previous study has shown a predominance of the zoonotic species *Cryptosporidium parvum* Tyzzer, 1912 in humans (see Soba and Logar 2008), and demonstrated that identical parasite subtypes circulate in livestock and humans, thus confirming the zoonotic potential of *C. parvum*. Rotavirus genotypes of bovine and porcine origin were detected in human infections (Steyer et al. 2008, 2010, 2013a) and, more recently, an orthoreovirus strain with high similarity to viruses detected in European bats was found in a child hospitalised with acute gastroenteritis (Steyer et al. 2013b).

In 2012, a study on *Clostridium difficile* showed a considerable overlap between ribotypes isolated from humans, animals and the environment, suggesting that pigs, cattle and poultry can be an important and emerging source of human infection or colonisation in Slovenia (Janežic et al. 2012). In contrast with those studies, only the non-zoonotic

assemblage E was found in 81 samples from Slovenian cattle by a real-time PCR assay (van Lith et al. 2015), suggesting a less important role of livestock in the transmission of giardiasis. Surveys of other animals will allow drawing more robust conclusions on zoonotic transmission of giardiasis in Slovenia.

However, the present study has some limitations. First, as stated above, the examined cases of human giardiasis were mostly from adults and children were under-represented. Given that carriage of intestinal protists is low in children living in Europe (Guarino et al. 2014), it seems plausible that paediatricians exclude parasites from the diagnostic scheme of gastroenteritis, thus contributing to the underdiagnosis of giardiasis in children. Moreover, giardiasis can occur as a mixed infection with other pathogens with more acute course and can therefore remain undiagnosed. The distribution of *G. duodenalis* assemblages and genotypes in Slovenian children may thus differ from that observed in this study. Second, we found that all 85 DNA from human stools tested positive for DNA of *G. duodenalis* using a TaqMan rt-PCR assay (Verweij et al. 2004), whereas only 51 (60%) resulted positive by conventional, nested PCR assays at three different loci. The rate of positivity, however, was much higher (85%) for more recently collected samples than for older one (19%). This may be explained by more extensive fragmentation of DNA extracted from stools stored for prolonged time at -20 °C, resulting in a template from which the multi-copy, 62 bp target of rt-PCR, is amplified more efficiently than the single copy, 500 bp targets of conventional PCR.

In conclusion, this study showed that assemblages and genotypes of *G. duodenalis* associated with human giardiasis in Slovenia are those that predominantly circulate in Europe, and confirmed that assemblage B isolates are difficult to genotype.

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