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ORIGINAL PAPER

Multilocus sequence typing of canine *Giardia duodenalis* from South Eastern European countries

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Abstract *Giardia duodenalis* is a worldwide occurring protozoan that can infect various mammalian hosts. While living conditions are getting closer between pet animals and owners, there is discussion whether dogs may contribute to the transmission of these pathogens to humans. The present study was conducted in order to identify the *Giardia* assemblages in dogs from South Eastern Europe. For this purpose, 1645 faecal samples of household and shelter dogs from Albania, Bulgaria, Hungary, Macedonia, Romania and Serbia were tested for *Giardia* coproantigen by enzyme-linked immunosorbent assay (ELISA). A subset of 107 faecal samples demonstrating *Giardia* cysts by direct immunofluorescence assay (IFA) or microscopy (15–22 per country) plus 26 IFA-positive canine faecal samples from Croatia were used for DNA extraction and multilocus sequence typing with nested PCRs targeting five different gene loci: SSU rRNA, ITS1-5.8S-ITS2, beta giardin (bg), glutamate dehydrogenase (gdh) and triosephosphate isomerase (tpi). One third (33.7 %) of the samples tested positive for *Giardia* antigen in the coproantigen ELISA. Shelter dogs were infected more frequently than household dogs (57.2 vs. 29.7 %, $p<0.01$). Amplification was obtained in 82.0, 12.8, 11.3, 1.5, and 31.6 %, of the investigated samples at the SSU rRNA, bg, gdh and tpi loci and the ITS1-5.8S-ITS2 region, respectively. The dog-specific assemblages C and D were identified in 50 and 68 samples, respectively. The results demonstrate that *G. duodenalis* should be considered as a common parasite in dogs from South Eastern Europe. However, there was no evidence for zoonotic *Giardia* assemblages in the investigated canine subpopulation.

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Introduction

Giardia duodenalis is a worldwide occurring protozoan parasite infecting mammals including humans. In both developing and industrialised countries, *G. duodenalis* belongs to the most frequently diagnosed parasites of the gastrointestinal tract (Cacciò et al. 2005). *Giardia* infections may cause intestinal malabsorption with diarrhoea but can also be asymptomatic (Ballweber et al. 2010). Transmission occurs directly by ingestion of intermittently shed and immediately infectious *Giardia* cysts. Additionally, contaminated water or food may be a source of infection (Adam 1991; Feng and Xiao 2011). The taxonomy of *G. duodenalis* is still under discussion because of the substantial genetic heterogeneity (Plutzer et al. 2010; Thompson and Monis 2012). Currently, eight different assemblages and several subassemblages that were defined based on molecular and isoenzyme analyses are recognised (Monis et al. 2009; Plutzer et al. 2010). The assemblages A and B are considered zoonotic and occur in a wide host spectrum including humans and various animal species. The other assemblages are mainly host-specific: assemblages C and D occur in dogs, assemblage E in ruminants, assemblage F in cats, assemblage G in rodents and assemblage H in marine mammals (Ballweber et al. 2010; Cacciò and Ryan 2008; Lasek-Nesselquist et al. 2010). There has been evidence that dogs may also harbour isolates of *Giardia* assemblages A and B (Covacin et al. 2011; Eligio-García et al. 2008; Traub et al. 2004). The question whether *Giardia* infected dogs must be considered a risk for the transmission of this parasite to humans or vice versa has been subject of previous research (Thompson and Monis 2012). Several studies have proven that dogs carry infections with *G. duodenalis* worldwide. Prevalence data for canine *Giardia* infections range from 4.0 % in the USA (microscopy) (Little et al. 2009), over 10.0 % in Portugal (microscopy) (Neves et al. 2014) and 19.0 % in Italy (enzyme-linked immunosorbent assay, ELISA) (Bianciardi et al. 2004) to 22.7 % in Belgium (immunofluorescence assay, IFA) (Claerebout et al. 2009). Up to the present, only scarce information exists on *Giardia* infections and the potential zoonotic risk of dogs in South Eastern European countries. In Albania, the prevalence for an infection with *Giardia* was 35.5 % in dogs (ELISA) and 11.2 % in humans (IFA) (Shukullari et al. 2013; Spinelli et al. 2006). According to a review from 2011, the prevalence for human *Giardia* infections detected in Serbia over the last decades was 6.1 % (Nikolić et al. 2011). Furthermore, an investigation of water supplies of Southern Russia, Bulgaria and Hungary revealed considerable contamination with *Giardia* cysts in drinking water resources (Karanis et al. 2006; Plutzer et al. 2008). To date, prevalence data on canine *Giardia* infections exist for Serbia (3.8 and 14.6 % for household, stray and/or military working dogs, based on microscopy), Romania (34.6 % for household, kennel and shelter dogs with ELISA) and Hungary (58.8 % for household and kennel dogs based on ELISA) (Mircean et al. 2012; Nikolić et al. 2008; Nikolić et al. 1993; Szénási et al. 2007). Some of the data from this region are based on microscopy only, which is not as sensitive as ELISA and IFA (Feng and Xiao 2011; Geurden et al. 2008). Genotyping of canine isolates from Croatia and Hungary revealed the presence of dog-specific assemblages C and D as well as the zoonotic assemblages A and B (Beck et al. 2012; Szénási et al. 2007). A publication on the distribution of human *Giardia* assemblages revealed the occurrence of assemblage B in 87.0 % and a mixture of assemblages AII and B in 13.0 % of the investigated patients from Bulgaria (Chakarova et al. 2011). Single locus genotyping of *G. duodenalis* reveals limited information on the assemblage level whereas multilocus sequence typing (MLST) provides necessary information for the identification of *Giardia* subassemblages (Beck et al. 2012; Plutzer et al. 2010). In order to further characterise the potential risk of *Giardia* transmission in countries from South Eastern Europe, the objectives of the present study were to identify the *Giardia* assemblages of dogs by MLST of five gene loci and to add information on the occurrence of *Giardia* infections in dogs.

Materials and methods

Sample origin

A total of 1671 faecal dog samples were collected in seven South Eastern European countries from 2010 to 2014 (Table 1). Samples from Albania, Bulgaria and Hungary derived from studies that were conducted to survey canine gastrointestinal parasitic infections including giardiasis. Samples from Macedonia, Romania and Serbia were collected for the purpose of this study as were 26 *Giardia* cyst (IFA)-positive samples from Croatia which were provided specifically for MLST. Faecal samples were collected from dogs of all ages, both sexes, various breeds and different life styles. Street, shelter and kennel dogs (summarised for analysis as 'shelter dogs') as well as household dogs visiting veterinary clinics for various reasons were included. The samples were processed in a close timely manner (storage at 7 °C) or were frozen at −20 °C until analysed.

Screening for *Giardia* infections with coproantigen ELISA

For the detection of *Giardia* coproantigen, faecal samples from all countries except Croatia were screened using the

Table 1 Description of canine faecal samples collected in six South Eastern European countries for MLST including screening results for *Giardia* by coproantigen ELISA

Origin (country)	Period of collection	Positive/total number of samples (percentage)			Reference
		total	shelter dogs	household dogs	
Albania (Tirana area)	2010–2011	214/602 (35.5 %)	0/0	214/602 (35.5 %)	(Shukullari et al. 2013)
Bulgaria (different regions)	2012–2013	89/294 (30.3 %)	16/32 (50.0 %)	73/262 (27.9 %)	(Kirkova et al. unpublished)
Hungary (Western Hungary)	2012–2013	53/296 (17.9 %)	8/35 (22.9 %)	45/261 (17.2 %)	(Capári et al. unpublished)
Macedonia (different regions)	2013–2014	45/136 (33.1 %)	7/15[a] (46.7 %)	37/117[a] (31.6 %)	This study
Romania (South-Eastern Romania)	2013–2014	66/183 (36.1 %)	20/27 (74.0 %)	46/156 (29.5 %)	This study
Serbia (Belgrade)	2013	88/134 (65.7 %)	88/134 (65.7 %)	0/0	This study
Total	2010–2014	555/1645 (33.7 %)	139/243[a] (57.2 %)	415/1398[a] (29.7 %)	

[a] The origin (shelter dog/household dog) was unknown for four samples

ProSpecT™ Giardia Microplate assay (Remel, Lenexa, USA) according to the manufacturer's instructions.

Detection of *Giardia* cysts via IFA/merthiolate-iodine-formalin concentration (MIFC) following screening with coproantigen ELISA

At least 25 ELISA-positive samples from Albania, Bulgaria, Hungary, Macedonia and Romania were selected for further analysis with the IFA Merifluor® Cryptosporidium/Giardia (Meridian Bioscience, Luckenwalde, Germany) following the manufacturer's instructions. This method was used to confirm the presence of *Giardia* cysts by visualisation of fluorescein isothiocyanate (FITC)-conjugated antibodies against specific *Giardia* cyst wall epitopes. All 134 samples from Serbia were screened for *Giardia* cysts by the MIFC technique as described previously (Pfister et al. 2013).

DNA extraction

Per country, 15 to 26 *Giardia* cyst-positive samples were chosen for DNA extraction using the QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's recommended protocol. To increase the purity of the DNA, after extraction, all extracted samples were further purified with the QIAquick® PCR Purification Kit (Qiagen, Hilden Germany). The DNA concentration and purity were measured with the Nanodrop™ ND 1000-Spectrometer (Peqlab Erlangen, Deutschland).

Nested PCR amplification, species identification, sequencing and translation of DNA sequences to amino acids

Multilocus sequence typing was performed with nested PCRs targeting five different loci of the *Giardia* genome (Ballweber et al. 2010; Beck et al. 2012; Monis et al. 2009). The conserved small ribosomal subunit (SSU rRNA) locus and the internal transcribed spacer (ITS1-5.8S-ITS2) region were selected (Cacciò et al. 2010; Wielinga and Thompson 2007). Additionally, three fragments of single-copy, protein-coding gene targets were investigated: beta giardin (bg), glutamate dehydrogenase (gdh) and triosephosphate isomerase (tpi). The latter three genes with a high degree of genetic polymorphism are suitable for both genotyping and subtyping (Feng and Xiao 2011) (for primers and cycling conditions, see Table 2). For the PCR amplification processes, the following equipment was used: the Eppendorf Mastercycler® thermocycler (MWG Biotech, Ebersberg, Germany), the Veriti® Thermal Cycler, the GeneAmp® PCR System 2700 (both from Applied Biosystems®, Darmstadt, Germany) and the ProFlex™ PCR System (Life Technologies, Carlsbad, USA). PCR products of SSU rRNA and ITS1-5.8S-ITS2 were analysed on 2 % agarose gels dyed with GelRed™ nucleic acid stain, 10.000× in water (both from Biotium, Hayward, USA). Gel images were visualised using a gel documentation system (Peqlab, Erlangen, Germany). PCR-positive samples underwent purification with QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Forward and reverse sequencing were performed by Eurofins MWG Operon (Ebersberg, Germany). For PCR products of bg, gdh and tpi loci, a capillary electrophoresis was performed (QIAxcel®, Qiagen, Hilden, Germany), and

Table 2 Primers and PCR conditions used for the multilocus sequence typing of *Giardia duodenalis* in dogs from South Eastern Europe

Locus	Length of amplification, primers included (bp)	Primer	Reaction volume and contents[b,c]	Cycle condition[g,h]	Reference
SSU rRNA	292	1st amplification: RH11 5-′CATCCGGTCGATCCTGCC-3′ RH4 5′-AGTCGAACCCTGATTCTCCGCCAGG-3′	Total volume 50 μl[d] Template DNA 2–3 μl RH11 0.2 μM RH4 0.2 μM DMSO[e] 2.5 μl	94 °C, 45 s 50 °C, 45 s 72 °C, 60 s →40× 72 °C, 10 min	(Hopkins et al. 1997) (Read et al. 2002)
	175	2nd amplification: GiarF 5′-GACGCTCTCCCCAAGGAC-3′ GiarR 5′-CTGCGTCACGCTGCTCG-3′	PCR product 5 μl (amplification 1) GiarF 0.2 μM GiarR 0.2 μM BSA[f] 0.5 μl	Identical cycling conditions to the first amplification	
Internal Transcribed Spacer Region (ITS1-5.8S-ITS2)	347	1st amplification: FW1[a] 5′-TGGAGGAAGGAGAAGTCGTAAC-3′ RV1[a] 5′-GGGCGTACTGATATGCTTAAGT-3′	Total volume 40 μl[d] Template DNA 2 μl FW1 0.2 μM RV1 0.2 μM Mastermix 20 μl DMSO[e] 2 μl	94 °C, 30 s 59 °C, 30 s 72 °C, 60 s →35×	(Cacciò et al. 2010)
	315	2nd amplification: FW2[a] 5′-AAGGTATCCGTAGGTGAACCTG-3′ RV2[a] 5′-ATATGCTTAAGTTCCGCCCGTC-3′	PCR product 5 μl (amplification 1) FW2 0.2 μM RV2 0.2 μM Mastermix 20 μl DMSO[e] 2 μl	Identical cycling conditions to the first amplification	
Beta Giardin (bg)	753	1st amplification: G7 5′-AAGCCCGACGACCTCACCCGCAGTGC-3′ G759 5′-GAGGCCGCCCTGGATCTTCGAGAC GAC-3′	Total volume 50 μl[d] Template DNA 2–3 μl G7 0.2 μM G759 0.2 μM	94 °C, 30 s 60 °C, 30 s 72 °C, 45 s →35×	(Lalle et al. 2005)
	515	2nd amplification: FW[a] 5′-GAACGAACGAGATCGAGGTCCG-3′ RV[a] 5′-CTCGACGAGCTTCGTGTT-3′	PCR product 5 μl (amplification 1) FW 0.2 μM RV 0.2 μM	94 °C, 30 s 53 °C, 30 s 72 °C, 30 s →40×	
Glutamate Dehydrogenase (gdh)	755	1st amplification: GDH1 5′-TTCCGTRTYCAGTACAACTC-3′ GDH2 5′-ACCTCGTTCTGRGTGGCGCA-3′	Total volume 50 μl[d] Template DNA 2–3 μl GDH1 0.2 μM GDH2 0.2 μM	94 °C, 45 s 50 °C, 45 s 72 °C, 45 s →35×	(Cacciò et al. 2008)
	530	2nd amplification: GDH3 5′-ATGACYGAGCTYCAGAGGCACGT-3′ GDH4 5′-GTGGCGCARGGCATGATGCA-3′	PCR product 5 μl (amplification 1) GDH3 0.2 μM GDH4 0.2 μM	94 °C, 30 s 55 °C, 30 s 72 °C, 30 s →40×	
Triosephosphate Isomerase (tpi)	605	1st amplification: AL3543 5′-AAATYATGCCTGCTCGTCG-3′	Total volume 50 μl[d] Template DNA 2–3 μl	94 °C, 45 s 50 °C, 45 s	(Sulaiman et al. 2003)[i]

Table 2 (continued)

Locus	Length of amplification, primers included (bp)	Primer		Reaction volume and contents[b,c]	Cycle condition[g,h]	Reference
		AL3546 5′-CAAACCTTYTCCGCAAACC-3′		AL3543 0.2 µM	72 °C, 45 s	
				AL3546 0.2 µM	→35×	
	563	2nd amplification:		PCR product 5 µl	94 °C, 30 s	
		AL3544 5′-CCCTTCATCGGNGGTAACTT-3′		(amplification 1)	50 °C, 30 s	
		AL3545 5′-GTGGCCACCACVCCCGTGCC-3′		AL3544 0.2 µM	72 °C, 30 s	
				AL3545 0.2 µM	→40×	

[a] Names given by the author of this study

[b] 2× GoTaq® Green Mastermix (Promega, Madison, USA), unless otherwise stated 25 µl were used in a total volume of 50 µl

[c] Water, Molecular Biology Reagent (Sigma Life Science, Taufkirchen, Germany), filled up to the total volume

[d] For both amplifications

[e] 5 % dimethyl sulfoxide (DMSO, Roth, Karlsruhe, Germany)

[f] Ultrapure bovine serum albumin (BSA) non-acetylated (1 % [50 mg/ml], Roth, Karlsruhe, Germany)

[g] Initial activation step was the same for all protocols: 94 °C for 2 min

[h] Final extension: 72 °C for 7 min was the same for all protocols

[i] Primers modified after Sulaiman et al. (2003)

the amplified samples were purified using the ExoSAP-IT®PCR Clean-Up Reagent (USB, Cleveland, USA). Forward and reverse sequencing were performed by Macrogen Inc. (Amsterdam, Netherlands). Reverse sequences were reversed, complemented, and aligned to the forward sequences using online tools (Reverse Complement: http://www.bioinformatics.org/sms/rev_comp.html, Clustal Omega: https://www.ebi.ac.uk/Tools/msa/clustalo). Database searches and sequence comparisons were done with BLAST provided by the National Center for Biotechnology Information (BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi). Additionally, sequences were assembled using SeqMan® (DNASTAR, Madison, USA). All interpretable nucleotide sequences of the bg, gdh and tpi loci were translated to amino acid sequences with an online translation tool (translate tool: http://web.expasy.org/translate) and aligned with respect to each other to recognise substitutions of particular amino acids.

Data analysis

The prevalence of infection with *Giardia* (ELISA) of household dogs and shelter dogs was compared with a χ^2-test using an online tool (Chi-square Calculator: http://socscistatistics.com/tests/chisquare/Default2.aspx). p values <0.05 were considered to be significant.

Results

Coproantigen ELISA

Approximately one third of the canine faecal samples from six South Eastern European countries tested positive for *Giardia* coproantigen (Table 1). Percentage of dogs tested positive ranged from 17.9 (Hungary) to 65.7 % (Serbia). The prevalence for shelter dogs was significantly higher compared to household dogs (139/243, 57.2 % vs. 415/1398, 29.7 %; $p<0.01$).

Detection of *Giardia* cysts via IFA/MIFC in *Giardia* coproantigen ELISA-positive samples

Giardia cysts were demonstrated for the majority of the ELISA-positive samples in the IFA: Albania 159 of 214 samples (74.3 %), Bulgaria and Hungary 25 of 25 samples each (100 %), Macedonia 22 of 25 samples (88.0 %); Romania 28 of 34 samples (82.4 %). Out of 88 ELISA-positive samples from Serbia, 57 showed *Giardia* cysts in the MIFC test (64.7 %). A total of 133 samples (15–26 samples per country), which contained *Giardia* cysts in the tested IFA or MIFC, were chosen for PCR analysis.

Genotyping at the SSU rRNA region

Amplification of the 175-bp fragment of the SSU rRNA region was obtained in 82.0 % (109/133) of the *Giardia* isolates (Table 3). Of the 109 PCR-positive samples, 104 (95.4 %) gave interpretable sequencing results. The sequence analysis of the amplification products revealed assemblage C in 46.2 % (48/104) and assemblage D in 53.8 % (56/104, Table 4). Forty-five isolates belonging to assemblage C showed 100 % homology with a sequence reported from an isolate of a dog from Japan (GenBank accession no. AB569372) while nucleotide (nt) substitutions were observed in three sequences (supplementary data, Table 1). Fifty-five isolates belonging to assemblage D were 100 % homologous to a dog isolate from Australia (GenBank accession no. AF199443). One isolate of assemblage D had a single nucleotide substitution (supplementary data, Table 1).

Sequences obtained at the SSU rRNA locus were deposited in GenBank under the following accession numbers: KP258238-KP258341.

Genotyping at the ITS1-5.8S-ITS2 region

In total, 31.6 % of the samples (42/133) showed amplicons at the 315-bp fragment encompassing the ITS1-5.8S-ITS2 region (Table 3). Forty sequences (95.2 %) belonged to assemblage D, whereas two samples did not give interpretable results (Table 4). Thirty-five isolates were 100 % homologous with a sequence of an isolate derived from a dog from Croatia (GenBank accession no. JN603692). Nucleotide substitutions were observed in five sequences, which were 99 % similar to assemblage D (supplementary data, Table 1).

Sequences obtained at the ITS1-5.8S-ITS2 region were deposited in GenBank under the following accession numbers: KP258356-KP258395.

Table 4 *Giardia* assemblages determined in MLST at five different loci in naturally infected dogs from seven different South Eastern European countries

Country	SSU rRNA			ITS1-5.8S-ITS2			bg			gdh			tpi		
	n[a]	C[b]	D[b]	n	C	D	n	C	D	n	C	D	n	C	D
Albania	17	5	12	8	0	8	2	1	1	1	1	0	0	0	0
Bulgaria	13	4	9	9	0	9	0	0	0	0	0	0	0	0	0
Croatia	16	6	10	7	0	7	3	2	1	2	1	1	1	1	0
Hungary	14	10	4	3	0	3	1	1	0	0	0	0	0	0	0
Macedonia	14	7	7	6	0	6	0	0	0	3	0	3	1	1	0
Romania	16	8	8	4	0	4	1	1	0	1	0	1	0	0	0
Serbia	14	8	6	3	0	3	0	0	0	0	0	0	0	0	0
Total	104	48	56	40	0	40	7	5	2	7	2	5	2	2	0

[a] *n*=PCR-positive samples with an interpretable sequencing result

[b] C=assemblage C; D=assemblage D

Genotyping at the beta giardin (bg) gene

The amplification of a 515-bp fragment of the bg gene was obtained from 12.8 % (17/133) of the *Giardia* isolates (Table 3). Seven of the 17 samples gave an interpretable sequencing result (41.2 %). Five isolates (71.4 %) belonged to assemblage C and two (28.6 %) belonged to assemblage D (Table 4). One sequence with assemblage C was 100 % homologous with a sequence of a dog from Croatia (GenBank accession no. JN416552). The other four isolates were all 99 % similar to assemblage C and revealed one nt substitution each (supplementary data, Table 1). Both isolates of assemblage D showed 100 % homology with sequences of the GenBank: one with a sequence of a dog from Nicaragua (GenBank accession no. EF455598) and the other one with a sequence of a dog from the UK (GenBank accession no. HM061152). Those two sequences differed in three nt positions from each other (supplementary data, Table 1). The translation of the nucleotide

Table 3 Results of the multilocus nested PCR performed at five different loci for 15 to 26 selected samples per country

Country	Number of samples for PCR	SSU rRNA[a]	ITS1-5.8S-ITS2[a]	bg[a]	gdh[a]	tpi[a]
Albania	17	17 (100 %)	8 (47.1 %)	2 (11.8 %)	2 (11.8 %)	0
Bulgaria	22	16 (72.7 %)	11 (50.0 %)	3 (13.6 %)	2 (9.1 %)	0
Croatia	26	16 (61.5 %)	7 (26.9 %)	4 (15.4 %)	4 (15.4 %)	1 (3.8 %)
Hungary	17	15 (88.2 %)	3 (17.6 %)	3 (17.6 %)	0	0
Macedonia	15	15 (100 %)	6 (40.0 %)	1 (6.7 %)	5 (33.3 %)	1 (6.7 %)
Romania	16	16 (100 %)	4 (25.0 %)	2 (12.5 %)	2 (12.5 %)	0
Serbia	20	14 (70.0 %)	3 (15.0 %)	2 (10.0 %)	0	0
Total	133	109 (82.0 %)	42 (31.6 %)	17 (12.8 %)	15 (11.3 %)	2 (1.5 %)

[a] Samples which were able to be sequenced with 93–100 % homology to *G. duodenalis* are defined as 'PCR-positive'

sequence to amino acid codons revealed silent nt substitutions within assemblages C and D. Of the 30 nt substitutions which were detected between assemblages C and D, one expressed substitution was detected (G208S).

Sequences obtained at the bg locus were deposited in GenBank under the following accession numbers: KP258342-KP258348.

Genotyping at the glutamate dehydrogenase (gdh) gene

Amplification of a 530-bp fragment of the gdh gene was obtained from 11.3 % (15/133) of the *Giardia* isolates (Table 3). Seven of them revealed interpretable sequencing results (46.7 %). Two isolates (28.6 %) belonged to assemblage C and five (71.4 %) to assemblage D (Table 4). The two assemblage C sequences were 100 % homologous with an isolate of a dog from Croatia (GenBank accession no. JN587394). Four assemblage D isolates were 100 % homologous with an isolate from a dog from Croatia (GenBank accession no. JN587398) while the other showed a deletion (supplementary data, Table 1). Translation of nucleotides into amino acids revealed silent nt substitutions within assemblage C. However, seven of the 56 nt substitutions expressed different amino acids in assemblage C compared to assemblage D (I586V, L795I, T829A, L835I, G863A, A901T, Q945H).

Sequences obtained at the gdh locus were deposited in GenBank under the following accession numbers: KP258349-KP258355.

Genotyping at the triosephosphate isomerase (tpi) gene

Amplification of a 563-bp fragment of the tpi gene was positive in 1.5 % (2/133) of the samples (Table 3). Both isolates gave an interpretable sequencing result belonging to assemblage C (Table 4). Between the two sequences, five nt substitutions were detected. One sequence showed a 100 % homology with a sequence of a dog from the USA (GenBank accession no. AY228641). The other sequence was 99 % similar to the latter sequence (supplementary data, Table 1). Translation of nucleotides into amino acids revealed that all substitutions were silent.

Sequences obtained at the tpi locus were deposited in GenBank under the following accession numbers: KP258396 and KP258397.

Combined genotyping results at five loci

Out of 109 samples with interpretable sequences two *Giardia* isolates (1.8 %) were amplified at four loci (Table 5). Amplifications at three and two loci were obtained from four (3.7 %) and 37 (33.9 %) samples, respectively. Single locus amplification was achieved in 66 (60.6 %) *Giardia* isolates. No sample could be amplified at all five loci. Assemblage C was detected in isolates of 50 dogs (46, one locus; 2, two loci; 1, three loci; 1, four loci). *Giardia* isolates from 68 dogs harboured assemblage D (37, one locus; 28, two loci; 2, three loci; 1, four loci). Sixteen shelter dogs were infected with *Giardia* assemblage C and 13 harboured *Giardia* assemblage D. In the group of household dogs, 34 and 55 samples with *Giardia* assemblages C or D, respectively, were detected.

Table 5 Combined genotyping results at five loci

Number of loci	SSU rRNA	ITS1-5.8S-ITS2	bg	gdh	tpi	Number of samples
4	X	X	X	X		1
	X		X	X	X	1
3	X	X	X			2
	X	X		X		1
	X		X	X		1
2	X	X				32
	X		X			1
	X			X		3
	X				X	1
1	X					61
		X				4
			X			1
Total	104	40	7	7	2	109

'Assemblage swapping' defined by the coexistence of two different assemblages within one sample at two loci was detected in nine isolates. Six isolates were typed as assemblage C at the SSU rRNA locus and as assemblage D at the ITS1-5.8S-ITS2 locus. Two isolates revealed assemblage C at the SSU rRNA locus and assemblage D at the gdh locus. One isolate had assemblage D at the SSU rRNA locus and the ITS1-5.8S-ITS2 locus and assemblage C at the bg locus.

Discussion

This study was performed since data on the occurrence and genotyping of *G. duodenalis* of dogs in South Eastern Europe are scarce. The presence of *G. duodenalis* in dogs was confirmed in all studied countries. The overall prevalence of canine infection with *G. duodenalis* in this study (33.7 %, ELISA) was higher than that in most of the surveys of Western Europe (Bianciardi et al. 2004; Claerebout et al. 2009; Epe et al. 2010; Overgaauw et al. 2009). A similar result was obtained in a study on intestinal parasites in shelter and hunting dogs from Spain (37.4 %, microscopy) (Ortuño et al. 2014). Although many prevalence studies on *Giardia* in dogs exist all over the world, data should be compared carefully since the methods used for *Giardia* detection possess different sensitivity. Microscopy has been demonstrated to be less sensitive compared to IFA and ELISA (Feng and Xiao 2011; Geurden et al. 2008; Maraha and Buiting 2000; Mircean

et al. 2012; Szénási et al. 2007; Tangtrongsup and Scorza 2010). Moreover, *Giardia* cysts are shed intermittently, which makes the coproantigen ELISA the most reliable method for detection of an infection with this protozoan parasite. A comparable result was observed in our study for the samples from Serbia. Only 57 of 134 samples were diagnosed positive for *Giardia* cysts using microscopy, whereas with ELISA 88 of 134 samples were *Giardia* positive.

The prevalence of *G. duodenalis* in dogs living in crowded environments or under poor hygienic and health conditions has been reported to be higher compared to household dogs (Ortuño et al. 2014; Tangtrongsup and Scorza 2010). Consequently, street, kennel and shelter dogs seem to be infected with *Giardia* more often (Mircean et al. 2012; Nikolić et al. 2008; Paz e Silva et al. 2012). In the present study, 57.2 % (139/243) of the shelter dogs were infected with *G. duodenalis* compared to 29.7 % (415/1398) of the household dogs, confirming previous studies.

To estimate the zoonotic potential of 133 of the *Giardia*-positive isolates, we performed multilocus sequence typing with nested PCR amplification of altogether five loci. The two highest amplification rates were achieved with 82.0 % at the conserved locus SSU rRNA and with 31.6 % at the ITS1-5.8S-ITS2 transcribed spacer region. The result might be explained by the multi-copy and conserved characteristics of the two targets. Compared to the SSU rRNA locus, the ITS1-5.8S-ITS2 region has the advantage of providing a higher level of polymorphism among *Giardia* isolates which facilitates their identification and enables the detection of subassemblages of assemblages A and B (Cacciò et al. 2010). The SSU rRNA locus has traditionally been used for species and assemblage level genotyping whereas the polymorphic loci bg, gdh and tpi are frequently used for subtyping clinical samples which is especially important for zoonotic isolates (Wielinga and Thompson 2007). Amplification of the latter targets could be achieved in a limited number of the investigated samples. The bg locus revealed positive PCR results in 12.8 %, the gdh locus in 11.3 % and at the tpi locus in 1.5 % of the 133 samples. Lower amplification rates at polymorphic loci compared to conserved regions have been reported in a number of studies elsewhere (Covacin et al. 2011; Johansen 2013; Ortuño et al. 2014; Pallant et al. 2015). A possible explanation might be that single-copy genes in the *Giardia* genome are more variable and consequently less reliable in the amplification process because they can cause mismatches in binding regions of the primers (Cacciò et al. 2010).

The genotyping of the isolates from dogs from South Eastern Europe revealed the dog-specific assemblages C and D, exclusively. Our results are in line with results from other studies on *Giardia* assemblages in the geographic region. A Hungarian study investigating the SSU rRNA locus revealed the dog-specific assemblages C and D in 40.0 and 66.7 %, respectively, including one mixed infection (Szénási et al. 2007). The predominance of non-zoonotic assemblages in both kennel and household dogs was also reported in an MLST study from Croatia investigating bg, gdh and tpi loci as well as the ITS1-5.8S-ITS2 region (Beck et al. 2012). Fifty-seven out of 96 samples contained at least one of the assemblages C or D (59.4 %), but in the same study, 16 isolates harboured the zoonotic assemblages A or B (16.7 %). Isolates containing both zoonotic and non-zoonotic assemblages occurred in 24.0 %; assemblage swapping of assemblages C and D occurred in 18.8 % which is more often, compared to the present study (8.2 %).

The predominance of dog-specific assemblages C and D over zoonotic assemblages A and B in canine *Giardia* isolates exists not only in South Eastern Europe but also in other countries worldwide. The occurrence of non-zoonotic assemblages C or D was 100 % at the SSU rRNA and 93.3 % at the bg locus in England (Upjohn et al. 2010), 98.7 % at the SSU rRNA, 97.3 % at the bg and 100 % at the gdh and tpi loci in Canada (McDowall et al. 2011), 88.6 % at the SSU rRNA locus in the USA (Johansen 2013) and 96.2 % at the SSU rRNA locus in Trinidad and Tobago (Mark-Carew et al. 2013). In general, assemblage D outweighed assemblage C in most studies on canine *Giardia* assemblages including the present study. There was no difference in the distribution of assemblages between shelter and household dogs in the present study. Nevertheless, potentially zoonotic assemblages have also been detected in dogs from different countries in other studies within the last years. The occurrence for assemblages A or B was 60 % at the SSU rRNA (plus 27.3 % mixed assemblages A and C) and 70 % at the gdh locus in Germany (Leonhard et al. 2007), 37.0 % at the bg locus in Belgium (Claerebout et al. 2009), 93.2 % at the SSU rRNA locus, 97 % at the bg and 72.2 % at the gdh locus in the USA (Covacin et al. 2011) and 84.1 % at the gdh and bg loci in Spain (Dado et al. 2012).

Regarding the distribution of assemblages within the dog population, close contact of household dogs with their owners is assumed to be responsible for infections with the zoonotic assemblages A and B whereas the transmission of assemblages C and D is more likely amongst dogs living in crowded environments (Claerebout et al. 2009). Differences in social and environmental conditions might contribute to the assemblage variations (Feng and Xiao 2011). However, shelter dogs might carry *Giardia* infections with zoonotic assemblages, and household dogs might harbour species-specific assemblages (Beck et al. 2012; Dado et al. 2012; Mark-Carew et al. 2013). It remains open whether assemblages C and D will outcompete assemblages A and B in dogs in the future due to an eventual superior adaption to the host (Cooper et al. 2010).

The translation of nucleotide sequences into amino acid sequences and their alignment revealed that substitutions within the assemblages C and D were all silent. However, nucleotide substitutions between the two dog-specific assemblages C and D revealed expressed changes in their amino

acid composition. Nucleotide differences within assemblages at all investigated loci might occur due to genetic exchanges or recombination events. Their existence strengthens the point that the genome of *G. duodenalis* is complex and that the mechanism of the reproduction is not clearly explored. The occurrence of sexual reproduction leading to variations in the *Giardia* genome is under discussion, but clear evidence is still missing (Cooper et al. 2007).

According to the results of the present study, *G. duodenalis* should be considered as a common parasite in dogs from South Eastern Europe. However, we did not find any evidence that the investigated dog population contributes to zoonotic transmission of *Giardia* infections in humans.

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Conflict of interest The authors declare that they have no conflict of interest. All marks are the property of their respective owners.

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