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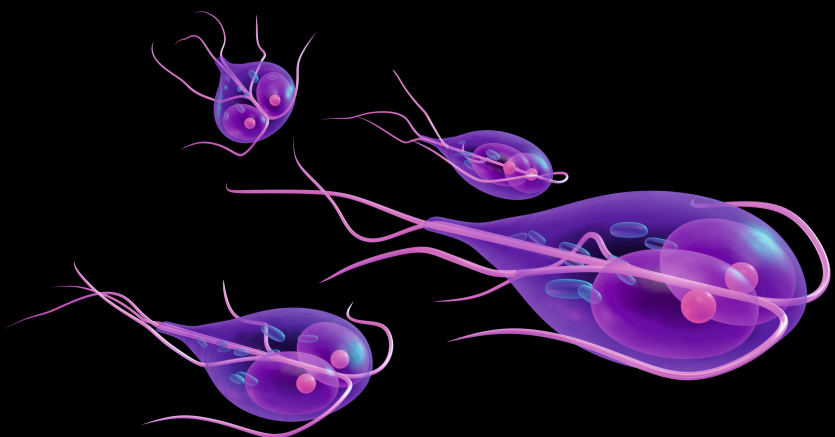
Marie Franziska Sommer



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Occurrence and genetic determination of *Giardia* in dogs from South Eastern Europe

von Marie Franziska Sommer
aus Tübingen

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Aus dem Veterinärwissenschaftlichen Department der Tierärztlichen
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Für meine Eltern

und für meinen Großvater, der ein Leben lang
von einem Hochschulstudium geträumt hat
(1921–2012)

So eine Arbeit wird eigentlich nie fertig, man muss sie für fertig erklären,
wenn man nach der Zeit und den Umständen das Möglichste getan hat.

JOHANN WOLFGANG VON GOETHE (1749–1832)

TABLE OF CONTENT

ABBREVIATIONS

bg	beta giardin
b.i.d.	bis in die (twice a day)
bp	base pair
BW	body weight
°C	degree Celsius
DFA	direct immunofluorescence assay
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ef-1	elongation factor 1-alpha
ELISA	enzyme linked immunosorbent assay
FITC	fluorescein isothiocyanate
G.	*Giardia*
g	gram
gdh	glutamate dehydrogenase
IFA	immunofluorescence assay
ITS	internal transcribed spacer
kg	kilogram
LAMP	loop mediated isothermal amplification
mg	milligram
MIFC	merthiolate iodine formalin concentration
MLST	multilocus sequence typing
MLG	multilocus genotype
nt	nucleotide
no	number
PCR	polymerase chain reaction
q.d.	quaque die (one a day)
q.i.d.	quater in die (four times a day)
qPCR	real-time quantitative PCR
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
SAF	Sodium acetate-acetic acid-formalin solution
s	second
s.i.d.	semel in die (once a day)
SNP	single-nucleotide polymorphism
spp.	species pluralis
SSU	small subunit
t.i.d.	ter in die (three times a day)
tpi	triosephosphate isomerase
WBC	Western Balkan Countries (Serbia, Bosnia and Herzegovina, Montenegro, Kosovo, Macedonia and Albania)
WHO	World Health Organisation
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
$ZnCl_2$	zinc chloride
$ZnSO_4$	zinc sulfate

I. INTRODUCTION

The protozoan parasite *Giardia duodenalis* was first described as 'very prettily moving animalcules' by Anthony van Leeuwenhoek in 1675 (Dobell, 1920; Lambl, 1859). Since the discovery of the primarily called *'Cercomonas dujardin'*, many researchers have contributed to a better understanding of the biology, taxonomy and epidemiology of the flagellated protozoan. To date, *G. duodenalis* belongs to the most frequently diagnosed parasites of the gastrointestinal tract in industrialised as well as in developing countries (Cacciò et al., 2005). Numerous vertebrate species were shown to harbour *Giardia* infections in nature (Thompson and Monis, 2012). Although many *Giardia* cases remain undetected during an asymptomatic course of disease, severe gastrointestinal illness might occur in both humans and animals (Adam, 1991; Tangtrongsup and Scorza, 2010).

After many years of uncertainty, the current research is heading towards a revised taxonomy of *G. duodenalis* which is now divided into two potentially zoonotic assemblages A and B and six host-specific genetic assemblages C–H and their correspondent subassemblages (Lasek-Nesselquist et al., 2010; Thompson, 2004; Thompson and Monis, 2012). Modern molecular techniques enable the genetic characterisation of *Giardia* isolated from different hosts and offer the capability for a better understanding of the different *Giardia* assemblages (Ballweber et al., 2010).

The distribution of zoonotic and host-specific assemblages in infected humans and animals and the associated question whether *Giardia* possesses zoonotic potential are subject of the current research (Feng and Xiao, 2011). Investigations of *Giardia* isolates have revealed the presence of zoonotic assemblages in a variety of animals as well as in humans (Lebbad et al., 2010). Data on the true frequency of the zoonotic transmission from animals to humans and vice versa is still limited and further effort is required for more detailed information on the transmission dynamics (Thompson, 2004). The role of dogs as a potential source for human *Giardia* infections is a broadly discussed topic since many of those companion animals live in close contact with their owners (Traub et al., 2004).

Even though various scientific studies from countries all over the world have provided results on canine *Giardia* infections, there are some regions with limited

information on this issue, for instance South Eastern Europe.

The present study focused on the South Eastern European countries Albania, Bulgaria, Croatia, Hungary, Macedonia, Romania and Serbia since information on genotyping of canine *Giardia* isolates from those countries is scarce. The determination of canine *Giardia* assemblages provides valuable information about the zoonotic potential and the possible transmission of the protozoan parasite to humans in this predisposed region. Thus, the aims of the present study were

1) to provide information on the occurrence of canine *Giardia* infections in South Eastern European countries.

2) to identify the *Giardia* assemblages by multilocus sequence typing of five different gene loci.

In the framework of a cooperation with researchers from the seven South Eastern European countries, this work contributes to an extended knowledge about the international distribution of *Giardia* assemblages in dogs.

II. LITERATURE REVIEW

1. *Giardia duodenalis*

1.1. Taxonomy and assemblages

The taxonomy of *Giardia duodenalis* has been under constant revision for over 100 years since the high genetic diversity of the intestinal parasite causes difficulties for a consistent classification (Sogin et al., 1989; Thompson and Monis, 2011). Major changes regarding the order and the family affiliations have been defined just recently (Thompson and Monis, 2012). According to the new classification, *Giardia* belongs to the phylum Metamonada, the subclass Diplozoa and the order Giardiida (Figure 1). However, a new taxonomic division of the protozoan parasite based on current molecular genotyping methods is still in progress (Thompson and Monis, 2011).

Kingdom Protozoa
Superphylum Eozoa (Cavalier-Smith 1996/7 emend. 1999 stat. nov.)
Phylum Metamonada (Grassé 1952 stat. nov. emend.)
Subphylum Trichozoa (Cavalier-Smith 1996/7 stat. nov. emend.)
Superclass Eopharyngia (Cavalier-Smith 1993 stat. nov.)
Class Trepomonadea (Cavalier-Smith 1993)
Subclass Diplozoa Dangeard (1910 stat. nov. Cavalier-Smith 1996)
Order Giardiida (Cavalier Smith 1996)
Genus *Giardia*

Figure 1: Taxonomy of *Giardia* (modified after Cavalier-Smith, 2003)

To date, there are six morphologically distinct species within the genus *Giardia* (Table 1). This classification is based on the shape of the trophozoite, the size of the ventral adhesive disc relative to the cell length and the shape of the median bodies (Filice, 1952). The *Giardia* species other than *G. duodenalis* have only been investigated in a limited number of studies and seem to be host-specific (Adams et al., 2004).

Table 1: Recognised species in the genus *Giardia* (modified after Monis et al., 2009)

Species	Hosts	Morphological characteristics	dimension of trophozoite	
			length	width
Giardia duodenalis	Various mammals, including humans	Pear-shaped trophozoites with claw-shaped median bodies	12–15 µm	6–8 µm
G. muris	Rodents	Rounded trophozoites with small round median bodies	9–12 µm	5–7 µm
G. microti	Rodents	Trophozoites similar to *G. duodenalis*. Mature cysts contain fully differentiated trophozoites.	12–15 µm	6–8 µm
G. ardeae	Birds	Rounded trophozoites with prominent notch in ventral disc and rudimentary flagellum. Median bodies round-oval to claw-shaped.	10 µm	6.5 µm
G. psittaci	Birds	Pear-shaped trophozoites, with no ventro-lateral flange. Claw-shaped median bodies.	14 µm	6 µm
G. agilis	Amphibians	Long, narrow trophozoites with club-shaped median bodies	20–30 µm	4–5 µm

Based on phylogenetic analysis and host-specificity, the morphologically uniform species *G. duodenalis* is divided into eight genetic assemblages A–H and numerous subassemblages (Monis et al., 2009; Plutzer et al., 2010). Assemblages A and B have the widest host-spectrum infecting various mammals including humans and are thus considered to contain zoonotic potential. In contrast, the other non-human assemblages are each associated with certain host species. Dogs are primarily infected with assemblages C and D, livestock with assemblage E, cats with assemblage F, rodents with assemblage G and marine vertebrates with assemblage H (Ballweber et al., 2010; Cacciò and Ryan, 2008; Lasek-Nesselquist et al., 2010). A novel *Giardia* genotype has been found in Australian marsupials

but has not yet been officially described (Adams et al., 2004). Within the assemblages of *G. duodenalis* further substructuring into subassemblages and subtypes exists. Especially for the zoonotic assemblages A and B, the information on the subtype level is important with regard to the potential for transmission to other species than humans (Feng and Xiao, 2011). Multiple subtypes of assemblage A have been detected via sequence analysis of the beta giardin (bg), glutamate dehydrogenase (gdh) and triosephosphate isomerase (tpi) genes (Table 2).

Table 2: Subtype nomenclature system for *Giardia* assemblage A (modified after Cacciò et al., 2008). The different subassemblages of *Giardia* assemblage A are assigned to multilocus genotypes (MLG) and subtypes based on multilocus sequence typing (MLST) analysis of the bg, gdh and tpi genes.

Sub-assemblage	MLG	Subtype			Host(s)
		gdh	bg	tpi	
AI	AI-1	A1	A1	A1	Humans, cattle, water buffalo, cat, pig, sheep
	AI-2	A5	A5	A5	Cat
AII	AII-1	A2	A2	A2	Human, cat
	AII-2	A3	A3	A2	Human
	AII-3	A3	A2	A2	Human
	AII-4	A4	A3	A2	Human
	AII-5	A3	A3	A1	Human
	AII-6	A3	A3	A3	Human
	AII-7	A3	A3	A4	Human
AIII	AIII-1	A6	A6	A6	Fallow dear, wild boar, cat

The substructuring of the genetically diverse assemblage B is still under revision as the high substitution rates restrain the determination of a true subassemblage pattern (Wielinga et al., 2011). Additionally, further research is required to estimate the substructure of assemblages C, D, F and G (Feng and Xiao, 2011).

In certain individual cases, it remains impossible to assign individual hosts unequivocally to one single assemblage because they carry mixtures of different assemblages with preferential PCR amplification of one assemblage over the other. Sequence chromatograms of *Giardia* isolates with such ’mixed

assemblages' show characteristic signatures of different assemblages within one sequence. A plausible explanation for this phenomenon would be the occurrence of recombinants carrying information from different *Giardia* assemblages or species (Cacciò and Sprong, 2010). Additionally, the term 'assemblage swapping' defines the coexistence of two different assemblages within one sample at two loci (Wielinga and Thompson, 2007).

With the intention to standardise the taxonomy of *Giardia*, a new nomenclature for species depending on the genotype has been recently suggested: within this new nomenclature, only assemblage A is referred to as *G. duodenalis* whereas the other assemblages are assigned to species names according to the particular host spectrum (Monis et al., 2009; Thompson and Monis, 2012) (Table 3). In the present study, the conventional nomenclature for *G. duodenalis* with its different assemblages and subassemblages is used.

Table 3: Suggestion for new genotypic groupings (assemblages) of *Giardia* (modified after Adams et al., 2004; Lasek-Nesselquist et al., 2010; Monis et al., 2009). New species names for *G. duodenalis* are assigned to the assemblages according to the host.

Species	**Assemblage**	**Host(s)**
Giardia duodenalis	A	Humans and other primates, dogs, cats, livestock, rodents and other wild mammals
G. enterica	B	Humans and other primates, dogs, some species of wild mammals
G. canis	C/D	Dogs, other canids
G. bovis	E	Cattle, other hoofed livestock
G. cati	F	Cats
G. simondi	G	Rodents
G.?	H	Marine vertebrates
G. muris	-	Rodents
G. microti	-	Rodents
G. ardeae	-	Birds
G. psittaci	-	Birds
G. agilis	-	Amphibians

1.2. Morphology

The infective cyst of *G. duodenalis* shed by an infected host is 8–14 µm long and 6–10 µm wide. Four nuclei, the crescentic fragments of the ventral disc and flagellar axonemes which are placed diagonally along the axis of the cyst can usually be identified (Smith and Mank, 2011) (Figure 2A).

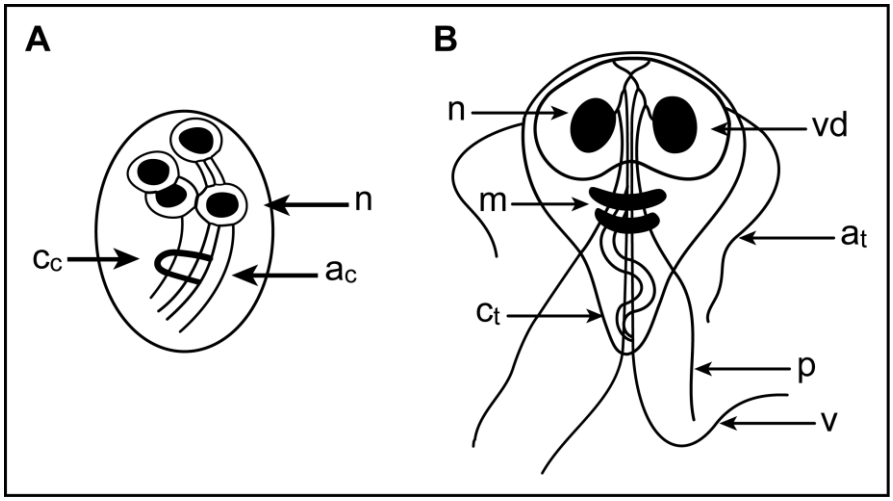

Figure 2: Line drawing of a *Giardia* cyst (A) and a *Giardia* trophozoite (B) with typical morphological characteristics. Key: axosytle (flagellar axoneme) (a_c), anterio-lateral flagellum (a_t), crescentic fragments of the ventral disc (c_c), caudal flagellum (c_t), median bodies (m), nucleus (n), posterior-lateral flagellum (p), ventral flagellum (v), ventral disc (vd) (modified after Smith and Mank, 2011).

The binucleated trophozoite of *G. duodenalis* is 12–18 µm long, 6–9 µm wide and 2–4 µm thick (Smith and Mank, 2011). The cytoskeleton consists of a median body, a concave surface on the anterior two-thirds of the ventral surface which is also referred as sucking, striated or ventral disk (Figure 2B). The latter element enables the trophozoite to attach to the wall of the small intestine (Adam, 1991). The median body has been used to distinguish different *Giardia* spp. (Filice, 1952). Four pairs of flagella arranged in bilateral symmetry (anterior, caudal, posterior and ventral) emerge from the basal bodies near the midline and antroventral to the nuclei (Adam, 1991). Compared to the trophozoite, organelles of the cyst are less identifiable (Smith and Mank, 2011).

1.3. Life cycle

The monoxenous life cycle of *G. duodenalis* includes two morphologically and biochemically distinct forms of the parasite (Lujan et al., 1997). The reproductive trophozoite is the vegetative form colonising the enterocytes of the proximal small

intestine and the environmentally resistant cyst is the infective form of *G. duodenalis* shed with the faeces. After ingestion, the cyst transforms into two trophozoites via excystation in the duodenum of the host stimulated by the presence of gastric acid, pancreatic enzymes and alkaline pH (Thompson et al., 2008) (Figure 3). Trophozoites divide by binary fission and might cause clinical symptoms through the strong attachment to the epithelial surface of the intestine. By encystation, some of the trophozoites transform into immediately infectious cysts, which are intermittently released with the faeces (Adam, 1991; Feng and Xiao, 2011). In dogs and cats the prepatent period is relatively short with 4–16 days whereas the patent period might last weeks to months (Deplazes et al., 2013).

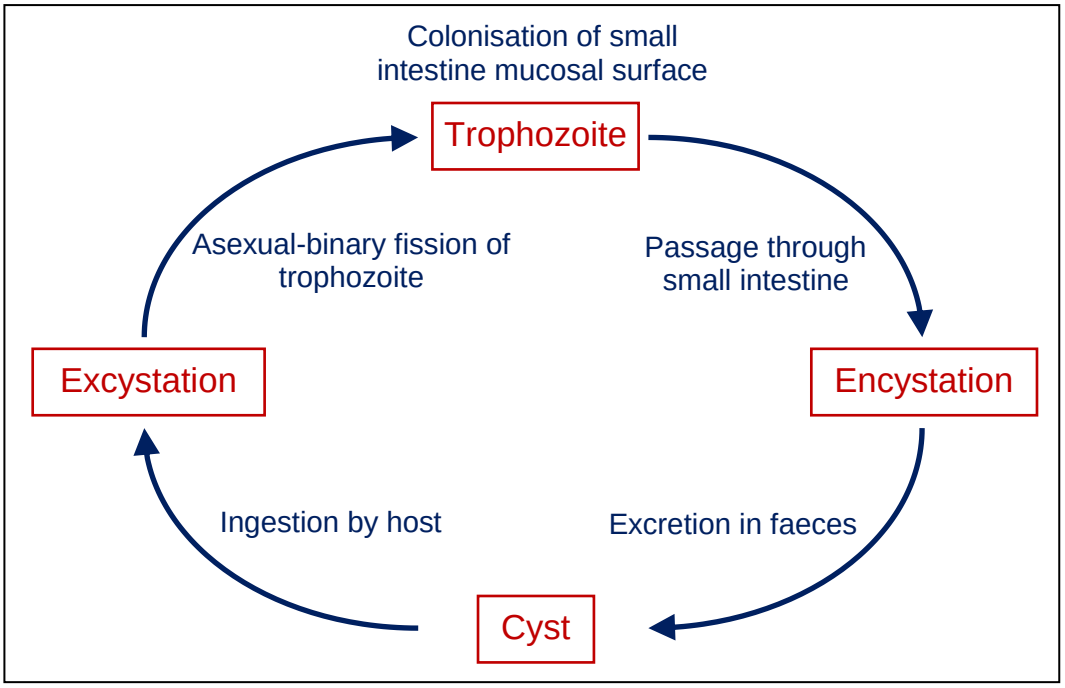

Figure 3: Life cycle of *Giardia duodenalis* (modified after Monis and Thompson, 2003)

1.4. Pathogenesis and clinical symptoms

Trophozoites attaching their ventral disk to the epithelium of the intestine are responsible for pathophysiological reactions including heightened rates of enterocyte apoptosis, small intestinal barrier dysfunction and activation of host lymphocytes. Furthermore, a shortening of brush border microvilli with or without villous atrophy, disaccharidase deficiencies, small intestinal malabsorption, anion hypersecretion and increased intestinal transit rates are assumed to contribute to the clinical picture (Cotton et al., 2011). However, the detailed pathophysiological mechanisms causing symptomatic *G. duodenalis* infections remain incompletely

understood (Adam, 1991; Chin et al., 2002; Thompson and Monis, 2012).

An infection with *G. duodenalis* may remain asymptomatic in many cases but can also cause acute or chronic infections (Ballweber et al., 2010). Even though *Giardia* does neither penetrate the intestinal epithelium or the surrounding tissues nor enter the blood stream, it might cause clinical symptoms (Buret, 2007). In humans and animals, typical symptoms are intermittent and self-limiting or continuing diarrhoea and malabsorption with abdominal cramps, bloating and weight loss (Adam, 1991; Ballweber et al., 2010; Feng and Xiao, 2011; Thompson et al., 2008). Both host and parasitic factors contribute to the development of clinical giardiosis (Cotton et al., 2011). In general, individual factors like age, immune competence, coexistent infections as well as hygienic and nutritional conditions of the host influence the clinical course of an infection with *G. duodenalis*. Young or immunocompromised individuals seem to have more severe clinical symptoms (Monis et al., 2009). Furthermore, in many cases reinfections may occur due to incomplete immune defence or antigenic variation of the protozoan parasite (Muller and von Allmen, 2005).

1.5. Epidemiology

Giardia is one of the most commonly identified intestinal pathogens of humans and other mammals worldwide (Thompson and Meloni, 1993). Moreover, it has been included in the World Health Organisation (WHO) Neglected Disease Initiative (Savioli et al., 2006). *Giardia* cysts are transmitted through contaminated food or water or through a direct faecal-oral route after contact with infected individuals (Adam, 1991). The minimal infective dose has been reported to be 10–100 cysts in humans and laboratory animals (Deplazes et al., 2013; Rendtorff and Holt, 1954).

Especially for breeding stations or shelters the elimination of *Giardia* cysts in the compounds is difficult because *Giardia* cysts are relatively resistant and might remain infectious for months in cold and moist environments as well as in water (Ortuño et al., 2014; Thompson et al., 2008). Temperatures over 60 °C generally stop the infectivity of *Giardia* cysts (Deplazes et al., 2013). Prevalence data on *Giardia* infections in dogs worldwide differ remarkably depending on the investigated dog population and the diagnostic test used and thus should be evaluated carefully (Bouzid et al., 2015; Thompson et al., 2008) (Table A1). The

utilisation of microscopy might cause lower prevalence rates because this method is not as sensitive as enzyme linked immunosorbent assay (ELISA) or immunofluorescence assay (IFA) (Feng and Xiao, 2011; Geurden et al., 2008). Shelter, stray or kennel dogs seem to be infected with *G. duodenalis* more often than household dogs (Huber et al., 2005; Ortuño et al., 2014; Tangtrongsup and Scorza, 2010). This fact might be explained by poor hygienic conditions in those facilities and a high concentration of animals including subclinical carriers causing permanent reinfections (Dubná et al., 2007; Tangtrongsup and Scorza, 2010). The latter compared the prevalence of gastrointestinal parasites of metropolitan household dogs to shelter dogs. *Giardia* was one of the most commonly found parasites in shelter dogs and there was a substantial increase in the prevalence for *Giardia* infection of dogs, which stayed in shelters for at least two months.

Besides the living conditions of investigated dogs, the age might have large impact on the prevalence and should not be underestimated (Itoh et al., 2015). In this regard, breeding kennel dogs might harbour *G. duodenalis* more frequently not only due to crowding of animals in restricted spaces but also due to the high percentage of puppies within this population. Batchelor et al. (2008) described in a study on endoparasites with zoonotic potential in dogs with gastrointestinal diseases in the UK that the prevalence of *Giardia* was significantly higher in dogs under one year of age. Almost one fifth of all symptomatic dogs under 6 months carried infections with the protozoan parasite. Furthermore, an empirical study on age-dependant prevalence of endoparasites in young dogs and cats from Germany showed that one month old dogs were more likely to be infected with *Giardia* (52.5 %) compared to older dogs (25.3 to 41.0 %) (Barutzki and Schaper, 2013). Similar observations had already been made 25 years earlier in a study on endoparasitic infections in pet dogs from the USA where *Giardia* infections were found significantly more often in dogs under two years of age, (Kirkpatrick, 1988).

1.6. Zoonotic potential

Giardia infections were categorised as a zoonosis by WHO in 1979 after their detection in wildlife such as beavers which had the potential to cause a waterborne transmission (WHO, 1979). Consumption of raw surface water provides a significant risk for giardiosis as it might be contaminated by infected

humans, companion animals, livestock or wildlife (Hoque et al., 2002; Karanis et al., 2006; Plutzer et al., 2008). Recent studies have focused on the role of companion animals and livestock for the zoonotic potential of *Giardia* (Thompson and Monis, 2011).

For many years, a clear understanding of the host range of different *Giardia* species (defining the zoonotic potential), their genotypes and their environmental maintenance has been hindered by the inconsistent taxonomy (Thompson et al., 2008). To date, the existence of host-specific assemblages and two zoonotic assemblages with broad host ranges has been confirmed by molecular characterisation of *Giardia* isolates from different species of mammalian hosts from all over the world (Thompson and Monis, 2012). The zoonotic assemblages A and B are equally distributed in humans from both industrialised and developing countries worldwide (Feng and Xiao, 2011). Due to the extensive substructuring within assemblages A and B, it is possible that some of the subgroups might carry a higher zoonotic potential than others (Thompson and Monis, 2012).

In dogs, genotyping studies have revealed inconsistent results for the distribution of *Giardia* assemblages. A study from Traub et al. (2004) revealed that inhabitants of rural areas in India harboured the same assemblages as their dogs and confirmed the suspicion of the zoonotic potential of *Giardia* for the first time. However, dogs from different countries all over the world carry zoonotic assemblages A and B (Claerebout et al., 2009; Covacin et al., 2011; Dado et al., 2012; Leonhard et al., 2007) as well as dog-specific assemblages C and D (Johansen, 2013; Mark-Carew et al., 2013; McDowall et al., 2011; Upjohn et al., 2010). Different cycles of transmission maintain host-specific and zoonotic assemblages of *Giardia* in nature (Figure 4): A/B by direct transmission between humans, E in livestock, C/D between dogs, F between cats and wildlife genotypes between wildlife species (Monis et al., 2009). Nevertheless, assemblages A and B (especially B) can also be transmitted to companion animals, livestock and wildlife (Thompson and Monis, 2011). To date, it remains unclear to what extent the different cycles interact between each other (Thompson et al., 2008).

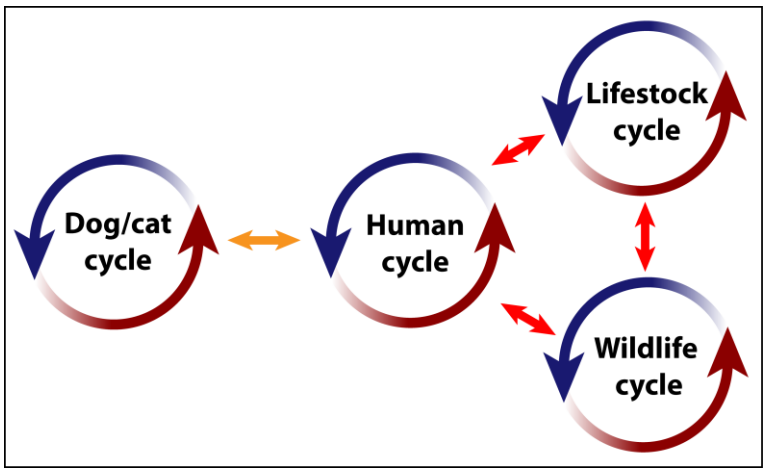

Figure 4: Major cycles of transmission of *G. duodenalis*. Blue arrows symbolise host-specific assemblages/species (→). Red arrows stand for zoonotic assemblages/species (→). The direct and occasionally waterborne transmission of zoonotic assemblages between the human and the dog/cat cycle is indicated by an orange arrow (↔), the transmission of zoonotic assemblages between the other cycles is possible direct and through water (↔). The frequency of transmission is unknown for all cycles (modified after Monis et al., 2009).

1.7. Diagnostics

The vegetative form of *Giardia* is rarely found in faecal samples since trophozoites normally remain in the small intestine. However, they might be detected in duodenal or jejunal fluid obtained by duodenoscopy or attached to gastrointestinal tissue during a pathology section (Smith and Mank, 2011) (Figure 5A). A direct method for the detection of *Giardia* cysts is the examination of the wet mount or material from a faecal concentrate with light microscopy (Adam, 1991). Flotation solutions with $ZnSO_4$ or $ZnCl_2$ are commonly used in the routine laboratory diagnostics, even though this method causes a deformation of the cysts (Deplazes et al., 2013; Zajac et al., 2002). This disadvantage can be avoided by using the merthiolate iodine formalin concentration method (MIFC) (Figure 5B) or the sodium acetate-acetic acid-formalin (SAF) method (Allen and Ridley, 1970; Pfister et al., 2013; Smith and Mank, 2011; Thornton et al., 1983). To increase the chance of verifying intermittently shed cysts, the collection of faecal samples over at least three consecutive days or a repetition of the faecal examination is suggested (Deplazes et al., 2013; Hiatt et al., 1995; Thompson et al., 2008) (Chapter II.1.3).

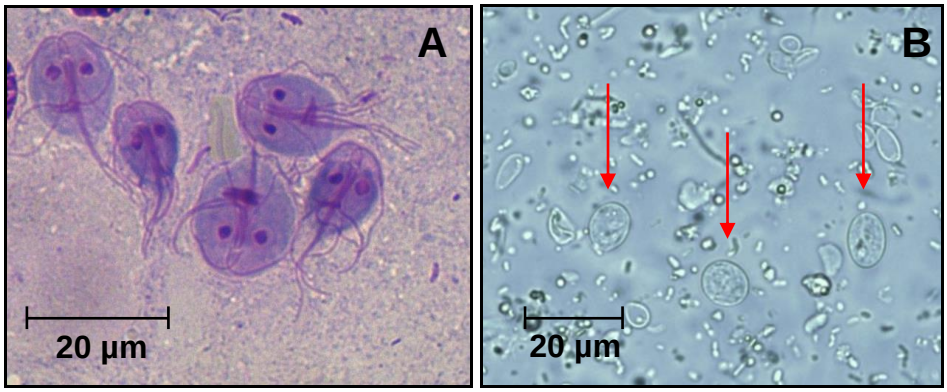

Figure 5: Trophozoites from an intestinal swab with Giemsa staining (A) and cysts from the MIFC technique (B) of *G. duodenalis*. Three *Giardia* cysts (B) are marked with red arrows (reference: Institute for Comparative Tropical Medicine and Parasitology, Munich).

Compared to microscopy, a direct immunofluorescence assay (IFA/DFA) for the detection of *Giardia* cysts has an improved sensitivity (up to 100 %) using fluorescein isothiocyanate (FITC)-marked monoclonal antibodies against *Giardia* cell wall antigens (Garcia and Shimizu, 1997; Geurden et al., 2008) (Chapter III.3.1).

Coproantigen enzyme linked immunosorbent assay (ELISA) is another highly sensitive method (sensitivity: 99–100 %, specificity: 96–99 %) with the advantage of not being dependent on the presence of *Giardia* cysts in the investigated samples (Maraha and Buiting, 2000; Rimhanen-Finne et al., 2007). It detects the *Giardia*-specific antigen (GSA 65) produced by trophozoites within the gastro-intestinal tract (Zimmerman and Needham, 1995) (Chapter III.2.1). Veterinary practices frequently use a *Giardia* SNAP® test, which is based on the ELISA principle with the advantage of a very rapid procedure (Carlin et al., 2006; Epe et al., 2010).

For the genetic characterisation of *Giardia* with conventional and nested polymerase chain reaction (PCR), various protocols are available investigating different gene loci with specific primers (Table A2). Adjacent sequencing of the amplification products enables the classification of the *Giardia* assemblages and subassemblages (Chapter II.1.1). Frequently investigated gene loci are SSU rRNA (Hopkins et al., 1997), beta-giardin (bg) (Lalle et al., 2005b), the elongation factor 1-alpha (ef-1) (Monis et al., 1999), the glutamate dehydrogenase (gdh) (Cacciò et al., 2008), the triosephosphate isomerase (tpi) (Sulaiman et al., 2003) and the ITS1-5.8S-ITS2 region (Cacciò et al., 2010). A multilocus PCR approach is

essential for the detection of subassemblages and mixed infections (Beck et al., 2012; Plutzer et al., 2010). Additionally, PCR protocols have successfully been combined with restriction fragment length polymorphism (RFLP) for a sensitive detection of assemblages, genotypic groups and for a reliable identification of mixed infections with *G. duodenalis* directly from faeces (Amar et al., 2002; Homan et al., 1998; Read et al., 2004). Furthermore, real-time PCR (qPCR) assays have been developed just recently as a promising method regarding specificity and sensitivity for the specific detection of assemblages A and B from human isolates (Almeida et al., 2010; Verweij et al., 2003). In 2009, a qPCR assay was developed to simultaneously detect *Giardia* infections and identify subgenotype A1 in canine faecal samples (Papini et al., 2009). The advantage over standard PCR approach is the possibility to distinguish between mixed infections and possible recombinants (Almeida et al., 2010). However, molecular analytical methods are still not viable for the daily routine diagnostics. Furthermore, there might be (sub)typing complications due to intra-isolate sequence heterogeneity and the unreliable assignment of isolates of *G. duodenalis* assemblages generated by different markers (Cacciò and Ryan, 2008).

1.8. Treatment of *Giardia* infections

Independent of the presence of clinical symptoms, all dogs shedding *Giardia* cysts should be treated because of the existing potential for a zoonotic transmission (Thompson et al., 2008). Even though some infections resolve spontaneously, a chronic development of the disease is also possible (Muller and von Allmen, 2005).

The treatment with the benzimidazole anthelmintic fenbendazole (50 mg/kg BW p.o., s.i.d. for 3–5 days) is suggested for dogs (Barr et al., 1994). Due to the high reinfection occurrence (especially in shelter dogs), the treatment should be repeated after 3–5 days (Beck and Arndt, 2014; Beelitz et al., 2006; Deplazes et al., 2013). In cases of treatment failure of fenbendazole, a good treatment outcome can be achieved with the nitroimidazole antibiotic medication Metronidazole (12.5–22 mg/kg BW p.o., b.i.d for 5 days with a repetition after 2–3 weeks, rededication for dogs required) (Schnieder, 2006; Tangtrongsup and Scorza, 2010). Furthermore, the antiprotozoal agent ronidazole (30–50 mg/kg BW p.o., b.i.d. for 7 days) in combination with environmental disinfection and shampooing of the dogs with chlorhexidine digluconate at the beginning and the end of

treatment might be effective for dogs infected with *G. duodenalis* (Fiechter et al., 2012). The drug combination of febantel-pyrantel-praziquentel (15/14.4/5 mg/kg BW p.o., q.d. for 5 days) might be administered in the case of a contemporaneous infection with *Giardia* and nematodes or cestodes in order to reduce the excretion of cysts (Miro et al., 2007; Tangtrongsup and Scorza, 2010). Since the benzimidazole anthelmintic albendazole (25 mg/kg BW p.o., b.i.d. for 2 days) might cause bone marrow suppression, it is no longer recommended for the treatment of *Giardia* infections in small animals (Beck and Arndt, 2014; Stokol et al., 1997). Besides the treatment with an adequate medication, it is essential to decrease the risk of a reinfection through decontamination of the environment. Kennels should be decontaminated with a steam cleaner and blankets need to be washed at 60 °C (Beck and Arndt, 2014). Shampooing of the animals to remove *Giardia* cysts in the fur has been reported to reduce the reinfection rate especially in long-haired animals.

Infected humans might be treated with the two nitroimidazoles metronidazole (250 mg/day p.o., t.i.d. for 5–10 days) or tinidazole (2 g/person, p.o., single dose) (Gardner and Hill, 2001; Savioli et al., 2006). The application of albendazole (200–400 mg/person p.o., q.i.d. for 5–10 days) is also effective for human patients (Gardner and Hill, 2001; Reynoldson et al., 1992).

2. *G. duodenalis* in South Eastern Europe.

2.1. Albania

Since the gastrointestinal parasite *G. duodenalis* is one of the most important non-viral infectious agents in humans worldwide, studies were conducted investigating healthy subjects and children in Albania (Berrilli et al., 2006; Spinelli et al., 2006) (Table 4). Clinically healthy adults were infected in 11.2 % (microscopy) and children in 5.6 % (microscopy). People originating from rural areas were significantly more often infected with *G. duodenalis.* The subsequent molecular analysis of faecal samples from children revealed assemblage A in 20.0 % and assemblage B in 24.0 %. The authors assumed that contact with infected animals or contaminated drinking water might be a possible source of transmission. The presence of microorganisms in drinking water has been confirmed in peripheral areas of Tirana (Palombi et al., 2001). *G. duodenalis* was not only verified in human samples, but also in 35.5 % (ELISA) of household dogs under veterinary

care from Tirana (Shukullari et al., 2013). Moreover, feline faecal samples collected in Tirana revealed *Giardia* coproantigen in 29.3 % (ELISA) (Knaus et al., 2014). However, information about the distribution of *Giardia* assemblages in dogs and cats is still missing.

2.2. Bulgaria

In 2011, results of the first study on the distribution of *Giardia* assemblages among human patients in Bulgaria were published (Chakarova et al., 2011) (Table 4). A total of 50 faecal samples were obtained after routine microscopic examination and a nested-PCR protocol targeting the tpi gene locus was performed. The majority of the samples carried assemblage B (87.2 %) with a high prevalence in the Stara Zagora region. Mixed infections with assemblages A (subassemblage AII) and B were observed in 12.7 %. Five years earlier, Karanis et al. (2006) reported about contaminated water supplies as a possible infection source for *Giardia* infections of the Bulgarian population. The presence of *Giardia* cysts was confirmed in 9.4 % (IFA) of tap, bottled, river, well and sewage water from Sofia District, Varna City and Varna Greater Area. Despite this finding, no reports about waterborne outbreaks of giardiosis exist in Bulgaria.

2.3. Croatia

In Croatia, several genotyping studies on *Giardia* assemblages in various animal species have been conducted within the last four years (Table 4). In order to gain information on the role of wild mammals as reservoir for *Giardia* infections, a large MLST study was performed (Beck et al., 2011b). Roe deer had the highest prevalence (24.0 %, IFA) whereas samples from bears and hares were free of *Giardia* cysts. According to the genotyping results of the ITS1-5.8S-ITS2 region, the SSU rRNA and tpi loci, assemblage A was predominant over assemblages B, C and D. Furthermore, the subtype A1 was detected more often than the subtype A2. A similar study on captive animals from the zoo of Zagreb revealed an overall prevalence of 29.0 % for a *Giardia* infection (Beck et al., 2011a). Phylogenetic analysis showed that *Giardia* isolates from those animals were genetically different from isolates of human or domestic animal origin. In the framework of a study on *Giardia* genotypes from household and kennel dogs, the zoonotic assemblages A and B were found in 16.7 % of the isolates (Beck et al., 2012). However, the majority of the dogs (59.4 %) carried the species-specific assemblages C and D.

2.4. Hungary

Hungarian researchers have put focus on the detection and characterisation of *G. duodenalis* in water samples and in aquatic birds (Table 4). An examination of raw and drinking water samples revealed the contamination with *Giardia* cysts in spring, raw, drinking and river water for the years 2000–2005 (Plutzer et al., 2007). Another publication about the investigation of 36 raw, surface and sewage water samples presented a prevalence of 69.4 % (Plutzer et al., 2008). The genetic characterisation of positive samples revealed mainly subassemblage AII, followed by assemblages BIII and BIV. According to this result, a human contamination was suspected as origin. However, current data show a prevalence of only 2.0 % (ELISA) in asymptomatic Hungarians from three distinct locations of the country (Plutzer et al., 2014). Since there was evidence for a contamination with *G. duodenalis* in Hungarian water supplies, the possible dissemination of human pathogenic *Giardia* cysts by aquatic birds was examined more closely (Plutzer and Tomor, 2009). Thirteen of 132 avian samples (9.8 %) were positive for *G. duodenalis* with IFA and PCR. Both assemblages A and B were detected. The question whether the infected aquatic birds actually carried zoonotic potential remained open due to the lack of information on the subassemblage level. In a preliminary study on the prevalence and genotype distribution of *G. duodenalis* in Hungarian household and kennel dogs, an overall prevalence of 58.8 % (ELISA) was generated (Szénási et al., 2007). Subsequently performed single-locus PCR revealed the canine assemblages C and D in all obtained sequences.

2.5. Macedonia

To date, research results on *G. duodenalis* in Macedonia have been published in Macedonian language, exclusively. For example, 15.5 % of 843 Macedonian children with gastrointestinal symptoms were screened positive for *Giardia* with microscopy in 2007 (Bojadžieva et al., 2007) (Table 4).

2.6. Romania

Comparisons of different methods for the detection of the protozoan parasite have been part of the current Romanian *Giardia* research (Table 4). Prevalence data for dogs varied remarkably between microscopy and ELISA. Three studies demonstrated *Giardia* infections in 34.6, 42.6 and 51.1 % of mixed canine populations with ELISA, whereas prevalence obtained by microscopy was lower

(Jarca et al., 2008; Mircean et al., 2012; Sorescu et al., 2014). Not only dogs from Romania have been subject of prevalence studies on *Giardia* but also cats from different rural districts of the country showing a prevalence of 27.9 and 47.4 % (ELISA) (Mircean et al., 2011; Sorescu et al., 2011). Both studies emphasised the role of age, origin and parasitic or non-parasitic coinfections influencing the prevalence. In order to gain information on the occurrence of *Giardia* in livestock, a total of 288 faecal samples from calves living in Western Romania were tested for *Giardia* coproantigen with ELISA (Ilie et al., 2011). The overall prevalence of 26.7 % implicated the presence of the intestinal parasite in cattle and emphasised the need for further research on the potential zoonotic transmission.

2.7. Serbia

Publications from 1993 until 2011 have confirmed that *G. duodenalis* is the most common intestinal protozoan parasite in dogs from the Belgrade area (Table 4). Faecal samples from household, stray, farm and military working (kennel) dogs were investigated in three different studies. The overall prevalence determined by microscopy ranged from 3.8 up to 14.6 % for those dog populations (Nikolić et al., 2008; Nikolić et al., 2002; Nikolić et al., 1993). Significantly higher infection rates were found in stray, farm and military working dogs. With the intention to evaluate the correlation of *Giardia* infections in household dogs and their owners, faecal samples of all family members of households accommodating *Giardia* positive dogs were also screened for *Giardia* cysts in two of the three studies. Two people living in one household with an infected dog carried an infection with *G. duodenalis* as well. The finding supports a possible transmission of *Giardia* infections between human and canine cycles. However, a molecular analysis of the concerned samples would have been essential for a further statement on the zoonotic potential and the transmission dynamics arising from the investigated dog population. Contemporaneous to a study on canine *Giardia* infections, a selection of 81 household cats from Belgrade was also tested for *Giardia* infections and showed a prevalence of 22.2 % (microscopy) (Nikolić et al., 2002). Human giardiosis is spread throughout Serbia with a higher incidence in the Northern part of the country (Nikolić et al., 2011). Compared to all other Western Balkan Countries (WBC), Serbia had the greatest number of *Giardia* cases per 100,000 population for each of the four years of the reporting period corresponding to a report of the WHO (1987).

Table 4: Summary of studies on *G. duodenalis* in the seven investigated South Eastern European countries Results for the prevalence are shown as absolute numbers and percentages. For performed PCRs, the occurring assemblages (ass.) are listed.

Country	No of samples (target species or material)	Method	Results (positive samples)	Reference
Albania	125 (human)	microscopy IFA PCR: SSU rRNA sequencing	7/125 (5.6 %) 10/50 (20.0 %) 22/50 (44.0 %) ass. A and B	(Berrilli et al., 2006)
	277 (human)	microscopy IFA in doubtful cases	31/277 (11.2 %)	(Spinelli et al., 2006)
	321 (human)	microscopy	35/321 (10.9 %)	(Sejdini et al., 2011)
	58 (feline)	ELISA	17/58 (29.3 %)	(Knaus et al., 2014)
Bulgaria	166 (water)	IFA	13/138 (9.4 %)	(Karanis et al., 2006)
	50 (human)	microscopy PCR: tpi RFLP	47/50 (94.0 %) 47/47 (100 %) 6/47 (ass. B) 41/47 (ass. A+B)	(Chakarova et al., 2011)
Croatia	832 (wild mammals)	IFA PCR: SSU rRNA ITS1-5.8S-ITS2 tpi sequencing	28/832 (3.4 %) 23/26 (88.5 %) 16/26 (61.5 %) 9/26 (34.6 %) ass. A, B, C, D	(Beck et al., 2011b)
	131 (mammalian zoo animals)	IFA PCR: SSU rRNA ITS1-5.8S-ITS2 tpi bg gdh sequencing	38/131 (29.0 %) 23/27 (85.2 %) 19/27 (70.4 %) 20/27 (74.1 %) 11/27 (40.7 %) 8/27 (29.6 %) ass. A, B, C, D	(Beck et al., 2011a)
	96 (canine)	PCR: bg ITS1-5.8S-ITS2 gdh tpi sequencing	52/96 (54.2 %) 56/96 (58.3 %) 46/96 (47.9 %) 62/96 (64.6 %) ass. A, B, C, D	(Beck et al., 2012)
Hungary	229 (canine)	microscopy ELISA PCR: SSU rRNA sequencing	14/187 (7.5 %) 110/187 (58.8 %) 15/15 (100 %) ass. C and D	(Szénási et al., 2007)

	76 (water)	IFA	27/76 (35.5 %)	(Plutzer et al., 2007)
	36 (water)	IFA PCR: gdh SSU rRNA sequencing	25/36 (69.4 %) 9/36 (25.0 %) 13/36 (36.1 %) ass. A and B	(Plutzer et al., 2008)
	132 (aquatic birds)	IFA PCR: SSU rRNA LAMP sequencing	4/132 (3.0 %) 5/132 (3.8 %) 5/132 (3.8 %) ass. A and B	(Plutzer and Tomor, 2009)
	300 (human)	ELISA PCR: SSU rRNA gdh sequencing	6/300 (2.0 %) 6/300 (2.0 %) 2/300 (0.7 %) ass. A and B	(Plutzer et al., 2014)
Macedonia	843 (human)	microscopy	131/843 (15.5 %)	(Bojadžieva et al., 2007)
Romania	184 (canine)	microscopy ELISA	3/184 (1.6 %) 94/184 (51.1 %)	(Jarca et al., 2008)
	183 (feline)	ELISA	51/183 (27.9 %)	(Mircean et al., 2011)
	76 (feline)	microscopy	36/76 (47.4 %)	(Sorescu et al., 2011)
	288 (bovine)	ELISA	77/288 (26.7 %)	(Ilie et al., 2011)
	614 (canine)	microscopy ELISA	52/614 (8.5 %) 144/416 (34.6 %)	(Mircean et al., 2012)
	183 (canine)	microscopy ELISA	77/183 (42.1 %) 78/183 (42.6 %)	(Sorescu et al., 2014)
Serbia	78 (canine)	microscopy	3/78 (3.8 %)	(Nikolić et al., 1993)
	5981 (human)	microscopy	407/5981 (6.8 %)	(Nikolić et al., 1998)
	167 (canine) 81 (feline)	microscopy	dogs: 24/167 (14.4 %) cats: 18/81 (22.2 %)	(Nikolić et al., 2002)
	151 (canine)	microscopy	22/151 (14.6 %)	(Nikolić et al., 2008)
	Review on the information available on the epidemiological characteristics of asymptomatic and symptomatic human giardiosis in Serbia			(Nikolić et al., 2011)

III. MATERIALS AND METHODS

1. Sample origin

From 2010 to 2014, a total of 1671 canine faecal samples were collected in seven South Eastern European countries (Figure 6).

Figure 6: Seven South European countries participating in the current study on the occurrence and genetic determination of *Giardia* in dogs from South Eastern Europe (Reference: www.stepmap.de).

Samples from Macedonia were collected in various regions all over the country. In Romania, mainly the South Eastern area including Bucharest, Buzau and Constanta were included in the collection process. The samples from Serbia were obtained from two different dog shelters in Belgrade. The Croatian samples were provided specifically for molecular genotyping and derived from 26 dogs that had been tested *Giardia* (IFA)-positive at the Department for Bacteriology and Parasitology of the Croatian Veterinary Institute in Zagreb. All samples from Albania, Bulgaria and Hungary originated from previously conducted studies

focusing on gastrointestinal parasitic infections of dogs living in those countries (Capári et al., unpublished; Kirkova et al., unpublished; Shukullari et al., 2013) (Table 5). Dogs of various breeds, all ages, both sexes and different life styles were included in the study. Household dogs had been visiting veterinary clinics for diverse reasons. All samples were collected immediately after natural defecation. For the analysis of prevalence data, the group of kennel, street and shelter dogs was combined into the term 'shelter dogs' due to assumed similar hygienic living conditions and compared to the group 'household dogs'. A subset of the faecal samples was stored at 7 °C after collection and screened for *Giardia* immediately afterwards. All other samples were frozen at –20 °C until they were further processed.

Table 5: Overview of faecal samples of dogs collected in seven South Eastern European countries for MLST

Country	Period of collection	Number of samples			Reference
		total	shelter dogs	household dogs	
Albania (Tirana)	2010–2011	602	0	602	(Shukullari et al., 2013)
Bulgaria (different regions)	2012–2013	294	32	262	(Kirkova et al., unpublished)
Croatia (Zagreb)	2013–2014	26	0	26	This study
Hungary (Western Hungary)	2012–2013	296	35	261	(Capári et al., unpublished)
Macedonia (different regions)	2013–2014	136[a]	15	117	This study
Romania (South-Eastern area)	2013–2014	183	27	156	This study
Serbia (Belgrade)	2013	134	134	0	This study
Total	2010–2014	1671[a]	243	1424	

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

2. Screening for *Giardia* positive samples

2.1. Enzyme linked immunosorbent assay (ELISA)

In order to detect *Giardia* positive samples, the ProSpecT™ *Giardia* Microplate assay (Remel, Lenexa, USA) was used according to the manufacturer's

instructions (Figure 7A). The screening was performed on the canine faecal samples from all investigated countries except from Croatia. The final spectrophotometric analysis was performed with the ELISA-reader (Deelux Labortechnik, Gödenstorf, Germany) at a wavelength of 450 nm. Samples with an optical density above 0.05 were classified as positive (Figure 7A). The ProSpecT™ *Giardia* Microplate assay has a sensitivity of 97 % and a specificity of 99.8 % (Zimmerman and Needham, 1995). The fact that the ELISA has the advantage of not being dependent on the excretion of cysts contributes to the high sensitivity of the method.

3. Screening for *Giardia* cysts

A positive result in the coproantigen ELISA does not guarantee the presence of *Giardia* cysts, which are necessary for the subsequent DNA extraction and molecular analysis. Against this background, a subset of ELISA-positive samples was further screened with IFA or MIFC.

3.1. Screening with immunofluorescence assay (IFA)

Analysis with the IFA Merifluor® *Cryptosporidium*/*Giardia* (Meridian Bioscience, Luckenwalde, Germany) was performed following the manufacturer's instructions. At least 25 ELISA-positive samples from Albania, Bulgaria, Hungary, Macedonia and Romania were investigated in order to confirm the presence of *Giardia* cysts by visualisation of fluorescein isothiocyanate (FITC)-conjugated antibodies against specific *Giardia* cyst wall-epitopes (Figure 7B). To date, Merifluor® *Cryptosporidium*/*Giardia* is the only available test operating also with frozen faecal samples. As the majority of the samples had been collected over several months or years, the freezing was inevitable. All 26 samples from Croatia were screened with IFA under the framework of the daily routine diagnostics of the Croatian Veterinary Institute in Zagreb.

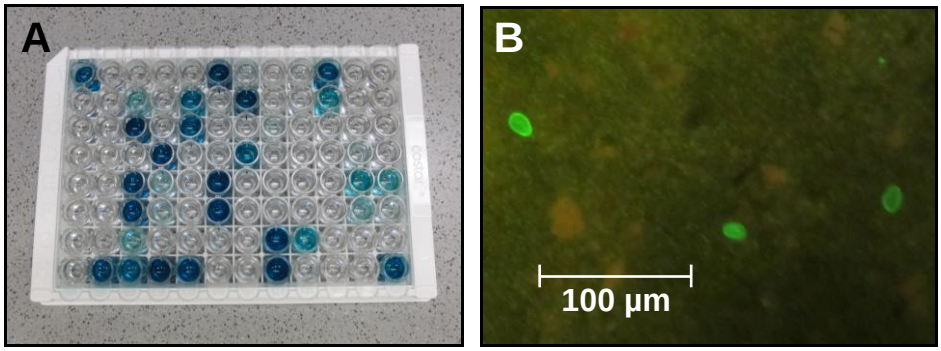

Figure 7: Diagnostic methods for the detection of *Giardia duodenalis*. Microwell plate of the ELISA (A): blue stained samples are positive for *G. duodenalis*. In the IFA (B) three *Giardia* cysts fluoresce apple green.

3.2. Screening with merthiolate iodine formalin concentration (MIFC)

Since it was possible to organise a straight transport to Munich directly after the collection period in two dogs shelters over two days, all 134 faecal samples from Serbia were screened for *Giardia* cysts by the MIFC technique which is only applicable for fresh faecal material (Pfister et al., 2013). Briefly, one to two grams of faeces per sample and 2.35 ml of MIF-solution were mixed in a beaker, sieved through a mesh (mesh width 300 µm) into a centrifuge tube, 1.5 ml of formaldehyde (37 %) added to the filtrate, the centrifuge tube was closed with a rubber plug and shaken firmly before the subsequent centrifugation (without the rubber plug) for five minutes (2000 U/min). During centrifugation, four layers developed within the centrifuge tube (Figure 8). If the layer of debris had accumulated at the interphase between the two liquids, it needed to be loosened by passing a swabstick gently round the circumference of the tube. The supernatant consisting of the top three layers was decanted and one drop of Lugol's solution was added to the sediment. One to two drops of the coloured sediment was placed on an object slide, covered with a cover slip and examined under a light microscope with 100–400× magnification.

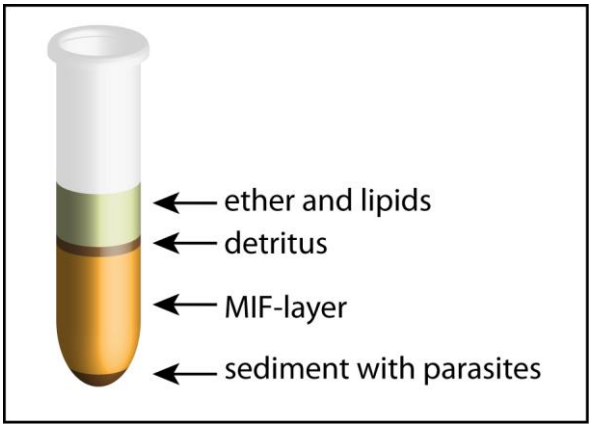

Figure 8: The separation of the different layers of a MIFC in a centrifuge tube after centrifugation.

4. DNA extraction

According to the result of the IFA or MIFC, 15 to 26 *Giardia* cyst-positive samples per country were chosen for DNA extraction. The QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used, following the manufacturer's recommended protocol with an initial incubation step at 95 °C for 15 minutes and two final DNA elution steps with 100 µl AE-buffer each. Since the IFA slides revealed mainly broken cyst walls, no additional wall-breaking steps to free the *Giardia* DNA were performed.

5. DNA purification

To increase the purity of the DNA after extraction, all extracted DNA samples were purified additionally with the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) including a final elution with 25 µl EB buffer as described previously (Beck et al., 2012).

6. Quality control of extraction and quantisation of DNA

For the determination of the DNA concentration and purity, 1.5 µl of each DNA sample were tested with the Nanodrop™ ND 1000-Spectrometer (Peqlab, Erlangen, Deutschland) (Figure 9). The method is based on the measurement of the 10 mm absorbance (A_{260}) of the extracted dissolved DNA at a wavelength of 260 nm. The DNA-concentration is determined as follows:

$$\text{DNA-concentration [µg/ml]} = A_{260}*50 \text{ (factor for DNA)}.$$

In order to verify the purity of the DNA the ratio A_{260}/A_{280} was measured. The

value for pure DNA varied between 1.8 and 2.0. A target ratio below 1.8 refers to the contamination with protein of the sample. An A_{260}/A_{280} ratio greater than 2.0 indicates DNA degradation and measurement of free nucleotides (RNA).

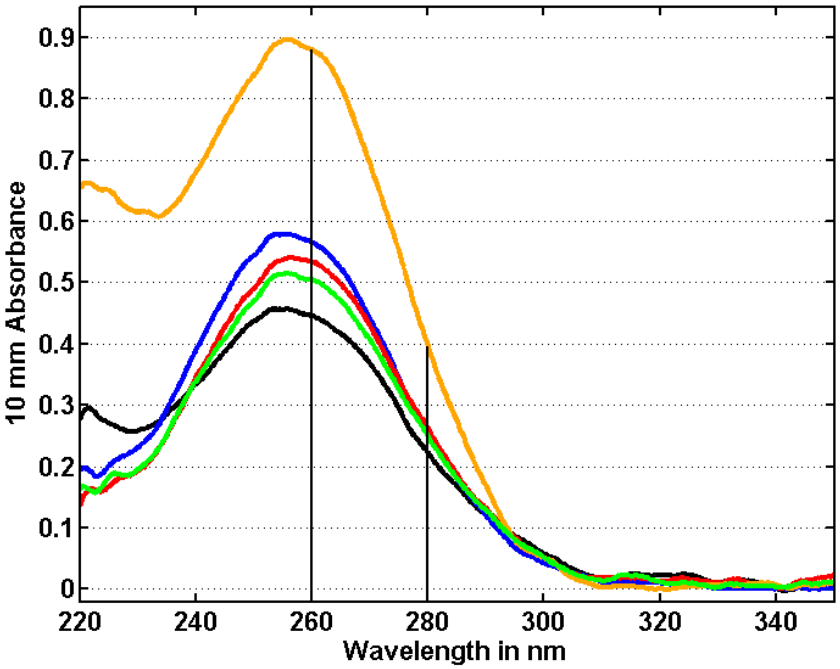

Figure 9: Absorbance of the DNA sample in dependence of the wavelength measured with the Nanodrop™ ND 1000-Spectrometer. Maximum absorbance of DNA occurs between 250 and 260 nm. The two vertical lines indicate the wavelengths utilised for analysis of the DNA concentration and purity. The different curves belong to five DNA samples originating from Macedonia with a DNA content ranging from 22.3 to 43.9 µg/ml and a DNA purity ranging from 2.01 to 2.55.

Subsequent to the PCR of five different *Giardia* gene loci, all samples were divided into a 'positive' and a 'negative' group according to the PCR result of each investigated gene locus. For each group, the average DNA concentration, the average DNA purity and the standard deviation of the DNA purity were calculated. In order to illustrate the exact distribution of the DNA concentrations and the DNA purity values, two histograms were generated for the PCR-positive and negative samples.

7. Polymerase Chain Reaction for detection of *Giardia* DNA

Five different loci of the *Giardia* genome were investigated with multilocus sequence typing (MLST). Nested polymerase chain reactions (PCR) were performed targeting the conserved small ribosomal subunit (SSU rRNA), the

internal transcribed spacer (ITS1-5.8S-ITS2) region, the structural protein-coding gene beta giardin (bg) and two housekeeping enzyme-coding genes, the glutamate dehydrogenase (gdh) and the triosephosphate isomerase (tpi). The latter three protein-coding genes have a high degree of genetic polymorphism and are commonly used for genotyping as well as for subgenotyping. The following equipment was used for the PCR amplification processes: 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).

7.1. Nested PCR for the detection of the SSU rRNA gene

The first reaction of the nested PCR was carried out using 2–3 µl of template DNA, 25 µl of 2x GoTaq® Green Mastermix (Promega, Madison, USA), 1 µl (0.2 µM) of each primer (10 µM, Eurofins MWG Operon, Ebersberg, Germany), 2.5 µl of 5 % dimethyl sulfoxide (DMSO, Roth, Karlsruhe, Germany) and water-ultra pure grade (Sigma Life Science, Taufkirchen, Germany), filled up to a total volume of 50 µl. The organic solvent DMSO was added in order to improve the amplification of the targeted GC-rich regions. The forward primer RH11 (5-’CATCCGGTCGATCCTGCC-3’) and the reverse primer RH4 (5’-AGTCGAACCCTGATTCTCCGCCAGG-3’) were used for the amplification of a 292 bp fragment of the SSU rRNA gene locus (Hopkins et al., 1997). The first round cycling conditions included an initial activation at 94 °C for 2 min, 40 denaturation/annealing/elongation cycles at 94 °C for 45 s, at 50 °C for 45 s and at 72 °C for 60 s, followed by the final elongation at 72 °C for 10 min.

The reaction volume for the nested PCR contained 5 µl of the template DNA of the first reaction, 25 µl of 2x GoTaq® Green Mastermix, 1 µl (0.2 µM) of each primer (10 µM), 0.5 µl ultrapure Bovine Serum Albumin (BSA, Roth, Karlsruhe, Germany) non-acetylated (1 % [50 mg/ml]) and water-ultra pure grade, filled up to a total volume of 50 µl. BSA was used as a coenhancer of DMSO stabilising the DNA polymerase and counteracting the potential inhibitory effects of high concentrations of organic solvents on DNA polymerase activity (Farell and Alexandre, 2012). Forward and reverse primers GiarF (5’-GACGCTCTCCCCAAGGAC-3’) and GiarR (5’-CTGCGTCACGCTGCTCG-3’) were used for the amplification of a 175 bp fragment (Figure 10) of the SSU

rRNA (Read et al., 2002). Cycling conditions for the nested-PCR reaction were identical to the conditions for the first reaction.

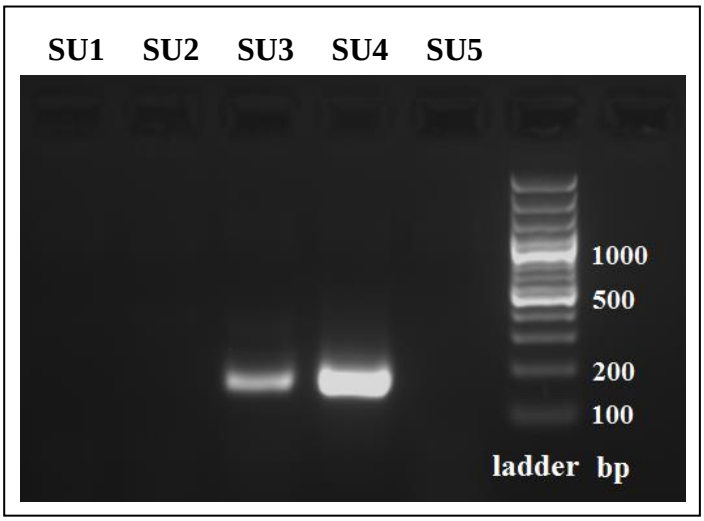

Figure 10: Gel electrophoresis of PCR-products of the SSU rRNA region. Right side: Gene ruler 100 bp Plus DNA ladder. SU5: negative control. SU4: positive control. SU3 is positive for *Giardia* showing a band of 175 bp. No amplification product was achieved for SU1 and SU2.

7.2. Nested PCR for the detection of the ITS1-5.8S-ITS2 region

For the first amplification, the reaction mix contained 2–3 µl of template DNA, 20 µl of 2x GoTaq® Green Mastermix, 0.8 µl (0.2 µM) of each primer (10 µl), 2 µl of 5 % DMSO and water-ultra pure grade, filled up to a total volume of 40 µl. For the amplification of a 347 bp fragment of the ITS1-5.8-ITS2 region, the forward primer FW1 (5'-TGGAGGAAGGAGAAGTCGTAAC-3') and the reverse primer RV1 (5'-GGGCGTACTGATATGCTTAAGT-3') were named and used as previously described (Cacciò et al., 2010). The cycling conditions were the same for both amplifications with 94 °C for 2 min for one cycle, 94 °C for 30 s, 59 °C for 30 s and 72 °C for 60 s for 35 cycles, followed by 72 °C for 7 min.

For the second amplification, 5 µl of the DNA template of the first reaction were used with identical reaction mix contents as in the first amplification. A 315 bp fragment of the ITS1-5.8S-ITS2 region (Figure 11) was obtained using forward primer FW2 (5'-AAGGTATCCGTAGGTGAACCTG-3') and the reverse primer RV2 (5'-ATATGCTTAAGTTCCGCCCGTC-3') as previously described (Cacciò et al., 2010).

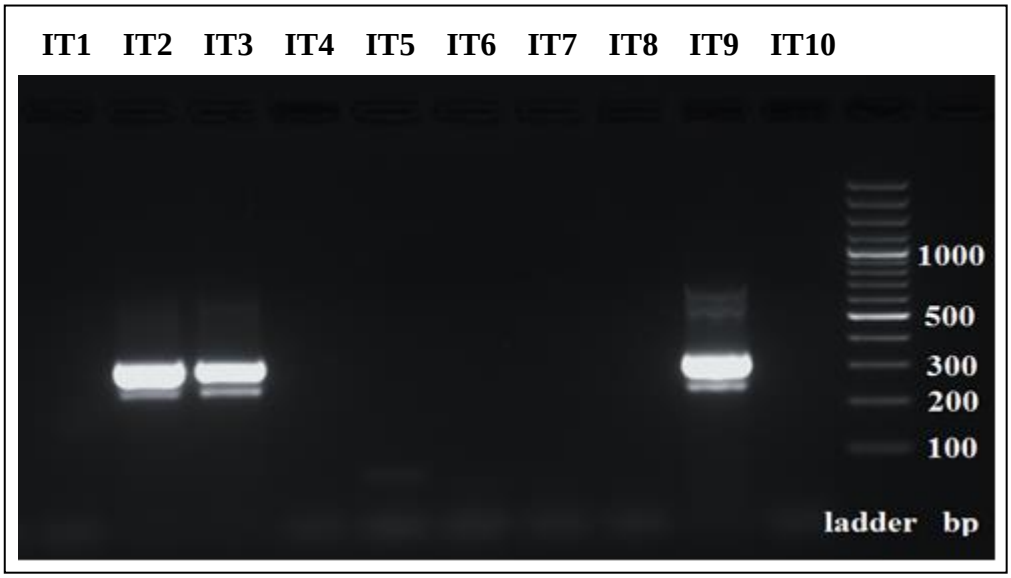

Figure 11: Gel electrophoresis of PCR-products of the ITS1-5.8S-ITS2 region. Right side: Gene Ruler 100bp Plus DNA ladder. IT10: negative control. IT9: positive control. Positive amplicons of IT2 and IT3 show bands of 315 bp.

7.3. Nested PCR for the detection of the beta giardin gene

Both primary and secondary reactions were performed in a 50 µl PCR reaction mix comprising 25 µl of 2x GoTaq® Green Mastermix, 1 µl (0.2 µM) of each primer (10 µM) and water-ultra pure grade, filled up to the total volume. In the first amplification, 2–3 µl of DNA were used while the second amplification used 5 µl of the reaction product. First forward and reverse primers amplifying a 753 bp long region of the bg gene locus were G7 (5'-AAGCCCGACGACCTCACCCGCAGTGC-3') and G759 (5'-GAGGCCGCCCTGGATCTTCGAGACGAC-3'). Primers for the second reaction were FW (5'-GAACGAACGAGATCGAGGTCCG-3') and RV (5'-CTCGACGAGCTTCGTGTT-3') which addressed a 515 bp fragment (Figure 12) of the bg gene locus (Lalle et al., 2005a). The cycling conditions for the first reaction were as follows with an initial 94 °C for 2 min for one cycle, 94 °C for 30 s, 60 °C, 30 s and 72 °C for 45s for 35 cycles, followed by 72 °C for 7 min.

For the nested reaction, the cycling conditions were 94 °C for 2 min for one cycle, 94 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s for 40 cycles, followed by 72 °C for 7 min.

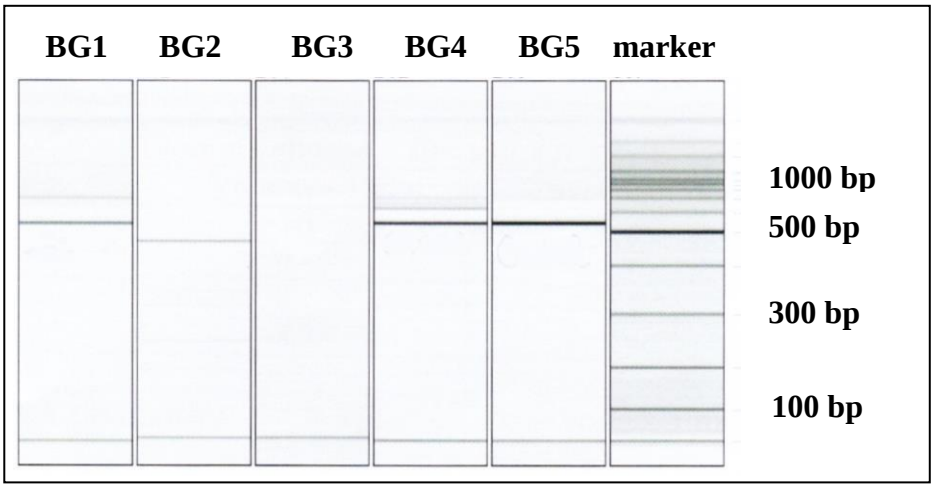

Figure 12: Capillary electrophoresis of PCR products of the bg gene locus. BG5: positive control. The samples BG1 and BG4 are positive for *Giardia* showing a band of approximately 515 bp. Sample BG2 shows a non-specific band under 500 bp. Alignment Marker (15 bp/1000 bp) and QX DNA size marker (100 bp–2500 bp) were used.

7.4. Nested PCR for the detection of the glutamate dehydrogenase gene

PCR reactions used 2–3 µl of the DNA template, 25 µl of 2x GoTaq® Green Mastermix, 1 µl (0.2 µM) of each primer (10 µl) and water-ultra pure grade, filled up to a final volume of 50 µl. Forward primer GDH1 (5'-TTCCGTRTYCAGTACAACTC-3') and reverse primer GDH2 (5'-ACCTCGTTCTGRGTGGCGCA-3') targeting a 755 bp long fragment of the gdh locus were used according to a previously conducted study (Cacciò et al., 2008). The first-round PCR conditions were 94 °C for 2 min for one cycle, 94 °C for 45 s, 50 °C for 45 s and 72 °C for 45 s for 35 cycles, followed by 72 °C for 7 min.

Five µl from the first-round reaction were used in the second-round PCR with forward and reverse primers GDH3 (5'-ATGACYGAGCTYCAGAGGCACGT-3') and GDH4 (5'-GTGGCGCARGGCATGATGCA-3') targeting a 530 bp long fragment (Figure 13) of the gdh locus (Cacciò et al., 2008). The second round PCR conditions were 94 °C for 2 min for one cycle, 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s for 40 cycles, followed by 72 °C for 7 min.

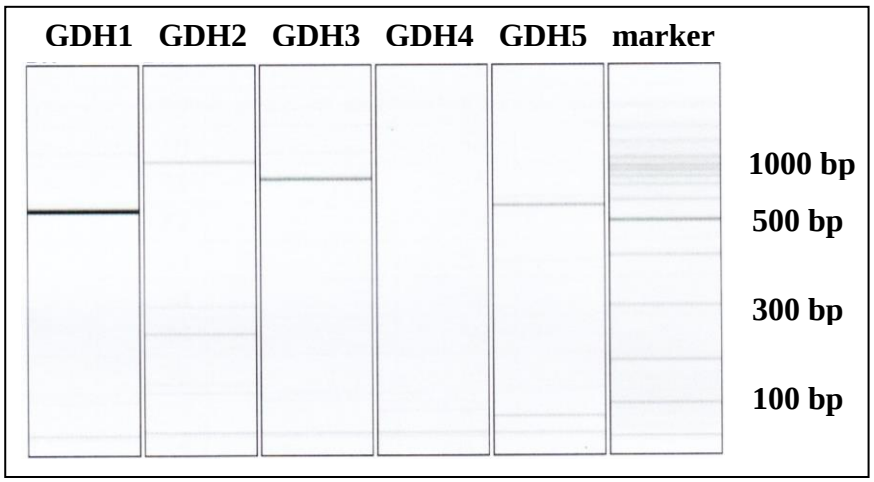

Figure 13: Capillary electrophoresis of PCR products of the gdh gene locus. GDH5: positive control. The sample GDH1 is positive for *Giardia* showing a band of approximately 530 bp. Samples GDH2 and GDH3 show non-specific bands of over 600 bp and under 300 bp. Alignment Marker (15 bp/1000 bp) and QX DNA size marker (100 bp–2500 bp) were used.

7.5. Nested PCR for the detection of the triosephosphate isomerase gene

Amplification of a 605 bp fragment of the tpi gene locus involved the use of a 50 µl suspension of the following reagents: 2–3 µl of the DNA template, 25 µl of 2x GoTaq® Green Mastermix, 1 µl (0.2 µM) of each primer (10 µl) and water-ultra pure grade, filled up to the total volume. Primers from Sulaiman et al. (2003) were modified after they had been tested for specificity with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The original primers contained the variable base inosine (I) which can pair with adenine, thymine, or cytosine and allows for the design of primers spanning a single-nucleotide polymorphism (SNP) without the polymorphism disrupting the primer's annealing efficiency (Table 6). According to the BLAST results, inosine was replaced by bases or base combinations with the intention to support a more precise primer-target binding (Table A3).

Table 6: Modification of primers from Sulaiman et al. for the tpi gene locus. I: Inosine pairs with adenine, thymine, or cytosine Y: pairs with pyrimidine bases (C, T). N: pairs with all four bases (A, C, G, T).

primer name	primer after Sulaiman et al. (5'-3')	modified primer (5'-3')
AL3543	AAAT**I**ATGCCTGCTCGTCG	AAAT**Y**ATGCCTGCTCGTCG
AL3546	CAAACCTT**I**TCCGCAAACC	CAAACCTT**Y**TCCGCAAACC
AL3544	CCCTTCATCGG**I**GGTAACTT	CCCTTCATCGG**N**GGTAACTT
AL3545	GTGGCCACCAC**I**CCCGTGCC	GTGGCCACCAC**V**CCCGTGCC

The modified primers AL3543 and AL3546 were used for the first reaction. Primary cycling conditions were 94 °C for 2 min for one cycle, 94 °C for 45 s, 50 °C for 45 s and 72 °C for 45 s for 35 cycles, followed by 72 °C for 7 min.

For the amplification of a 563 bp fragment (Figure 14) of the tpi locus in the second reaction, the identical reaction volume contents were used with the exception of the usage of 5 µl of the first reaction product. Modified primers AL3544 and AL3545 were used for the second reaction. Secondary cycling conditions were 94 °C for 2 min for one cycle, 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s for 40 cycles, followed by 72 °C for 7 min.

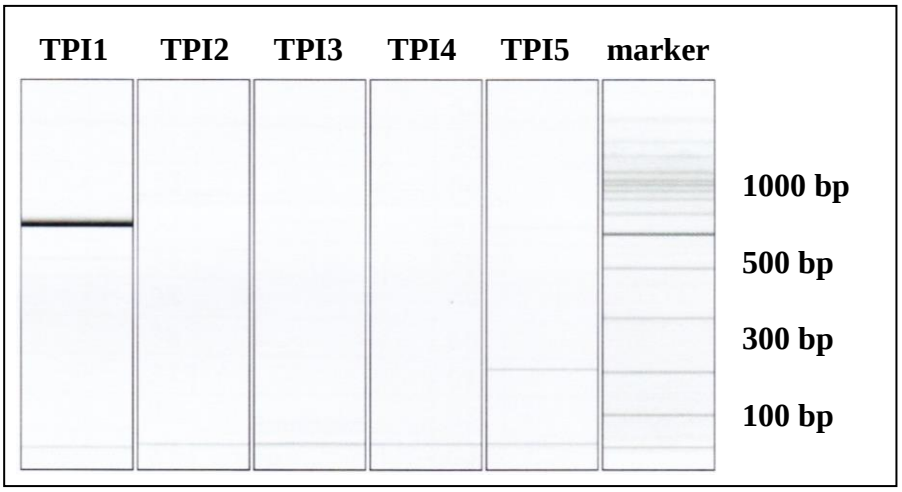

Figure 14: Capillary electrophoresis of PCR products of the tpi gene locus. The sample TPI1 is positive for *Giardia* showing a band of approximately 563 bp. No amplification product was obtained from samples TPI2–TPI4. Sample TPI5 shows a non-specific band of 200 bp. Alignment Marker (15 bp/1000 bp) and QX DNA size marker (100 bp–2500 bp)were used

8. Visualisation of PCR products

8.1. Agarose gel electrophoresis

PCR products of SSU rRNA and ITS1-5.8S-ITS2 were analysed on 2 % Top Vision Agarose gels (Fermentas, St. Leon-Rot, Germany) produced with TAE buffer 50× (Qiagen, Hilden, Germany) and TBE buffer 10× (Fermentas, St. Leon-Rot, Germany). The agarose was dyed with GelRed™ nucleic acid stain, 10.000× in water (Biotium, Hayward, USA) and a Gene Ruler 100bp Plus DNA ladder (Fermentas, St. Leon-Rot, Germany) was added to every agarose gel. A gel documentation system was used for visualising gel images under UV light (Peqlab, Erlangen, Germany).

8.2. Capillary electrophoresis

Capillary electrophoresis was performed for PCR products of bg, gdh and tpi loci (QIAxcel®, Qiagen, Hilden, Germany). QX wash buffer, QX separation buffer, QX DNA Alignment Marker (15 bp/1000 bp) and QX DNA size marker (100 bp–2500 bp) were utilised according to the manufacturer's instructions. The fluorescence of nucleotides was excited by UV-light, further processed by a photomultiplier and converted into an electronic signal.

9. DNA purification

PCR products obtained from the SSU rRNA locus and ITS1-5.8S-ITS2 region were purified using QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany). Purification of the amplified samples from bg, gdh and tpi loci was performed with the ExoSAP-IT® PCR Clean-Up Reagent (USB, Cleveland, USA). Both purification kits were used according to the manufacturer's instructions.

10. Sequencing and sequence analysis: determination of assemblages

For PCR-positive products of the SSU rRNA locus and ITS1-5.8S-ITS2 region, forward and reverse sequencing were performed by Eurofins MWG Operon (Ebersberg, Germany). For amplicons of bg, gdh and tpi loci, Macrogen Inc. (Amsterdam, Netherlands) conducted forward and reverse sequencing. Obtained 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). The obtained sequences were compared against the GenBank (BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table A4). Additionally, sequences were also assembled using SeqMan® (DNASTAR, Madison, USA).

11. Translation of nucleotide sequences into amino acids

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.

12. Statistical analysis

Differences in prevalence data between household dogs and shelter dogs were tested by Chi-squared analysis using an online tool (Chi-square Calculator: http://socscistatistics.com/tests/chisquare/Default2.aspx). *p* values <0.05 were considered to be significant.

IV. RESULTS

The results of the study were published in an international, peer-reviewed journal.

A supplement to table 5 of the paper illustrating the combined genotyping results including the assemblages at all five loci is available in the annex (Table A5).

1. Publication

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

M. F. Sommer[1,*], R. Beck[2], M. Ionita[3], J. Stefanovska[4], A.Vasić[5], N. Zdravković[5], D. Hamel[6], S. Rehbein[6], M. Knaus[6], I. L. Mitrea[3], E. Shukullari[7], Z. Kirkova[8], D. Rapti[7], B. Capári[9], C. Silaghi[1, 10]

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[1]Institute of Comparative Tropical Medicine and Parasitology, Ludwig-Maximilians-University, Munich, Germany
[2]Department for Bacteriology and Parasitology, Croatian Veterinary Institute, Zagreb, Croatia
[3]Faculty of Veterinary Medicine, UASVM Bucharest, Bucharest, Romania
[4]Department of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, University'Ss.Cyril & Methodius', Skopje, Macedonia
[5]Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Serbia
[6]Kathrinenhof Research Centre, Merial GmbH, Rohrdorf-Lauterbach, Germany
[7]Faculty of Veterinary Medicine, Agricultural University of Tirana, Tirana, Albania
[8]Division of Epidemiology and Medical Parasitology, Trakia University, Stara Zagora, Bulgaria
[9]Kapriol Bt., Városlal ut. 5, 8330 Sümeg, Hungary
[10]Present Address: National Reference Centre for Vector Entomology, Institute of Parasitology, University of Zurich, Zurich, Switzerland

Corresponding author:

Marie Franziska Sommer
Comparative Tropical Medicine and Parasitology, Ludwig-Maximilians-Universität
Leopoldstr. 5 80802 Munich, Germany
Franziska.Sommer@tropa.vetmed.uni-muenchen.de
Telephone: +49 (0)89 2180 – 3622
Fax: +49 (0)89 2180 – 3623

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.

Key words: *Giardia duodenalis*; Dog; Multilocus genotyping; Assemblages; South Eastern Europe

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.

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.

Screening for Giardia infections with coproantigen ELISA

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

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′-GAGGCCGCCCTGGATCTTCGAGACGAC-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 Dehydro-genase (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×	
Triose-phosphate Isomerase (tpi)	605	1st amplification: AL3543 5′-AAATYATGCCTGCTCGTCG-3′ AL3546 5′-CAAACCTTYTCCGCAAACC-3′	Total volume 50 µl[d] Template DNA 2–3 µl AL3543 0.2 µM AL3546 0.2 µM	94 °C, 45 s 50 °C, 45 s 72 °C, 45 s → 35×	(Sulaiman et al. 2003)[i]
	563	2nd amplification: AL3544 5′-CCCTTCATCGGNGGTAACTT-3′ AL3545 5′-GTGGCCACCACVCCCGTGCC-3′	PCR product 5 µl (amplification 1) AL3544 0.2 µM AL3545 0.2 µM	94 °C, 30 s 50 °C, 30 s 72 °C, 30 s → 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)

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.

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'

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 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.

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 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.

‘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.

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

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.

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Supplementary data

Table 1

Nucleotide substitutions of sequences obtained in the present study in comparison to selected reference sequences from GenBank

locus	assemblage	reference sequence[a]	sequence with substitution[a,b]	reference → substitution	bp	expression in amino acid sequence
SSU	C	AB569372	KP258271	C → A	62	-
			KP258264	C → T	64	-
			KP258334	G → A	94	-
	D	AF199443	KP258313	G → A	139	-
ITS	D	JN603692	KP258389	C → T	36	-
				C → T	252	-
			KP258388	G → T	88	-
			KP258383	G → A	193	-
			KP258362	C → G	196	-
			KP258393	G → A	254	-
BG	C	JN416552	KP258347	C → G	121	silent
			KP258341	C → G	121	silent
			KP258345	C → G	121	silent
			KP258344	G → A	205	silent
	D	EF455598 KP258343	HM061152 KP258346	A → G	19	silent
				G → A	91	silent
				A → C	97	silent
GDH	D	JN587398	KP258355	A → deletion	339	frame shift
TPI	C	AY228641	KP258396	T → C	100	silent
				C → A	124	silent
				C → T	202	silent
				T → C	316	silent
				C → T	508	silent

[a]GenBank accession number

[b]Sequences whose accession numbers are not listed in this column were 100 % homologous to the reference sequence.

2. Further results

The results obtained by the Nanodrop™ ND 1000-Spectrometer measurement as described in chapter III.6 were organised into a data table and two histograms.

The average DNA concentration of the positive samples was higher, compared to the negative samples, with one exception at the tpi locus (Table 7). The average ratio for the DNA purity for both positive and negative samples was located between 1.8 and 2.0 with no pronounced difference. However, the standard deviation was higher for the PCR-negative samples at all loci.

Table 7: Overview of DNA concentrations and purities for all five loci. At each locus, the PCR-positive and PCR-negative samples are evaluated separately. For both groups the average DNA concentration is calculated, as well as the average and the standard deviation of the purity (A_{260}/A_{280}). The last row includes all loci as shown in Figure 15 and Figure 16.

Locus	PCR result	Average DNA concentration in µg/ml	Average ratio of DNA purity	Standard deviation DNA purity
SSU rRNA	positive	40.0	1.98	0.33
	negative	35.8	1.90	0.59
ITS1-5.8S-ITS2	positive	48.3	2.03	0.24
	negative	35.3	1.93	0.45
bg	positive	94.2	2.03	0.24
	negative	36.5	1.96	0.41
gdh	positive	90.3	2.04	0.25
	negative	36.3	1.96	0.41
tpi	positive	32.1	2.09	0.28
	negative	39.2	1.96	0.40
All loci	positive[a]	41.5	1.97	0.34
	negative	29.6	1.92	0.60

[a]The label ‘positive’ for the row ‘all loci’ implies a positive PCR result at one locus minimum.

To gain better insight into the distribution of the DNA concentrations, a histogram was created including both PCR-positive and negative samples (Figure 15). The group of negative samples is located around the lowest DNA concentrations whereas the group of positive samples is reaching towards proportionally higher DNA concentrations. Even though some samples contained DNA in concentrations over 100 µg/ml, a successful PCR amplification was not achieved

in all cases.

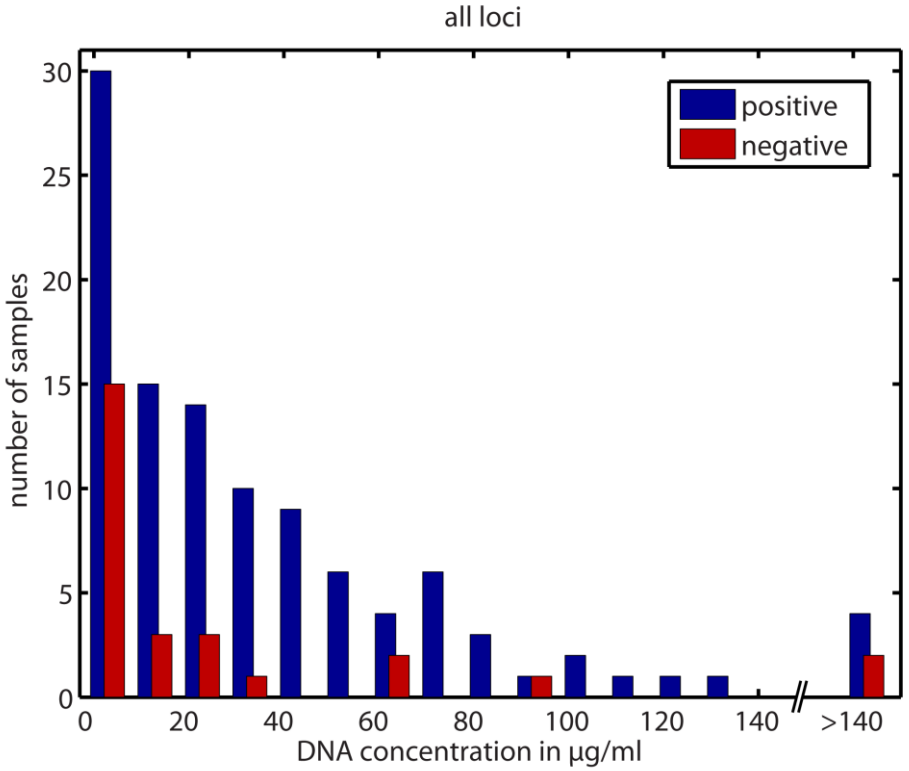

Figure 15: Histogram of DNA concentration for 109 PCR-positive and 24 PCR-negative samples. The DNA concentration is measured by Nanodrop™ ND 1000-Spectrometer and grouped into 10 µg/ml bins. The red columns denote negative samples which could not be amplified at any locus, while the blue bars indicate samples which were positive at one or more loci (SSU rRNA, ITS1-5.8S-ITS2, bg, gdh and tpi).

For a better understanding of the correlation between the purity of the DNA and the PCR success, a second histogram was created (Figure 16). The ratio of DNA purity ranged from 0.8 to 2.5 for all samples. The majority of the PCR-positive samples had a ratio A_{260}/A_{280} around 2.0.

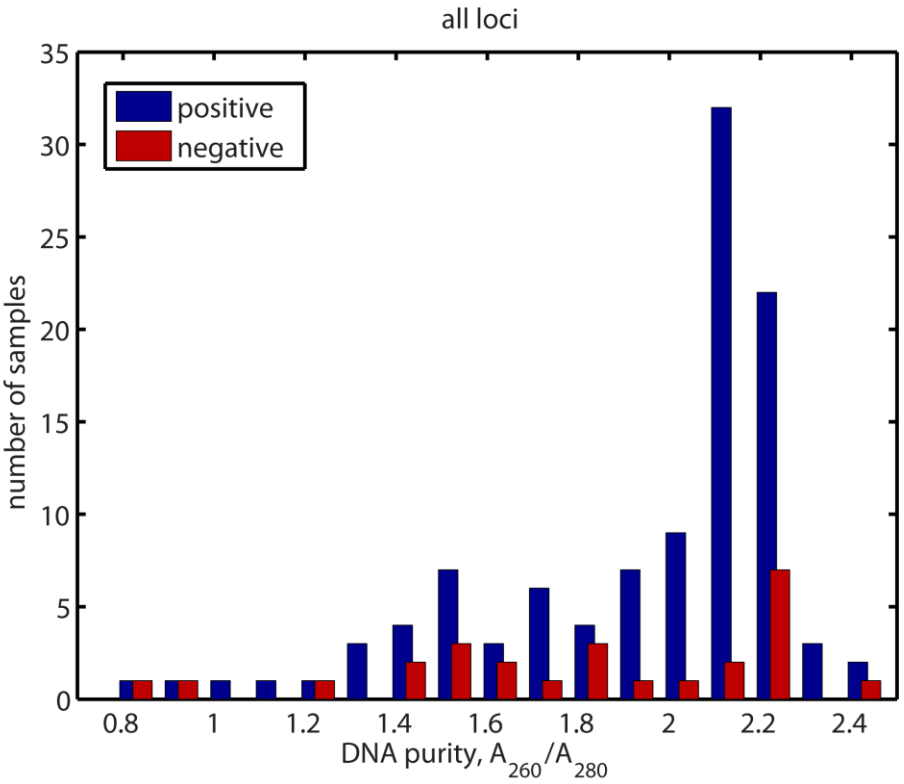

Figure 16: Histogram of DNA purity for 109 PCR-positive and 24 PCR-negative samples. The DNA purity is calculated by the ratio A_{260}/A_{280} and grouped into bins with 0.1 width. The red columns denote negative samples which could not be amplified at any locus, while the blue bars indicate samples which were positive at one or more loci (SSU rRNA, ITS1-5.8S-ITS2, bg, gdh and tpi).

V. DISCUSSION

In all considered countries from South Eastern Europe, at least 17.9 % of the canine faecal samples were positive for *G. duodenalis* with an overall prevalence of 33.7 %. In direct comparison of the obtained results to other studies it is important to consider that the prevalence for *Giardia* infections might be influenced by different factors like the detection method used, the quality of the material, as well as the age, the existence of clinical symptoms and the origin of the investigated canine population (Bouzid et al., 2015).

In the present study, a difference in the prevalence caused by different methods was observed in the samples from Serbia. They were primarily screened for *Giardia* cysts with microscopy and secondly with ELISA. By microscopy, *Giardia* cysts were detected in 57 of 134 samples (42.5 %) whereas 88 samples (65.7 %) were positive with ELISA. These results confirm the frequent observation that microscopy is less sensitive than coproantigen ELISA or IFA (Feng and Xiao, 2011; Geurden et al., 2012; Jarca et al., 2008; Maraha and Buiting, 2000). A study on prevalence and risk factors of *G. duodenalis* in dogs from Romania revealed a prevalence of 8.5 % for an infection with *Giardia* by microscopy and a prevalence of 34.6 % by ELISA (Mircean et al., 2012). Prevalence data obtained by coproantigen ELISA was thirty times higher (51.1 %) than prevalence data obtained by microscopy (1.6 %) in an investigation of canine faecal samples from Satu-Mare County, Romania (Jarca et al., 2008). On the one hand, those results might be explained by the fact that microscopy is a direct detection method for intermittently shed *Giardia* cysts in the faeces whereas the ELISA bases on the indirect detection of the coproantigen GSA 65 produced during the binary fission of trophozoites in the small intestine. Especially in cases of light infections, the cyst burden might be very small and cysts might not be found in every obtained faecal sample while the coproantigen is more likely to be present. On the other hand, microscopy requires an experienced examiner, particularly when cysts are destroyed or occur only sporadically in a sample. However, a very recent study on prevalence of *Giardia* species and other intestinal parasites in shelter dogs from Romania has revealed comparable results for microscopy and ELISA with 42.1 and 42.6 %, respectively (Sorescu et al., 2014). A possible explanation for this finding is that the majority (76.9 %) of the investigated dogs showed gastrointestinal symptoms such as diarrhoea, vomiting

and anorexia. Assumed clinical giardiosis might have been caused by high infection pressure resulting in a high cyst count in microscopy.

Besides the appearance of clinical symptoms, the prevalence might also be influenced by the way the investigated dogs were kept. In the present study, shelter dogs were significantly more often infected with *Giardia* (57.2 %) compared to household dogs (29.7 %; $p < 0.01$). This finding is in line with other studies on dogs living in crowded environments like shelters or kennels. In a comparison of infections with *Giardia* infections in dogs from Brazil, a significant difference ($p < 0.001$) was observed between household dogs (12.3 %) and shelter dogs (45.0 %) via microscopy (Huber et al., 2005). In an investigation of intestinal parasites in different dog populations from Belgium, 9.3 % of household dogs were positive for *Giardia* in the IFA compared to 43.9 % of infected kennel dogs (Claerebout et al., 2009). In a recently conducted study on canine giardiosis in Italy, 17.9 % of household dogs revealed *Giardia* cysts in the microscopic examination versus 35.8 % of positive kennel dogs (Pipia et al., 2014). A high prevalence for *Giardia* infections in shelter and kennel dogs might not only be caused by overcrowded living conditions but also by poor hygienic conditions leading to permanent reinfections of the animals (Itoh et al., 2015; Ortuño et al., 2014; Tangtrongsup and Scorza, 2010). Consequently, the treatment and a proper elimination of *G. duodenalis* in shelter and kennel dogs might be protracted and unsatisfactory (Beck and Arndt, 2014).

With respect to formerly published prevalence data from South Eastern Europe, the comparison is limited to three countries. For dogs from Hungary, the result of the present study (36.1 %) was lower than in previously conducted studies on canine *Giardia* infections from the same country with 51.1 % (ELISA) and 42.6 % (ELISA) (Jarca et al., 2008; Sorescu et al., 2014). Recent prevalence data for *Giardia* infections in dogs from Romania varied from 34.6 over 42.6 to 51.1 %, depending on the investigated dog population. In the present study, the overall prevalence for a mixture of shelter and household dogs was 36.1 %, which is similar to an investigation of Romanian kennel, shelter, shepherd and household dogs revealing *Giardia* infections in 34.6 % with ELISA (Mircean et al., 2012). The higher occurrence of *Giardia* infections (51.1 and 42.6 %) in two other studies might be explained by the fact that the majority of the dogs was either under two years of age or living in a dog shelter (Jarca et al., 2008; Sorescu et al.,

2014). Of all investigated countries, dogs from Serbia had the highest prevalence rate with 65.7 %, which differs from other publications from the same region (microscopy, 3.8–14.6 %) (Nikolić et al., 1993, 2002, 2008). However, in the other studies mixed dog populations of household, shelter and military working dogs were investigated whereas samples for the present study were obtained from two dog shelters, exclusively. No comparable studies on the occurrence of *Giardia* in canine faecal samples were found for Albania and Bulgaria. For Croatia, information on the distribution of canine *Giardia* assemblages has been gained but data on the general prevalence is still unavailable (Beck et al., 2012).

The obtained overall prevalence of 33.7 % in the present study is relatively high in comparison with prevalence studies conducted worldwide on canine *Giardia* infections. A limited number of studies have revealed a comparable prevalence of 37.8 % in hunting and shelter dogs and 31.3 % in household and shelter dogs both determined with microscopy (Huber et al., 2005; Ortuño et al., 2014). However, a consistent and valid comparison with the present study should rely on the same detection method, namely ELISA. Prevalence studies investigating *Giardia* isolates from symptomatic or asymptomatic household and shelter dogs were performed for instance in Asia, Europe and North America. Prevalence data obtained by ELISA ranged from 8.3 to 21.0 % (Barutzki and Schaper, 2003; Bianciardi et al., 2004; Carlin et al., 2006; Itoh et al., 2011; Olson et al., 2010; Overgaauw et al., 2009; Upjohn et al., 2010). The finding that most international studies show a lower prevalence for canine *Giardia* infections compared to the investigated South Eastern European countries might be explained by deviant husbandry conditions.

Multilocus sequence typing was performed for 133 canine samples with the intention to determine the canine *Giardia* assemblages of the investigated dog population. The SSU rRNA amplification success rate is in line with other studies in which 60.0 % to 95.9 % of the samples could be amplified at this conserved locus (Leonhard et al., 2007; McDowall et al., 2011; Pallant et al., 2015; Upjohn et al., 2010). Even though the SSU rRNA locus has limitations for gaining information at the subassemblage level, it is still very useful for the detection of mixed assemblages (Lebbad et al., 2010; Pallant et al., 2015) (Chapter II.1.1). The ITS1-5.8S-ITS2 region is less often investigated compared to the SSU rRNA, the bg, the gdh and the tpi loci. However, it is highly suitable for genotyping also with

regard to subassemblages due to its high level of polymorphism among *Giardia* isolates (Cacciò et al., 2010). Compared to the results of the present study, an amplification percentage of 58.0 % at this region was achieved in a previously conducted study on canine *Giardia* assemblages from Croatia (Beck et al., 2012). The bg, gdh and tpi genes, which are all characterised by a high intraassemblage discrimination capability were also included in the MLST protocol because they are suitable for genotyping *Giardia* assemblages and subassemblages of animals (Lebbad et al., 2010).

Regarding the amplification success of the bg locus in 12.8 % of the investigated samples, divergent results exist from previous studies. The amplification success rate at the bg locus ranged from 5.6 to 48.7 % in studies on the molecular characterisation of canine *Giardia* isolates from Arizona, Germany and Spain (Johansen, 2013; Ortuño et al., 2014; Pallant et al., 2015).

Regarding the amplification success rate at the gdh locus of 11.3 % of the investigated samples, comparable results exist in the current literature. In a study on the genetic characterisation of dogs from the USA, the gdh locus provided limited results with genotype information in 7.1 % whereas the amplification at the SSU rRNA locus was positive in 31.1 % (Covacin et al., 2011). Just recently, an amplification rate of 5.7 % was obtained in an investigation of household dogs from Germany (Pallant et al., 2015). A survey on canine *Giardia* genotypes from Croatia achieved higher amplification rates at all loci compared to the present study. However, in comparison with the other investigated loci, the amplification of the gdh locus was the least successful with 48.0 % (Beck et al., 2012). In the present study, the amplification of a fragment of the tpi gene locus was successful in 1.5 % of the canine samples. The number of equivalent studies using the same tpi primers for an investigation of canine *Giardia* isolates is limited. Beck at al. (2012) were able to amplify 64.5 % of the investigated samples at the tpi locus. In the latter study, additional assemblage D specific tpi primers were utilised for the second amplification following the same PCR conditions as for the nested PCR with conventional tpi primers. Positive results were obtained in 55.0 % of the samples. In an investigation of canine *Giardia* assemblages from Spain, the same assemblage D specific tpi primers doubled the percentage of positive samples (Ortuño et al., 2014). Possibly, the genotyping results of protein coding targets might vary by PCR assay, due to the fact that some sets of oligonucleotide

primers might amplify some assemblages preferentially (Cacciò and Ryan, 2008). The finding that only two of 133 isolates were amplified at the tpi locus in the present study might be explained be the assumption that primers from Sulaiman et al. (2003) are not specific for the amplification of assemblage D which was detected in the majority of the samples (Scorza et al., 2012). Although the SSU rRNA, ITS1-5.8S-ITS2, bg and gdh primers are supposed to detect all *Giardia* assemblages, amplification failure for some samples might occur due to mismatches in the binding regions of the primers (Beck et al., 2012).

The quantity of the DNA might also influence the PCR outcome. Low numbers of cysts in the investigated samples could be a possible reason for amplification failure (Paz e Silva et al., 2012). In order to avoid PCR failure due to the absence of *Giardia* cysts and subsequently *Giardia* DNA, IFA or MICF were performed additionally to the ELISA in the present study. As a result, samples containing *Giardia* cysts were selected for genotyping, exclusively. Despite that, some samples revealed a high cyst-count in the IFA or the MIFC and a DNA concentration of at least 50 µg/ml but could not be amplified at any gene locus. On average, PCR-positive samples contained about 40 % more DNA compared to PCR-negative samples. However, contamination of the DNA samples and other DNA sources besides *Giardia* might influence the measurement of the DNA content of faecal samples.

Besides the quantity of the DNA, the quality of the DNA contributes to the outcome of the PCR. The mean value for the purity of the DNA obtained by Nanodrop™ ND 1000-Spectrometer was 1.96 and the standard deviation was 0.4. Thus, samples with a high DNA concentration might have been negative in the PCR amplifications due to inadequate DNA purity values. The quality of the investigated DNA might have been reduced by freezing after collection, shipment, storage at –20° C for months or years, thawing and refreezing. Meanwhile, the proposition that the PCR outcome might be better with freshly extracted DNA from unfrozen faecal samples has been proven wrong in some investigations (Pallant et al., 2015).

The sequencing results of the amplified PCR products of all five gene loci revealed the exclusive presence of dog-specific *Giardia* assemblages in the investigated dog population. The predominance of assemblages C and D coincides with the results of the previously conducted surveys from South Eastern Europe.

In a study on the genotype distribution of *G. duodenalis* in Hungarian dogs, sequencing of products of the SSU rRNA PCR revealed assemblage C in 40.0 % and assemblage D in 66.7 % of the investigated kennel and household dogs (Szénási et al., 2007). In the investigation of bg, gdh and tpi loci and the ITS1-5.8S-ITS2 region, the majority of canine samples (59.4 %) from Croatia contained at least one of the dog-specific assemblages C or D (Beck et al., 2012). Unlike the results of the present study, the zoonotic assemblages A or B were also found (16.7 %). The simultaneous occurrence of zoonotic and species-specific assemblages at different loci underlined the importance of the MLST approach of the Croatian study since single locus PCR would have missed one of the two assemblages. The presence of two different assemblages within one sample might be due to a coexisting multiple infection or genetic recombination (Pallant et al., 2015).

In a global context, conflicting results exist for the distribution of *Giardia* assemblages in dogs. A number of studies investigating different gene loci have predominantly revealed the species-specific assemblages C and D whereas others mainly detected the zoonotic assemblages A and B. It is impossible to assign a distribution pattern of canine *Giardia* assemblages to particular regions of the world.

Within Europe, a just recently conducted MLST study on the *Giardia* genotypes of dogs from Germany revealed assemblage D in 56.1 % and assemblage C in 42.2 % by the investigation of SSU rRNA, bg and gdh loci (Pallant et al., 2015). The minority of the samples harboured zoonotic assemblages. In shelter dogs from England, mainly the assemblages C and D were detected by SSU rRNA and bg PCRs (Upjohn et al., 2010). Likewise, 63.0 % of a mixed dog population from Belgium was infected with *Giardia* assemblages C and D (Claerebout et al., 2009). The present study investigating canine samples from South Eastern Europe revealed a comparable distribution of assemblages at all gene loci.

An opposed distribution of *Giardia* assemblages on the same continent was observed in a study investigating *Giardia* isolates from German dogs (Leonhard et al., 2007). Almost two thirds of the isolates harboured the zoonotic *Giardia* assemblage A at the SSU rRNA and gdh loci whereas assemblages C and D were only detected in 12.7 %. Similarly, a genotyping study from Spain revealed mainly zoonotic assemblages in the examined dogs at the bg and gdh loci (Dado

et al., 2012).

The same comparison of the canine *Giardia* assemblage distribution can be drawn for American countries. An investigation of household dogs originating from the USA exhibited the canine assemblages C or D at the SSU rRNA and bg loci in all samples (Johansen, 2013). Accordingly, the majority of kennel and shelter dogs from Trinidad and Tobago revealed host-specific assemblages C and D in a study targeting the SSU rRNA locus (Mark-Carew et al., 2013). An MLST study evaluating the zoonotic potential of *Giardia* from dogs and cats in Ontario, Canada detected assemblages C and D in almost 100 % of the samples at the SSU rRNA, bg, gdh and tpi loci (McDowall et al., 2011). The very same distribution of assemblages C and D was observed in a molecular characterisation of *Giardia* at the SSU rRNA, bg and gdh loci in dogs from Brazil (Paz e Silva et al., 2012).

In contrast, another publication from the USA has stated the predominant detection of the zoonotic *Giardia* assemblages A and B (69.0 %) in canine samples at the SSU rRNA, bg and gdh loci (Covacin et al., 2011).

Various theories exist regarding the distribution and occurrence of host-adapted and zoonotic assemblages within different dog populations. On the one hand, there is the hypothesis that the friendly nature of well-socialised household dogs facilitates an increased close contact of dogs amongst each other during an encounter in public areas leading to a distribution of dog-specific assemblages C and D (Wang et al., 2012). On the other hand, close contact of owners with their household dogs is assumed to promote canine *Giardia* infections with human assemblages A and B (Claerebout et al., 2009). Correspondingly, shelter or kennel dogs which are living in close contact with their conspecifics are supposed to distribute dog-specific assemblages C and D among each other (Simonato et al., 2015; Uehlinger et al., 2013). According to this estimation, zoonotic assemblages A and B might be outcompeted by dog-specific assemblages C and D in the future (Cooper et al., 2010; Thompson et al., 1996). To date, conflicting results of genotyping studies prevent a clear understanding of the distribution of assemblages within different dog populations. Some household dogs harbour zoonotic assemblages (Claerebout et al., 2009; Eligio-García et al., 2008; Lalle et al., 2005a; Traub et al., 2004) whereas other dogs with the same origin carry infections with dog-specific assemblages only (Johansen, 2013; McDowall et al., 2011; Pallant et al., 2015; Paz e Silva et al., 2012). Concurrently, shelter or kennel

dogs might be infected with zoonotic *Giardia* assemblages (Dado et al., 2012) or dog-specific assemblages (Mark-Carew et al., 2013; Ortuño et al., 2014; Upjohn et al., 2010). In the present study, both shelter and household dogs harboured assemblages C and D.

Sequences obtained from genotyping of the bg, gdh and tpi loci were translated into their amino acid codon in order to gain information on the impact of the nucleotide substitutions detected in the alignment of the sequences (Chapter XII.11). As most *Giardia* genes do not contain introns, the determination of the amino acid codon frame of each of the consensus sequence alignments from the start codon of that gene was possible (Wielinga and Thompson, 2007). According to the results of the translation into amino acids, all nucleotide substitutions occurring within the dog-specific assemblages C and D were silent. The occurrence of unexpressed intraassemblage substitutions at the bg locus might rather be caused by the aging process of the gene than by changes in the gene function (Wielinga and Thompson, 2007). On the contrary, nucleotide substitutions detected between assemblages C and D resulted in a change of amino acid sequences as expected. Further investigation of the impact of nucleotide substitutions on the amino acid codon could provide valuable information for the classification of assemblages C and D into subassemblages. In order to find reasons for the extensive genetic heterogeneity of the protozoan parasite, the question whether *Giardia is* capable of sexual reproduction has been raised (Birky, 2010; Ramesh et al., 2005). Even though five genes with the capability to function during meiosis have been proven to be present in *Giardia*, the subject is currently still under debate.

VI. CONCLUSION

G. duodenalis should be considered as a common enteric parasite in dogs originating from Albania, Bulgaria, Croatia, Hungary, Macedonia, Romania and Serbia. The prevalence for a *Giardia* infection was significantly higher for dogs originating from shelters compared to dogs living in private households. Multilocus sequence typing (MLST) of five different gene loci revealed an overall amplification rate of 27.8 % with the highest success rate at the SSU rRNA locus (82.0 %). The importance of the application of an MLST approach was verified since some isolates showed different assemblages at different gene loci. This finding would have been missed by a single locus sequence typing approach. Sequencing revealed dog-specific assemblages C and D, exclusively. According to the results of the present study, there was no evidence for the presence of zoonotic assemblages in the investigated canine samples.

VII. SUMMARY

To date, worldwide investigations of *Giardia duodenalis* have contributed to a better understanding of the biology, pathogenesis, epidemiology and complex taxonomy of the protozoan parasite harbouring zoonotic potential. Modern genotyping tools like multilocus sequence typing (MLST) of different loci of the *Giardia* genome enable the discrimination of zoonotic assemblages A and B and non-zoonotic assemblages C to H of *Giardia*, which are species-specific. Nevertheless, numerous questions regarding the transmission cycles between infected animals and humans or vice versa remain unanswered. Since dogs serve humans as companion animals comprising close interaction between each other, the determination of the *Giardia* assemblages in dogs is of major importance in consideration of the possible zoonotic potential arising from canine *Giardia* infections.

The aims of the present study were to determine the *Giardia* assemblages of household and shelter dogs from seven South Eastern European countries via multilocus sequence typing (MLST) and to gain information on the occurrence of *Giardia* infections in the investigated dog populations from Albania, Bulgaria, Hungary, Macedonia, Romania and Serbia. For this reason, 1671 faecal samples were collected over a period of five years from 2010 to 2014. Enzyme-linked immunosorbent assay (ELISA) was utilised for the detection of *Giardia* infections for 1645 faecal samples. Additionally, a subset of samples containing *Giardia* coproantigen in the ELISA was further tested for the presence of *Giardia* cysts via merthiolate iodine formalin concentration (MIFC) or immunofluorescence assay (IFA). A total of 107 faecal samples demonstrating *Giardia* cysts in the MIFC or IFA and 26 IFA-positive samples from Croatia were selected for DNA extraction and subsequent MLST. Nested PCR protocols were used targeting five different genetic loci: the SSU rRNA, the ITS1-5.8S-ITS2, the beta giardin (bg), the glutamate dehydrogenase (gdh) and the triosephosphate isomerase (tpi).

According to the ELISA results, infections with *G. duodenalis* were present in 33.7 % of the investigated dogs.

In the present study, the prevalence was 35.5 % in Albania, 30.3 % in Bulgaria, 17.9 % in Hungary, 33.1 % in Macedonia, 36.1 % in Romania and 65.7 % in

Serbia. Shelter dogs were significantly more often infected with 57.2 % compared to 29.7 % for household dogs ($p < 0.01$). Most comparable internationally conducted studies using the same detection method have revealed a lower percentage of canine *Giardia* infections.

Positive PCR results were obtained in 82.0 % at the SSU rRNA locus, in 31.6 % at the ITS1-5.8S-ITS2 region, in 12.8 % at the bg locus, in 11.3 % at the gdh locus and in 1.5 % at the tpi locus. Sequencing of the PCR products revealed the dog-specific assemblage C in 50 samples and the dog-specific assemblage D in 68 samples. Zoonotic assemblages A and B were not detected in the investigated dog population. In nine isolates, the coexistence of two different assemblages within one sample at two different gene loci was found (‘assemblage swapping’).

In conclusion, *G. duodenalis* was present in dogs from all investigated South Eastern European countries. Since the MLST did neither detect *Giardia* assemblage A nor B, there was no evidence for the presence of a zoonotic potential arising from the investigated canine population.

VIII. ZUSAMMENFASSUNG

Bis heute haben weltweite Studien über *Giardia duodenalis* zu einem besseren Verständnis der Biologie, der Pathogenese, der Epidemiologie und vor allem auch der komplexen Taxonomie des protozoären Parasiten mit zoonotischem Potential beigetragen. Moderne Genotypisierungsmethoden wie die Sequenzbestimmung verschiedener Genloci (multilocus sequence typing, MLST) des Giardiengenoms ermöglichen es heutzutage, die zoonotischen Giardien Assemblages A und B von den nicht-zoonotischen, speziesspezifischen Giardien Assemblages C bis H zu unterscheiden. Dennoch sind auch weiterhin viele Fragen bezüglich des Übertragungszyklus zwischen infizierten Tieren und Menschen oder auch zwischen infizierten Menschen und Tieren ungeklärt. Es ist von besonderer Bedeutung, die Giardien Assemblages bei Hunden zu bestimmen, da sie als Begleittiere in engem Kontakt mit Menschen stehen und von ihnen möglicherweise ein zoonotisches Potential ausgeht.

Die vorliegende Studie hatte das Ziel, die Giardien Assemblages von Hunden aus privaten Haushalten und Tierheimen in sieben südosteuropäischen Ländern mittels MLST zu bestimmen und Informationen zum Vorkommen von Giardieninfektionen in den untersuchten Hundepopulationen zu gewinnen. Zu diesem Zweck wurden in Albanien, Bulgarien, Ungarn, Mazedonien, Rumänien und Serbien 1671 Kotproben über einen Zeitraum von fünf Jahren von 2010 bis 2014 gesammelt. Zum Nachweis von Giardieninfektionen in 1645 Kotproben wurde ein Antikörper basiertes Nachweisverfahren (Enzyme-linked immunosorbent assay, ELISA) verwendet. Ein Teil der ELISA-positiven Proben wurde entweder mittels der Merthiolat-Iodine-Formalin-Concentration Methode (merthiolate iodine formalin concentration, MIFC) oder mit einem Immunofluoreszenz Test (immunofluorescence assay, IFA) zusätzlich auf Giardien Zysten geprüft. Insgesamt 107 Kotproben, die in der MIFC oder im IFA Giardien Zysten aufwiesen und 26 zusätzliche IFA-positive Proben aus Kroatien wurden für die DNA-Extrahierung und anschließende MLST ausgewählt. Die folgenden fünf Genloci wurden mit verschiedenen nested PCR Protokollen untersucht: SSU rRNA, ITS1-5.8S-ITS2, Beta Giardin (bg), Glutamatdehydrogenase (gdh) und Triosephosphat Isomerase (tpi).

Mittels ELISA ließ sich bei 33,7 % der untersuchten Hunde eine Giardieninfektion nachweisen.

Im Rahmen dieser Studie wurden in den einzelnen Ländern die folgenden Prävalenzen festgestellt: 35,5 % in Albanien, 30,3 % in Bulgarien, 17,9 % in Ungarn, 33,1 % in Mazedonien, 36,1 % in Rumänien und 65,7 % in Serbien. In Tierheimen lebende Hunde waren mit 57,2 % signifikant häufiger infiziert als privat gehaltene Hunde mit 29,7 % ($p < 0,01$). Vergleichbare internationale Studien ergaben unter Verwendung gleicher Untersuchungsmethoden niedrigere Prävalenzen.

Positive PCR Ergebnisse konnten in 82,0 % am SSU rRNA Locus, in 31,6 % an der ITS1-5.8S-ITS2 Region, in 12,8 % am bg Locus, in 11,3 % am gdh Locus und in 1,5 % am tpi Locus erzielt werden. Die Sequenzierung der PCR Produkte ergab den hundespezifischen Assemblage C in 50 Proben und den hundespezifischen Assemblage D in 68 Proben. Die zoonotischen Assemblages A und B wurden in der untersuchten Hundepopulation nicht nachgewiesen. Neun Isolate enthielten an zwei verschiedenen Genloci jeweils zwei verschiedene Assemblages (‚assemblage swapping‘).

Zusammenfassend konnte *G. duodenalis* bei Hunden aus allen untersuchten südosteuropäischen Ländern nachgewiesen werden. Da in der Sequenz-bestimmung keine der zoonotischen Assemblages A oder B nachgewiesen wurden, gab es keinen Beweis dafür, dass von der untersuchten Hundepopulation ein zoonotisches Potential ausgeht.

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X. FIGURES

XI. TABLES

XII. ANNEX

1. Global prevalence data of *G. duodenalis*

Table A1: Overview of worldwide prevalence data of canine *G. duodenalis*

Country	Investigated dog population	Method	Positive/total (prevalence)	Reference
Europe				
Belgium	symptomatic, household, kennel	IFA	263/1159 (22.7 %)	(Claerebout et al., 2009)
England	symptomatic	microscopy	380/4526 (8.4 %)	(Batchelor et al., 2008)
	shelter	ELISA	184/878 (21.0 %)	(Upjohn et al., 2010)
Finland	household	IFA, ELISA	8/150 (5.3 %)	(Rimhanen-Finne et al., 2007)
Germany	household	MIFC	28/1281 (2.2 %)	(Epe et al., 2004)
	household	MIFC, ELISA	1393/8438 (16.5 %)	(Barutzki and Schaper, 2003)
Greece	herding, hunting	microscopy	12/281 (4.3 %)	(Papazahariadou et al., 2007)
Italy	kennel, household	microscopy	17/113 (15.0 %)	(Berrilli et al., 2004)
	household	ELISA	20/105 (19.0 %)	(Bianciardi et al., 2004)
	kennel	PCR	26/127 (20.5 %)	(Scaramozzino et al., 2009)
	kennel, household	microscopy	172/655 (26.3 %)	(Pipia et al., 2014)
	kennel	microscopy	48/318 (15.1 %)	(Simonato et al., 2015)
Netherlands	household	ELISA	14/152 (9.2 %)	(Overgaauw et al., 2009)
Norway	≤ 12 months	IFA	73/887 (8.2 %)	(Hamnes et al., 2007)
Portugal	household	microscopy	37/368 (10.1 %)	(Neves et al., 2014)

Spain	shelter	microscopy	18/1800 (1.0 %)	(Martinez-Carrasco et al., 2007)
	shelter	microscopy	82/1161 (7.1 %)	(Miro et al., 2007)
	shelter	microscopy	99/604 (16.4 %)	(Dado et al., 2012)
	shelter, hunting	microscopy	64/169 (37.9 %)	(Ortuño et al., 2014)
America				
Argentinia	household	microscopy	195/2193 (8.9 %)	(Fontanarrosa et al., 2006)
Brazil	household, stray, kennel	microscopy	52/300 (17.3 %)	(Paz e Silva et al., 2012)
	household, shelter	microscopy	52/166 (31.3 %)	(Huber et al., 2005)
	household, kennel, stray	microscopy	119/410 (29.0 %)	(Mundim et al., 2007)
	household	microscopy	43/254 (16.9 %)	(Katagiri and Oliveira-Sequeira, 2008)
	household, shelter	microscopy	33/200 (16.5 %)	(Meireles et al., 2008)
Canada	≤ 6 months	microscopy	11/9486 (0.1 %)	(Shukla et al., 2006)
	household, symptomatic	ELISA	241/1871 (12.9 %)	(Olson et al., 2010)
	> 1 year of age, shelter, petshop, household	IFA	61/209 (29.2 %)	(Uehlinger et al., 2013)
USA	household	microscopy	35172/519585 (6.8 %)	(Covacin et al., 2011)
	symptomatic, household	ELISA	2506/16064 (15.6 %)	(Carlin et al., 2006)
	household	microscopy	216/6555 (3.3 %)	(Gates and Nolan, 2009)
	household	IFA	9/129 (7.0 %)	(Wang et al., 2012)

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Asia				
Japan	kennel	ELISA	118/316 (37.3 %)	(Itoh et al., 2005)
	household	microscopy	137/1105 (12.4 %)	(Itoh et al., 2009)
	household	ELISA	196/2365 (8.3 %)	(Itoh et al., 2011)
	kennel	ELISA	147/573 (25.7 %)	(Itoh et al., 2015)
China	household, stray	PCR	12/267 (4.5 %)	(Li et al., 2015)
South Korea	household	ELISA	53/472 (11.2 %)	(Liu et al., 2008)
Thailand	Household	microscopy	18/229 (7.9 %)	(Inpankaew et al., 2007)
Australia	Household	microscopy	130/1400 (9.3 %)	(Palmer et al., 2008)

2. Frequently used genes for molecular typing of *G. duodenalis*

Table A2: Overview of frequently investigated genes and used primers for the genetic determination of *G. duodenalis*.

Gene	Function	Primer (5'-3')	references
SSU rRNA	Small subunit of the ribosome	RH11 CATCCGGTCGATCCTGCC RH4 AGTCGAACCCTGATTCTCCGCCAGG GiarF GACGCTCTCCCCAAGGAC GiarR CTGCGTCACGCTGCTCG	(Hopkins et al., 1997; Read et al., 2002)
		G18S2 TCCGGTYGATTCTGCC G18S3 CTGGAATTACCGCGGCTGCT	(Monis et al., 1999)
		Gia2029 AAGTGTGGTGCAGACGGACTC Gia2150c CTGCTGCCGTCCTTGGATGT RH11 CATCCGGTCGATCCTGCC RH4 AGTCGAACCCTGATTCTCCGCCAGG	(Appelbee et al., 2003)
		AL4303 ATCCGGTCGATCCTGCCG AL4305 AGGATCAGGGTTCGACT AL4304 CGGTCGATCCTGCCGGA AL4306 GGCGGAGGATCAGGGT	(Sulaiman et al., 2003)
ITS1-5.8S-ITS2	ribosomal	FW1 TGGAGGAAGGAGAAGTCGTAAC RV1 GGGCGTACTGATATGCTTAAGT FW2 AAGGTATCCGTAGGTGAACCTG RV2 ATATGCTTAAGTTCCGCCCGTC	(Cacciò et al., 2010), (Beck et al., 2012)
bg	structural protein	G7 AAGCCCGACGACCTCACCCGCAGTGC G759: GAGGCCGCCCTGGATCTTCGAGACGAC FW GAACGAACGAGATCGAGGTCCG RV CTCGACGAGCTTCGTGTT	(Lalle et al., 2005b)
gdh	housekeeping enzyme	GDH1 TTCCGTRTYCAGTACAACTC GDH2 ACCTCGTTCTGRGTGGCGCA GDH3 ATGACYGAGCTYCAGAGGCACGT GDH4 GTGGCGCARGGCATGATGCA	(Cacciò et al., 2008)
		GDHeF TCAACGTYAAYCGYGGYTTCCGT GDHiR GTTRTCCTTGCACATCTCC GDHiF CAGTACAACTCYGCTCTCGG	(Read et al., 2004)
		GDH1 ATCTTCGAGAGGATGTTGAG GDH4 ATGACGCGACGCTGGGATACT	(Homan et al., 1998)
tpi	housekeeping enzyme	AL3543 AAATIATGCCTGCTCGTCG AL3546 CAAACCTTITCCGCAAACC AL3544 CCCTTCATCGGIGGTAACTT AL3545 GTGGCCACCACICCCGTGCC	(Sulaiman et al., 2003)
		TPIGENF ATCGGYGGTAAYTTYAARTG TPIGENR CACTGGCCAAGYTTYTCRCA TPI16F CCCTTCATCGGYGGTAAC TPI533R CCCGTGCCRATRGACCACAC TPI572R ACRTGGACYTCCTCYGCYTGCTC	(Monis et al., 1999)

		TPIDF CCGTTCATAGGTGGCAACTT TPIDR GTAGCC ACTACA CCAGTTCC	(Lebbad et al., 2010)
		RTTPIF ATYAAGAGCCACGTRGCGKC RTTPIR CCATGATTCTRCGYCTTTCAG	(Traub et al., 2004)
ef-1	component of the trans-lational apparatus	EF1AR AGCTCYTCGTGRTGCATYTC GLONGF GCTCSTTCAAGTACGCGTGG GLONGR GCATCTCGACGGATTCSACC	(Monis et al., 1999)
		RTef1-aF GCCGAGGAGTTCGACTACATC RTef1-aR GACGCCSGAGATCTTGTAGAC	(Traub et al., 2004)

3. Nomenclature for incompletely specified bases in nucleic acid sequences

Table A3: Summary of single-letter code recommendations of the Nomenclature Committee of the International Union of Biochemistry (NC-IUB, http://www.chem.qmul.ac.uk/iubmb/misc/naseq.html)

Symbol	description	Bases represented			
A	**a**denosine	A			
C	**c**ytidine		C		
G	**g**uanosine			G	
T	**t**hymidine				T
U	**u**ridine				U
W	**w**eak	A			T
S	**s**trong		C	G	
M	a**m**ino	A	C		
K	**k**eto			G	T
R	pu**r**ine	A		G	
Y	p**y**rimidine		C		T
B	not A (**B** comes after A)		C	G	T
D	not C (**D** comes after C)	A		G	T
H	not G (**H** comes after G)	A	C		T
V	not T (**V** comes after T and U)	A	C	G	
N	a**N**y base (not a gap), primer mixture	A	C	G	T

4. Sequence comparison with GenBank

Table A4: GenBank numbers of isolates used for a comparison of obtained sequences. C = assemblage C, D = assemblage D.

SSU rRNA		ITS1-5.8S-ITS2		bg		gdh		tpi	
C	AB569372	D	JN603692	C	JN416552	C	JN587394	C	AY228641
D	AF199443			D	EF455598	D	JN587398		
				D	HM061152				

5. Combined genotyping results

Table A5: Overview of genotyping results of all five loci. The table shows all isolates with interpretable sequencing results. The assemblages at each locus are denoted as capital letters C or D.

number of loci	SSU rRNA	ITS1-5.8S-ITS2	bg	gdh	tpi	number of samples	
four	D	D	D	D		1	2
	C		C	C	C	1	
three	D	D	C			1	4
	D	D	D			1	
	D	D		D		1	
	C		C	C		1	
two	C	D				6	37
	D	D				26	
	C		C			1	
	C			D		2	
	D			D		1	
	C				C	1	
one	C					36	66
	D					25	
		D				4	
			C			1	
total	104	40	7	7	2	109	109

6. Equipment

ELISA-reader	Deelux Labortechnik GmbH, Gödenstorf, Germany
Nanodrop™ ND 1000-Spectrometer	Peqlab, Erlangen, Germany

Thermocycler Mastercycler gradient	Eppendorf, Hamburg, Germany
Veriti® Thermal Cycler	Applied Biosystems®, Darmstadt, Germany
GeneAmp® PCR System 2700	Applied Biosystems®, Darmstadt, Germany
ProFlex™ PCR System	Life Technologies, Carlsbad, USA
Gel chambers in different sizes	Peqlab, Erlangen, Germany
Gel documentation system (UV-Light)	Peqlab, Erlangen, Germany
QIAxcel® Advanced System	Qiagen, Hilden, Germany

7. Kits

ProSpecT™ *Giardia* Microplate Assay	Sekisui Virotech, Rüsselsheim, Germany
ELISA Merifluor *Cryptosporidium*/ *Giardia*	Meridian Bioscience, Luckenwalde, Germany
QIAamp DNA Stool Mini Kit	Qiagen, Hilden, Germany
QIAquick PCR Purification kit	Qiagen, Hilden, Germany
GoTaq Green Mastermix	Promega, Madison, USA
QIAxcel DNA Screening kit (2400)	Qiagen, Hilden, Germany
ExoSAP-IT® PCR Clean-Up Reagent	USB, Cleveland, USA

8. Chemicals

MIFC-solution without thiomersal	Pharmacy of the clinical centre of the LMU, Munich, Germany
37 % formaldehyde	Roth, Karlsruhe, Germany
Glycerine	Merck Millipore, Darmstadt, Germany
H_2O of the reverse osmosis system	Millipore GmbH, Schwalbach, Germany
99.5 % diethyl ether	Roth, Karlsruhe, Germany
1 % Lugols's iodine	Roth, Karlsruhe, Germany
Microbiological H_2O	Sigma-Aldrich Chemistry, Munich, Germany
Dimethyl sulfoxide (DMSO)	Roth, Karlsruhe, Germany
Ultrapure Bovine Serum Albumin (BSA), non-acetylated	Roth, Karlsruhe, Germany
Ethanol, denatured	Roth, Karlsruhe, Germany

Sodium acetate buffer	Sigma-Aldrich Chemistry, Munich, Germany

9. Nucleotides and primers

RH11, RH4	Eurofins MWG Operon, Ebersberg, Germany
GiarF, GiarR	Eurofins MWG Operon, Ebersberg, Germany
FW1, RV1	Eurofins MWG Operon, Ebersberg, Germany
FW2, RV2	Eurofins MWG Operon, Ebersberg, Germany
G7, G759	Eurofins MWG Operon, Ebersberg, Germany
FW, RV	Eurofins MWG Operon, Ebersberg, Germany
GDH1, GDH2	Eurofins MWG Operon, Ebersberg, Germany
GDH3, GDH4	Eurofins MWG Operon, Ebersberg, Germany
AL3543, AL3546	Eurofins MWG Operon, Ebersberg, Germany
AL3544, AL3545	Eurofins MWG Operon, Ebersberg, Germany

10. Buffer and solution for agarose gel electrophoresis

Top Vision Agarose	Fermentas, St. Leon-Rot, Germany
TAE buffer 50×	Qiagen, Hilden, Germany
TBE buffer 10×	Fermantas, St. Leon-Rot, Germany
Gel Red™ Nucleid Acid stain, 10,000× in water	Biotium, Hayward, USA
Gene Ruler 100bp Plus DNA ladder	Fermantas, St. Leon-Rot, Germany

11. Sequencing Data

11.1. SSU rRNA sequence comparison of *G. duodenalis*

11.1.1. Alignment of nucleotide sequences

AB569372: reference sequence *Giardia* assemblage C

AF199443: reference sequence *Giardia* assemblage D

KP258238-KP258341: sequences obtained in the present work

C: *Giardia* assemblage C

D: *Giardia* assemblage D

Nucleotides with black frame: mark for interassemblage substitution

Nucleotides with yellow frame: mark for intraassemblage substitution

```
C  1: AB569372, KP258250, KP258251, KP258252, KP258253, KP258254, KP258255,
      KP258256, KP258267, KP258272, KP258278, KP258279, KP258281, KP258282,
      KP258285, KP258286, KP258288, KP258290, KP258292, KP258293, KP258295,
      KP258299, KP258301, KP258305, KP258306, KP258307, KP258310, KP258311,
      KP258312, KP258315, KP258320, KP258321, KP258323, KP258324, KP258325,
      KP258326, KP258327, KP258328, KP258329, KP258332, KP258333, KP258336,
      KP258337, KP258338, KP258339, KP258341
   2: KP258271
   3: KP258334
   4: KP258264
D  5: AF199443, KP258238, KP258239, KP258240, KP258241, KP258242, KP258243,
      KP258244, KP258245, KP258246, KP258247, KP258248, KP258249, KP258257,
      KP258258, KP258259, KP258260, KP258261, KP258262, KP258263, KP258265,
      KP258266, KP258268, KP258269, KP258270, KP258273, KP258274, KP258275,
      KP258276, KP258277, KP258280, KP258283, KP258284, KP258287, KP258289,
      KP258291, KP258294, KP258296, KP258297, KP258298, KP258300, KP258302,
      KP258303, KP258304, KP258308, KP258309, KP258314, KP258316, KP258317,
      KP258318, KP258319, KP258322, KP258330, KP258331, KP258335, KP258340
   7: KP258313
```

```
1      ACAAGCCATGCATGCCCGCACACCCGGGAGGCGGCGGACGGCTCAGGACAACGGTTGCAC 60
2      ACAAGCCATGCATGCCCGCACACCCGGGAGGCGGCGGACGGCTCAGGACAACGGTTGCAC 60
3      ACAAGCCATGCATGCCCGCACACCCGGGAGGCGGCGGACGGCTCAGGACAACGGTTGCAC 60
4      ACAAGCCATGCATGCCCGCACACCCGGGAGGCGGCGGACGGCTCAGGACAACGGTTGCAC 60
5      ACAAGCCATGCATGCCCGCACACCCGGGAAGCGGCGGACGGCTCAGGACAACGGTTGCAC 60
6      ACAAGCCATGCATGCCCGCACACCCGGGAAGCGGCGGACGGCTCAGGACAACGGTTGCAC 60
       ****************************** ******************************

1      CCCCCGCGGCGGTCCCTGCTAGCCGGACACCGCTGGCAACCCGGCGCCAAGACGTGCGCG 120
2      CACCCGCGGCGGTCCCTGCTAGCCGGACACCGCTGGCAACCCGGCGCCAAGACGTGCGCG 120
3      CCCCCGCGGCGGTCCCTGCTAGCCGGACACCGCTGACAACCCGGCGCCAAGACGTGCGCG 120
4      CCCTCGCGGCGGTCCCTGCTAGCCGGACACCGCTGGCAACCCGGCGCCAAGACGTGCGCG 120
5      CCCCCGCGGCGGTCCCTGCTAGCCGGACACCGCTGGCAACCCGGCGCCAAGACGTGCGCG 120
6      CCCCCGCGGCGGTCCCTGCTAGCCGGACACCGCTGGCAACCCGGCGCCAAGACGTGCGCG 120
       * * ****************************** ************************

1      CAAGTGCGGGCGCCCGCGGG 140
2      CAAGTGCGGGCGCCCGCGGG 140
3      CAAGTGCGGGCGCCCGCGGG 140
4      CAAGTGCGGGCGCCCGCGGG 140
5      CAAGTGCGGACGCCCGCGGG 140
6      CAAGTGCGGACGCCCGCGAG 140
       ********* ******** *
```

11.2. ITS1-5.8S-ITS2 sequence comparison of *G. duodenalis*

11.2.1. Alignment of nucleotide sequences

JN603692: reference sequence *Giardia* assemblage D

KP258356-KP258395: sequences obtained in the present work

D: *Giardia* assemblage D

Nucleotides with yellow frame: mark for intraassemblage substitution

```
    ┌ 1: JN603692, KP258356, KP258357, KP258358, KP258359, KP258360, KP258361,
    │    KP258363, KP258364, KP258365, KP258366, KP258367, KP258368, KP258369,
    │    KP258370, KP258371, KP258372, KP258373, KP258374, KP258375, KP258376,
    │    KP258377, KP258378, KP258379, KP258380, KP258381, KP258382, KP258384,
    │    KP258385, KP258386, KP258387, KP258390, KP258391, KP258392, KP258394,
D   ┤    KP258395
    │  2: KP258393
    │  3: KP258388
    │  4: KP258383
    │  5: KP258362
    └  6: KP258389

1     CGGATGGATCCCTCGCGTGCCCCGCGTGTCGCCCCCGCGGCCCGGTCGGCGAGAGAGCCC 60
2     CGGATGGATCCCTCGCGTGCCCCGCGTGTCGCCCCCGCGGCCCGGTCGGCGAGAGAGCCC 60
3     CGGATGGATCCCTCGCGTGCCCCGCGTGTCGCCCCCGCGGCCCGGTCGGCGAGAGAGCCC 60
4     CGGATGGATCCCTCGCGTGCCCCGCGTGTCGCCCCCGCGGCCCGGTCGGCGAGAGAGCCC 60
5     CGGATGGATCCCTCGCGTGCCCCGCGTGTCGCCCCCGCGGCCCGGTCGGCGAGAGAGCCC 60
6     CGGATGGATCCCTCGCGTGCCCCGCGTGTCGCCCCTGCGGCCCGGTCGGCGAGAGAGCCC 60
      ********************************** ************************

1     CGCGCCGGCGGATGCCTCGGCCCGGGTGTCGACGAAGAGCGCGGCGGAGCGCGAGACGCG 120
2     CGCGCCGGCGGATGCCTCGGCCCGGGTGTCGACGAAGAGCGCGGCGGAGCGCGAGACGCG 120
3     CGCGCCGGCGGATGCCTCGGCCCGGGTTTCGACGAAGAGCGCGGCGGAGCGCGAGACGCG 120
4     CGCGCCGGCGGATGCCTCGGCCCGGGTGTCGACGAAGAGCGCGGCGGAGCGCGAGACGCG 120
5     CGCGCCGGCGGATGCCTCGGCCCGGGTGTCGACGAAGAGCGCGGCGGAGCGCGAGACGCG 120
6     CGCGCCGGCGGATGCCTCGGCCCGGGTGTCGACGAAGAGCGCGGCGGAGCGCGAGACGCG 120
      *************************** ********************************

1     GTGCGGACCCGCACGCCCCGAGAAGCACCGACCCTCGAACGCAGCGCGCCACGGCGCCGC 180
2     GTGCGGACCCGCACGCCCCGAGAAGCACCGACCCTCGAACGCAGCGCGCCACGGCGCCGC 180
3     GTGCGGACCCGCACGCCCCGAGAAGCACCGACCCTCGAACGCAGCGCGCCACGGCGCCGC 180
4     GTGCGGACCCGCACGCCCCGAGAAGCACCGACCCTCGAACGCAGCGCGCCACGGCGCCGC 180
5     GTGCGGACCCGCACGCCCCGAGAAGCACCGACCCTCGAACGCAGCGCGCCACGGCGCCGC 180
6     GTGCGGACCCGCACGCCCCGAGAAGCACCGACCCTCGAACGCAGCGCGCCACGGCGCCGC 180
      ************************************************************

1     CGCCTCGGAGCCGGCCGTGTGCCGCGCGCCGCGCCGCAGAGAGAGCCCCGCGGCGGTCCT 240
2     CGCCTCGGAGCCGGCCGTGTGCCGCGCGCCGCGCCGCAGAGAGAGCCCCGCGGCGGTCCT 240
3     CGCCTCGGAGCCGGCCGTGTGCCGCGCGCCGCGCCGCAGAGAGAGCCCCGCGGCGGTCCT 240
4     CGCCTCGGAGCCAGCCGTGTGCCGCGCGCCGCGCCGCAGAGAGAGCCCCGCGGCGGTCCT 240
5     CGCCTCGGAGCCGGCGGTGTGCCGCGCGCCGCGCCGCAGAGAGAGCCCCGCGGCGGTCCT 240
6     CGCCTCGGAGCCGGCCGTGTGCCGCGCGCCGCGCCGCAGAGAGAGCCCCGCGGCGGTCCT 240
      ************ ** ******************************************

1     GCCGGGATGCGCGGCCCGAGGCGGCGGGGAC 271
2     GCCGGGATGCGCGACCCGAGGCGGCGGGGAC 271
3     GCCGGGATGCGCGGCCCGAGGCGGCGGGGAC 271
4     GCCGGGATGCGCGGCCCGAGGCGGCGGGGAC 271
5     GCCGGGATGCGCGGCCCGAGGCGGCGGGGAC 271
6     GCCGGGATGCGTGGCCCGAGGCGGCGGGGAC 271
      *********** * *****************
```

11.3. Beta giardin sequence comparison of *G. duodenalis*

11.3.1. Alignment of nucleotide sequences

JN416552: reference sequence for *Giardia* assemblage C from GenBank

EF455598 and HM061152: reference sequences for *Giardia* assemblage D from GenBank

KP258342-KP258348: sequences obtained in the present work

C: *Giardia* assemblage C

D: *Giardia* assemblage D

Nucleotides with black frame: mark for interassemblage substitution

Nucleotides with yellow frame: mark for intraassemblage substitution

```
    ⌈1: JN416552, KP258342
  C ⊣2: KP258344
    ⌊3: KP258345, KP258347, KP258348
    ⌈4: EF455598, KP258343
  D ⊣5: HM061152, KP258346
    ⌊

1     CCGCGTCGACGACGACACGCGCGTCAAGATGATCAAGGACGCCATCGCTCACCTGGACAG 60
2     CCGCGTCGACGACGACACGCGCGTCAAGATGATCAAGGACGCCATCGCTCACCTGGACAG 60
3     CCGCGTCGACGACGACACGCGCGTCAAGATGATCAAGGACGCCATCGCTCACCTGGACAG 60
4     CCGCGTCGACGATGACACACGTGTCAAGATGATCAAGGATGCCATCGCACACCTTGACAG 60
5     CCGCGTCGACGATGACACGCGTGTCAAGATGATCAAGGATGCCATCGCACACCTTGACAG 60
      ************ ***** ** ***************** ******** ***** *****

1     GCTCATCCAGACCGAGTCGAGGAAGCGCCAGGGCTCGTTCGAGGACATCCGCGAGGAGGT 120
2     GCTCATCCAGACCGAGTCGAGGAAGCGCCAGGGCTCGTTCGAGGACATCCGCGAGGAGGT 120
3     GCTCATCCAGACCGAGTCGAGGAAGCGCCAGGGCTCGTTCGAGGACATCCGCGAGGAGGT 120
4     GCTCATTCAGACGGAGTCGAGGAAGCGCCAGAGCTCATTCGAGGACATCCGCGAGGAGGT 120
5     GCTCATTCAGACGGAGTCGAGGAAGCGCCAAAGCTCCTTCGAGGACATCCGCGAGGAGGT 120
      ****** ***** ***************** **** ************************

1     CAAGAAGTCCGCCGACAACATGTACCTGACGATCAAGGAGGAAATCGACACCATGGCCGC 180
2     CAAGAAGTCCGCCGACAACATGTACCTGACGATCAAGGAGGAAATCGACACCATGGCCGC 180
3     GAAGAAGTCCGCCGACAACATGTACCTGACGATCAAGGAGGAAATCGACACCATGGCCGC 180
4     AAAGAAGTCCGCTGACAACATGTATCTGACGATCAAGGAGGAGATTGACACAATGGCCGC 180
5     AAAGAAGTCCGCTGACAACATGTATCTGACGATCAAGGAGGAGATTGACACAATGGCCGC 180
       *********** *********** ***************** ** ***** ********

1     GAACTTCCGCAAGTCCCTTGCCGAGATGGGCGAGACCCTCAACAACGTCGAGACAAACCT 240
2     GAACTTCCGCAAGTCCCTTGCCGAAATGGGCGAGACCCTCAACAACGTCGAGACAAACCT 240
3     GAACTTCCGCAAGTCCCTTGCCGAGATGGGCGAGACCCTCAACAACGTCGAGACAAACCT 240
4     AAACTTCCGCAAGTCCCTCGCAGAGATGGGCGAGACGCTCAACAACGTCGAGACAAACCT 240
5     AAACTTCCGCAAGTCCCTCGCAGAGATGGGCGAGACGCTCAACAACGTCGAGACAAACCT 240
       ***************** ** ** *********** ************************

1     CCAGAACCAGATCGCCATCCACAACGACGCCATCGCGGCCCTCAGGAAGGAGGCCCTCAA 300
2     CCAGAACCAGATCGCCATCCACAACGACGCCATCGCGGCCCTCAGGAAGGAGGCCCTCAA 300
3     CCAGAACCAGATCGCCATCCACAACGACGCCATCGCGGCCCTCAGGAAGGAGGCCCTCAA 300
4     CCAGAACCAGATCGCCATCCACAACGACGCCATCGCAGCTCTCAGGAAGGAGGCCCTCAA 300
5     CCAGAACCAGATCGCCATCCACAACGACGCCATCGCAGCTCTCAGGAAGGAGGCCCTCAA 300
      ********************************** ** ********************

1     GAGCCTGAACGACCTCGAGACCGGCATCGCCACGGAGAACGCCGAGAGGAAGAAGATGTA 360
2     GAGCCTGAACGACCTCGAGACCGGCATCGCCACGGAGAACGCCGAGAGGAAGAAGATGTA 360
3     GAGCCTGAACGACCTCGAGACCGGCATCGCCACGGAGAACGCCGAGAGGAAGAAGATGTA 360
4     GAGCCTGAACGACCTTGAGACCGGCATCGCTACGGAGAACGCCGAGAGGAAGAAGATGTA 360
5     GAGCCTGAACGACCTTGAGACCGGCATCGCTACGGAGAACGCCGAGAGGAAGAAGATGTA 360
      *************** ************** ****************************

1     CGACCAGCTCAACGAGAAGGTCGCAGAGGGATTCGCCCGCATCTCCGCCGCCATCGAGAA 420
2     CGACCAGCTCAACGAGAAGGTCGCAGAGGGATTCGCCCGCATCTCCGCCGCCATCGAGAA 420
3     CGACCAGCTCAACGAGAAGGTCGCAGAGGGATTCGCCCGCATCTCCGCCGCCATCGAGAA 420
4     CGACCAGCTCAACGAGAAGGTCGCAGAGGGATTCGCCCGTATTTCCGCTGCCATCGAGAA 420
5     CGACCAGCTCAACGAGAAGGTCGCAGAGGGATTCGCCCGTATTTCCGCTGCCATCGAGAA 420
      ************************************** ** ***** ***********

1     GGAGACGATCGCCCGCGAGAGGGCCGTCAGCGCAGCCACGACCGAGGCGCTCACA 475
2     GGAGACGATCGCCCGCGAGAGGGCCGTCAGCGCAGCCACGACCGAGGCGCTCACA 475
3     GGAGACGATCGCCCGCGAGAGGGCCGTCAGCGCAGCCACGACCGAGGCGCTCACA 475
4     GGAGACGATCGCCCGCGAGAGAGCCGTCAGCGCAGCCACAACAGAGGCTCTCACA 475
5     GGAGACGATCGCCCGCGAGAGAGCCGTCAGCGCAGCCACAACAGAGGCTCTCACA 475
      ********************* ***************** ** ***** ******
```

11.3.2. Alignment of amino acids

```
1+2+3   RVDDDTRVKMIKDAIAHLDRLIQTESRKRQGSFEDIREEVKKSADNMYLTIKEEIDTMAA 60
4+4     RVDDDTRVKMIKDAIAHLDRLIQTESRKRQSSFEDIREEVKKSADNMYLTIKEEIDTMAA 60
        ****************************.****************************

1+2+3   NFRKSLAEMGETLNNVETNLQNQIAIHNDAIAALRKEALKSLNDLETGIATENAERKKMY 120
4+5     NFRKSLAEMGETLNNVETNLQNQIAIHNDAIAALRKEALKSLNDLETGIATENAERKKMY 120
        ************************************************************

1+2+3   DQLNEKVAEGFARISAAIEKETIARERAVSAATTEALT 158
4+5     DQLNEKVAEGFARISAAIEKETIARERAVSAATTEALT 158
        ************************************
```

11.4. Glutamate dehydrogenase sequence comparison of *G. duodenalis*

11.4.1. Alignment of nucleotide sequences

JN587394: reference sequences for assemblage C from GenBank

JN587398: reference sequences for assemblage D from GenBank

KP258349-KP258355: sequences obtained in the present work

C: *Giardia* assemblage C

D: *Giardia* assemblage D

Nucleotides with black frame: mark for interassemblage substitution

Nucleotides with yellow frame: mark for intraassemblage substitution

```
C   1: JN587394, KP258349, KP258350
D  ┌2: JN587398, KP258351, KP258352, KP258353, KP258354
   └3: KP258355

1      CGGCGCTGACACCGACGTTCCTGCTGGCGACATTGGTGTCGGCGGTCGCGAGATCGGCTA 60
2      CGGCGCTGACACTGACGTTCCTGCTGGTGACATTGGCGTCGGAGCCCGCGAGATCGGTTA 60
3      CGGCGCTGACACTGACGTTCCTGCTGGTGACATTGGCGTCGGAGCCCGCGAGATCGGTTA 60
       ************ ************** ******** ***** ** *********** **

1      CCTGTTTGGGCAGTACAAGCGCCTCAGGAACGAGTTCACAGGGGTCCTCACTGGTAAGAA 120
2      CCTGTTTGGCCAGTACAAGCGCCTCAGGAACGAGTTCACAGGAGTTCTCACTGGCAAGAA 120
3      CCTGTTTGGCCAGTACAAGCGCCTCAGGAACGAGTTCACAGGAGTTCTCACTGGCAAGAA 120
       ********* ********************************** ** ******** *****

1      CGTCAAGTGGGGCGGTTCCCTCATCAGGCCAGAGGCCACCGGATATGGCGCTGTCTACTT 180
2      CATCAAGTGGGGCGGATCCCTCATCAGGCCAGAGGCCACGGGCTATGGAGCCGTCTACTT 180
3      CATCAAGTGGGGCGGATCCCTCATCAGGCCAGAGGCCACGGGCTATGGAGCCGTCTACTT 180
       * ************* ************************ ** ***** ** ********

1      CCTCGAGGAGATGTGCAAGGACAACAACACCATAATCAGGGGTAAGAACGTCCTCCTCTC 240
2      CCTTGAGGAGATGTGCAAGGACAACAACACCATAATCAGGGGCAAGAACGTCCTGCTCTC 240
3      CCTTGAGGAGATGTGCAAGGACAACAACACCATAATCAGGGGCAAGAACGTCCTGCTCTC 240
       *** ************************************ *********** *****

1      CGGGTCCGGCAACGTTGCCCAGTTCGCGTGCGAGAAGCTCATCCAGCTCGGCGCAAAGGT 300
2      TGGTTCTGGAAACGTCGCTCAATTCGCGTGCGAGAAACTCCTTCAGCTAGGCGCAAAAGT 300
3      TGGTTCTGGAAACGTCGCTCAATTCGCGTGCGAGAAACTCCTTCAGCTAGGCGCAAAAGT 300
        ** ** ** ***** ** ** ************** *** * ***** ******** **

1      CCTCACCTTCTCTGACTCCAACGGAACCATCGTCGACAAGGATGGCTTCAACGAGGAGAA 360
2      GCTTACATTCTCTGACTCTAACGGAACCATCGTCGATAAGGATGGCTTCAACGAGGAGAA 360
3      GCTTACATTCTCTGACTCTAACGGAACCATCGTCGATA-GGATGGCTTCAACGAGGAGAA 359
```

```
        ** ** ********** ***************** * ********************

1       GCTTGCCCACATCAAGTATCTTAAGAACGAGAAGCGCGCTCGCATCTCTGAGTTCAAGGA 420
2       ACTTACTCACCTCAAGTACCTCAAGAACGAGAAGCGTGGCCGTATCTCCGAGTTCAAGGA 420
3       ACTTACTCACCTCAAGTACCTCAAGAACGAGAAGCGTGGCCGTATCTCCGAGTTCAAGGA 419
         *** * *** ******* ** ************** *  ** ***** **********

1       CAAGTATCCCAGTGTCACGTACTACGAAAACAAGAAGCCCTGGGAGTGCTTCGAGGGCCA 480
2       CAAGTATCCTAGCGTCGCGTACTACGAGAACAAGAAGCCATGGGAATGCTTTGAGGGGCA 480
3       CAAGTATCCTAGCGTCGCGTACTACGAGAACAAGAAGCCATGGGAATGCTTTGAGGGGCA 479
        ********* ** *** ********** *********** ***** ***** ***** **

1       TGTGGAC 487
2       AGTGGAC 487
3       AGTGGAC 486
         ******
```

11.4.2. Alignment of amino acids

Amindo acids: KP258355 was not aligned towards the other sequences because it contains a frame shift at bp position 339. Besides that it is equal to all other sequences with assemblages D.

```
1       GADTDVPAGDIGVGAREIGYLFGQYKRLRNEFTGVLTGKNVKWGGSLIRPEATGYGAVYF 60
2       GADTDVPAGDIGVGAREIGYLFGQYKRLRNEFTGVLTGKNIKWGGSLIRPEATGYGAVYF 60
        ************************************.******************

1       LEEMCKDNNTIIRGKNVLLSGSGNVAQFACEKLIQLGAKVLTFSDSNGTIVDKDGFNEEK 120
2       LEEMCKDNNTIIRGKNVLLSGSGNVAQFACEKLLQLGAKVLTFSDSNGTIVDKDGFNEEK 120
        *********************************.************************

1       LAHIKYLKNEKRARISEFKDKYPSVTYYENKKPWECFEGHVD 162
2       LTHLKYLKNEKRGRISEFKDKYPSVAYYENKKPWECFEGQVD 162
        *.*.********.************.*************.**
```

11.5. Triosephosphate isomerase sequence comparison of *G. duodenalis*

11.5.1. Alignment of nucleotide sequences

AY228641: reference sequence from GeneBank

KP258396 and KP258397: sequences of *Giardia* assemblage C obtained in the present study at the tpi locus

C: *Giardia* assemblage C

Nucleotides with yellow frame: mark for intraassemblage substitution

```
  C {1: AY228641
     2: KP258397
     3: KP258396

1     TCCCTTCATCGGGGGTAACTTTAAGTGCAACGGGTCGCTTGACTTTATCAAAAGCCATGT 60
2     TCCCTTCATCGGGGGTAACTTTAAGTGCAACGGGTCGCTTGACTTTATCAAAAGCCATGT 60
3     ------------------------------------------------------------ATGT 4
                                                                   ****

1     AGCGGCCATCGCGTCCCACAAGATTCCCGACTCTGTTGATGTGATCATCGCCCCCTCGTC 120
2     AGCGGCCATCGCGTCCCACAAGATTCCCGACTCTGTTGATGTGATCATCGCCCCCTCGTC 120
```

```
3    AGCGGCCATCGCGTCCCACAAGATTCCCGACTCTGTTGACGTGATCATCGCCCCCTCGTC 64
     ************************************ ********************

1    CGTGCATCTGTCTACGGCCATCGCAGCGAACACATCGAAGCAGCTGAAGATAGCAGCGCA 180
2    CGTGCATCTGTCTACGGCCATCGCAGCGAACACATCGAAGCAGCTGAAGATAGCAGCGCA 180
3    CGTACATCTGTCTACGGCCATCGCAGCGAACACATCGAAGCAGCTGAAGATAGCAGCGCA 124
     *** ************************************************************

1    GAATGTGTACCTCGAGGGAAACGGCGCATGGACGGGCGAGACAAGTGTTGAGATGCTTCA 240
2    GAATGTGTACCTCGAGGGAAACGGCGCATGGACGGGCGAGACAAGTGTTGAGATGCTTCA 240
3    GAATGTGTACCTCGAGGGAAATGGCGCATGGACGGGCGAGACAAGTGTTGAGATGCTTCA 184
     ******************** ****************************************

1    GGACATGGGCCTGAGTCACGTGATAGTAGGGCACTCTGAAAGACGTAGGATCATGGGCGA 300
2    GGACATGGGCCTGAGTCACGTGATAGTAGGGCACTCTGAAAGACGTAGGATCATGGGCGA 300
3    GGACATGGGCCTGAGTCACGTGATAGTAGGGCACTCTGAAAGACGTAGGATCATGGGCGA 244
     ************************************************************

1    GACCAACGAGCAGAGTGCCAAGAAGGCTAAGCGTGCTCTGGAGAAGGGCATGATGGTCAT 360
2    GACCAACGAGCAGAGTGCCAAGAAGGCTAAGCGTGCTCTGGAGAAGGGCATGATGGTCAT 360
3    GACCAACGAGCAGAGCGCCAAGAAGGCTAAGCGTGCTCTGGAGAAGGGCATGATGGTCAT 304
     *************** ********************************************

1    CTTCTGCACTGGGGAGACACTGGACGAGCGCAAGGCCAACAAGACTATGGATGTGAACAT 420
2    CTTCTGCACTGGGGAGACACTGGACGAGCGCAAGGCCAACAAGACTATGGATGTGAACAT 420
3    CTTCTGCACTGGGGAGACACTGGACGAGCGCAAGGCCAACAAGACTATGGATGTGAACAT 364
     ************************************************************

1    TGGACAGCTCGAGGCCCTTAAGAAGGAAGTCGGTGACGCTAAGGCGCTCTGGAAGAGTGT 480
2    TGGACAGCTCGAGGCCCTTAAGAAGGAAGTCGGTGACGCTAAGGCGCTCTGGAAGAGTGT 480
3    TGGACAGCTCGAGGCCCTTAAGAAGGAAGTCGGTGACGCTAAGGCGCTCTGGAAGAGTGT 424
     ************************************************************

1    CGTCATCGCCTACGAGCCCGTGTGGTCCATCGGCACGGGCGTGGTGGCCACA 532
2    CGTCATCGCCTACGAGCCCGTGTGGTCCATCGGCACGGGCGTGGTGGCCAC- 531
3    CGTCATCGCCTACGAGCCCGTGTGGTCTATCGGCACGGG------------- 463
     *************************** ************
```

11.5.2. Alignment of amino acids

```
1    PFIGGNFKCNGSLDFIKSHVAAIASHKIPDSVDVIIAPSSVHLSTAIAANTSKQLKIAAQ 60
2    PFIGGNFKCNGSLDFIKSHVAAIASHKIPDSVDVIIAPSSVHLSTAIAANTSKQLKIAAQ 60
3    ------------------VAAIASHKIPDSVDVIIAPSSVHLSTAIAANTSKQLKIAAQ 41
                       ****************************************

1    NVYLEGNGAWTGETSVEMLQDMGLSHVIVGHSERRRIMGETNEQSAKKAKRALEKGMMVI 120
2    NVYLEGNGAWTGETSVEMLQDMGLSHVIVGHSERRRIMGETNEQSAKKAKRALEKGMMVI 120
3    NVYLEGNGAWTGETSVEMLQDMGLSHVIVGHSERRRIMGETNEQSAKKAKRALEKGMMVI 101
     ************************************************************

1    FCTGETLDERKANKTMDVNIGQLEALKKEVGDAKALWKSVVIAYEPVWSIGTGVVAT 177
2    FCTGETLDERKANKTMDVNIGQLEALKKEVGDAKALWKSVVIAYEPVWSIGTGVVA- 176
3    FCTGETLDERKANKTMDVNIGQLEALKKEVGDAKALWKSVVIAYEPVWSIGT----- 153
     ***************************************************
```

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