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PROTOZOOLOGY - ORIGINAL PAPER

Molecular prevalence and genotyping of *Giardia duodenalis* in cattle in Central Anatolia Region of Turkey

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Abstract

The molecular prevalence and genotypes of *Giardia duodenalis* in cattle were investigated. A total of 450 fecal samples were collected from cattle in three provinces of Central Anatolia from August 2017 to July 2019. Genomic DNA was extracted from the fecal samples and used in molecular analysis carried out by nested PCR analyses of the *β-giardin* (*bg*) gene of *G. duodenalis*. Positive samples were further analyzed by nested PCR at two gene loci (*triosephosphate isomerase* (*tpi*) and *glutamate dehydrogenase* (*gdh*)) for genotyping of *G. duodenalis* isolates. PCR analyses of the *bg* gene indicated that the overall prevalence of *G. duodenalis* was 30.2%. However, lower rates were determined with PCR analyses for *gdh* and *tpi* loci. The sequence analyses of the *bg*, *gdh*, and *tpi* genes revealed the presence of zoonotic assemblage A and livestock-specific assemblage E. Combined-sequence analyses revealed that assemblage E was the most common in the study area. Our study provides the first data on the wide prevalence of livestock-specific assemblages E in cattle in Turkey. The prevalence of assemblage A in cattle also reveals the importance of cattle for zoonotic transmission of giardiasis in Turkey.

Keywords Assemblage · *β-Giardin* · Cattle · Central Anatolia · *Giardia duodenalis* · *Glutamate dehydrogenase* · Molecular prevalence · *Triosephosphate isomerase*

Introduction

Giardia species are some of the most prevalent flagellated protozoan parasites that infect many hosts worldwide, including mammals, amphibians, and birds (Ryan and Cacciò 2013). One species of *Giardia*, *Giardia duodenalis*, causes giardiasis in humans and most mammals (Feng and Xiao 2011). Transmission is generally by the fecal-oral route (Conn et al. 2007; Zhao et al. 2014; Akinkuotu et al. 2019). Infection is mainly associated with the consumption of contaminated food and water with *Giardia* cysts (Smith et al. 2006; Feng and Xiao 2011; Li et al. 2017; Zhong et al. 2018).

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Giardia duodenalis causes significant economic losses in cattle due to weight loss, growth retardation, production losses, and mortality (Geurden et al. 2010; Abeywardena et al. 2012; Shin et al. 2015; Akinkuotu et al. 2019; Kim et al. 2019; Dan et al. 2019). Recent studies of *G. duodenalis* have focused on the potential of cattle as a source for zoonotic transmission (Gultekin et al. 2017a; Bartley et al. 2019; Lichtmannsperger et al. 2019; Wang et al. 2019; Lee et al. 2019). Close contacts with infected cattle may be significant for zoonotic transmission of *G. duodenalis* (Feng and Xiao 2011; Santin et al. 2012).

Evidence from several studies has indicated that *G. duodenalis* is a species complex of at least eight distinct genetic groups, referred to as assemblages A to H (Monis et al. 2009; Feng and Xiao 2011). Some of these assemblages have different degrees of host specificity (Zhang et al. 2012; Koehler et al. 2014; Heyworth 2016; Utaaker et al. 2017). Assemblages A and B are able to infect numerous hosts, including humans, livestock (pigs, sheep, goat, and cattle), and companion animals (cats, dogs, and horses) (Maikai et al. 2012; Abeywardena et al. 2015; Xie et al. 2018; Akinkuotu et al. 2019). The other assemblages are either host-specific or

have a narrow host spectrum (Abeywardena et al. 2015). However, sporadic human cases with assemblages C, D, E, and F have been reported (Zahedi et al. 2017).

Giardiasis is one of the most important and common parasitic infections causing gastrointestinal disorder with chronic diarrhea, especially in immune-compromised individuals and children in Turkey (Degerli et al. 2012). In regard to zoonotic transmission routes, there have been limited detailed surveys on animal hosts, such as cattle, and environmental sources (Degerli et al. 2005a; Goz et al. 2006; Koloren et al. 2016; Gultekin et al. 2017a, b; Ayan et al. 2019; Demircan et al. 2019). In addition, genetic information on *G. duodenalis* assemblages responsible for host-specific or zoonotic infections is also missing. Surveys on the prevalence of giardiasis in cattle in Turkey are limited. Infection rates have been reported in the range of 3.7 to 64.7%, using conventional techniques and molecular assays (Degerli et al. 2005; Goz et al. 2006; Gultekin et al. 2017a; Ayan et al. 2019). Zoonotic assemblages A and B have been reported in calves and cattle in two studies with small-scale sampling using the *bg* gene as a diagnostic marker (Gultekin et al. 2017a; Ayan et al. 2019). Gaps still exist in the molecular epidemiology of *G. duodenalis* in cattle in Turkey. Therefore, we aimed to investigate the prevalence and variants in assemblages of *G. duodenalis* in cattle in Central Anatolia Region of Turkey, using multilocus genotyping targeting three gene loci (*bg*, *gdh*, and *tpi*). We also aimed to draw attention to the potential risk of cattle for zoonotic transmission of *G. duodenalis* within the context of public health.

Materials and methods

Study area and collection of fecal samples

This study was carried out in Kayseri, Nevsehir, and Sivas provinces of Central Anatolia Region between August 2017 and July 2019. A total of 450 fecal samples (150 from each province) were collected from droppings on the ground and placed in separate sterile plastic containers, labeled with the age, breed, sex, geographical origin, and date of collection. All samples were then transported immediately to the laboratory and stored at 4 °C until DNA extraction.

DNA extraction from fecal samples

A total of 100 mg fecal sample was mixed with 1.4 mL ASL buffer in a microcentrifuge tube and the tube was vortexed until the sample was thoroughly homogenized. Samples were subsequently mixed with 200 mg acid-washed glass beads (0.5 mm; Merck KGaA, Darmstadt, Germany) and were processed on a TissueLyser (Qiagen, Hilden, Germany). Lysate was disrupted with 3 freeze-thaw cycles (freezing in liquid nitrogen for 1 min followed by boiling in the heating block in a 90 °C for 1 min) before genomic DNA (gDNA) extraction. Finally, gDNA was extracted from disrupted samples by the QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction and eluted in 50 μL of AE buffer. The quantity and quality of the extracted genomic DNA were determined using the Qubit Fluorometric Quantitation (Thermo Fisher Scientific, USA) spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The gDNA was stored at −20 °C until PCR analyses.

PCR amplification

Genomic DNA samples were initially amplified with nested PCR (nPCR) for the *bg* gene region to detect *G. duodenalis* in cattle. The nPCR-positive samples were subsequently screened with *gdh*- and *tpi*-specific nPCRs. The primers and annealing temperatures in the nPCR assay of these markers are summarized in Table 1. The PCR reactions for each gene region were performed in 25-μL volume containing 12.5 μL of 2x Dream Taq PCR master mix (Thermo Fisher Scientific, Waltham, MA, USA), 1.25 μL from each primer (10 μM), 8 μL of ddH2O, and 2 μL of gDNA sample as the template (10–30 ng/μL) in a C1000 Thermal Cycler (Bio-Rad, USA). In the second PCR step, 1 μL of the first amplified PCR product was used as the DNA template. The PCR amplification protocols were applied to amplify a 511-bp fragment of *bg* (Lalle et al. 2005), 530 bp of *gdh* (Cacciò et al. 2008), and 532 bp of *tpi* (Sulaiman et al. 2003). For each PCR analysis, a *G. duodenalis*–positive DNA sample and distilled water were used as the positive and negative control, respectively. All secondary PCR amplicons were run at 135 V for 30 min and then monitored by electrophoresis in 2% agarose gels stained with SYBR™ Safe DNA (Invitrogen™, USA), and images were captured using a Fusion FX imaging system (Vilber Lourmat, France).

. DNA sequence analysis

The second PCR amplicons of each three-gene region were purified using a commercial kit (GeneJet Gel Extraction Kit, Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. The gel-purified PCR products were sequenced in both directions with the second PCR primers (Macrogen, the Netherlands) for each gene region. The obtained nucleotide sequences were assembled using Geneious Prime 2020.0.3 software (https://www.geneious.com). To determine the *Giardia* assemblages, BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/) and multiple alignments using MAFFT v7.450 (Katoh and Standley 2013) were used for comparison of the nucleotide sequences obtained in our work with reference sequences available in GenBank. Genetic variants were determined by polymorphisms at each gene locus.

Table 1 Primers used in nested PCR analyses of *bg*, *gdh*, and *tpi* gene region of *G. duodenalis* in Central Anatolia Region, Turkey

Genetic locus	Primer name	Primer sequence (5′ to 3′)	Annealing temp (°C)	Reference
bg	G7 G759	AAGCCCGACGACCTCACCCGCAGTGC GAGGCCGCCCTGGATCTTCGAGACGAC	64	Lalle et al. 2005
	2005F 2005R	GAACGAACGAGATCGAGGTCCG CTCGACGAGCTTCGTGTT	55	
tpi	AL3543 AL3546	AAATIATGCCTGCTCGTCG CAAACCTTITCCGCAAACC	53	Sulaiman et al. 2003
	AL3544 AL3545	CCCTTCATCGGIGGTAACTT GTGGCCACCACICCCGTGCC	57	
gdh	GDH1 GDH2	TTCCGTRTYCAGTACAACTC ACCTCGTTCTGRGTGGCGCA	50	Cacciò et al. 2008
	GDH3 GDH4	ATGACYGAGCTYCAGAGGCACGT GTGGCGCARGGCATGATGCA	50	

The isolate was referred to as a new variant even if a single polymorphism and/or indels were present. The sequences obtained were deposited in GenBank under accession numbers MN276321-MN276324 for the *bg* gene, MN447713-MN447716 for the *gdh* gene, and MN419028-MN419030 for the *tpi* gene.

Statistical analysis

The prevalence of *G. duodenalis* was compared based on sex, age group, and breed, using a chi-square (χ^2) test with the software SPSS 21.0 for Windows (IBM Corp. New York, NY). p values < 0.05 were considered statistically significant.

Results

Prevalence of *G. duodenalis*

The *bg*-nPCR revealed *G. duodenalis* in 30.2% (136/450) of the fecal samples. The prevalence of *G. duodenalis* infection associated with research areas and age, sex, and breed of cattle is presented in Table 2. The distribution of infection was statistically similar ($p > 0.05$) across the study areas. The highest prevalence rate (46.7%) was detected in the < 1-year-old age group, followed by the 1–3-year-old (36.7%) and > 3-year-old (13.6%) age groups. A significant ($p < 0.001$) difference was found between the > 3-year-old group and < 1-year-old and 1–3-year-old groups. The infection rate in females was similar to the rate in males, and slightly higher and lower infections were found in Brown Swiss and Anatolian Black breeds, respectively, than in other breeds. However, there was no significant ($p > 0.05$) association of *Giardia* infection with sex or breed of cattle (Table 2).

Detection of *G. duodenalis* by *gdh* and *tpi* amplification

The 136 *bg* PCR-positive DNA samples were subsequently subjected to PCR amplification of *G. duodenalis* with *gdh* and *tpi* genes. A total of 52 and 26 of the *bg* samples were positive for *gdh* and *tpi*, respectively.

Molecular characterization of *G. duodenalis* isolates

Sequence analysis of the 136 *bg*-positive samples revealed the presence of assemblages E (89.0%, 121/136) and A (11.0%, 15/136). All *gdh* and *tpi* locus-positive samples were successfully sequenced and both identified only assemblage E (Table 2). Sequences obtained from all isolates were compared with each other and the sequences in GenBank to determine genetic polymorphism within each gene loci.

The nucleotide sequence analyses of 121 isolates of the *bg* gene revealed three closely related variants (*bg*TE1 to *bg*TE3; T for Turkey) in assemblage E, exhibiting 0.2–0.6% genetic difference from each other. The remaining 15 isolates were determined in assemblage A, representing a single new variant (*bg*TA1). The most common variant was *bg*TE1, including 62 isolates, followed by *bg*TE2 and *bg*TE3 with 36 and 23 isolates each, respectively.

All isolates at the *gdh* and *tpi* loci were identified in assemblage E and revealed the presence of three and two closely associated variants, respectively. The variants in *gdh* were named *gdh*TE1, *gdh*TE2, and *gdh*TE3 and included 21, 18, and 13 isolates, respectively. These variants showed 0.2–0.78% genetic difference from each other. The variants in *tpi* were named *tpi*TE1 and *tpi*TE2 and included 20 and 6 isolates, respectively. These variants showed 0.2% genetic difference from each other.

The SNPs compared with the reference sequences within each gene region are shown in Table 3. The identified *bg* sequences of assemblages E and A had 1 to 3 nucleotide

Table 2 Prevalence and distribution of assemblages of *G. duodenalis* infecting cattle based on amplification and sequence analyses of the *bg* gene in Central Anatolia Region, Turkey

bg-nested PCR				Distribution of assemblages (%)			
Parameters	All	No of positives (%)	*p* value	E		A	
				n	% (95% CI)	*n*	% (95% CI)
Study site							
Kayseri	150	45 (30.0)	0.677	41	27.3 (23.4–31.3)	4	2.7 (2.0–3.4)
Nevsehir	150	42 (28.0)		36	24.0 (20.6–27.4)	6	4.0 (3.4–4.6)
Sivas	150	49 (32.7)		44	29.3 (25.4–33.1)	5	3.3 (2.6–4.0)
Sex							
Male	298	90 (30.2)	0.989	79	26.5 (23.7–29.2)	11	3.7 (3.2–4.1)
Female	152	46 (30.3)		42	27.6 (23.8–31.4)	4	2.6 (1.8–3.3)
Age groups							
< 1 year	150	70 (46.7)[a]	0.000	62	41.3 (37.3–45.2)	8	5.3 (4.6–5.9)
1–3 years	109	40 (36.7)[a]		34	31.1 (27.0–35.1)	6	5.6 (4.8–6.3)
> 3 years	191	26 (13.6)[b]		25	13.0 (8.6–17.3)	1	0.5 (0.0–0.9)
Breed group							
Holstein	120	35 (29.2)	0.971	31	25.9 (20.7–31.0)	4	3.3 (2.7–3.8)
Brown Swiss	172	55 (32.0)		47	27.3 (23.0–31.6)	8	4.7 (4.2–5.1)
Simmental	94	28 (29.8)		26	27.6 (21.7–33.4)	2	2.1 (1.5–2.6)
Crossbreed	52	15 (28.8)		14	27.0 (21.4–32.5)	1	2.0 (1.2–2.8)
Anatolian Black	12	3 (25.0)		3	25.0 (12.3–37.7)	0	0.0 (−)
Total	450	136 (30.2)		121	26.9 (25.1–28.6)	15	3.3 (3.0–3.5)

Groups indicated by different letters in columns were significantly different ($p < 0.001$)

Table 3 Genetic variants in assemblages of *G. duodenalis* for the *bg*, *gdh*, and *tpi* genes in cattle in Central Anatolia Region, Turkey

Genetic Variants	GenBank Accession No	No. of isolates	Nucleotide position and substitutions			
			64	412	484	
bg gene						
E (Ref.seq)	KT369772		T	C	G	
*bg*TE1	MN276322	62	*	*	*	
*bg*TE2	MN276321	36	C	*	A	
*bg*TE3	MN276323	23	*	T	*	
			415	423	587	
A (Ref.seq)	AY072723		C	T	A	
*bg*TA1	MN276324	15	T	C	C	
gdh gene			2	131	463	470
E (Ref.seq)	KF843925		T	A	G	G
*gdh*TE1	MN447714	21	G	G	*	*
*gdh*TE2	MN447715	18	G	*	*	*
*gdh*TE3	MN447716	13	*	*	A	A
			71	137		
tpi gene						
E (Ref.seq)	KT369762		T	A		
*tpi*TE1	MN419029	20	C	*		
*tpi*TE2	MN419030	6	C	G		

Nucleotide positions are numbered according to the references (ref.seq) with the first nucleotide as position 1. The *bg*TE1 to *bg*TE3 correspond to positions 11–501 of reference sequence KT369772; *bg*TA1 to positions 103–593 of reference sequence AY072723; *gdh*TE1 to *gdh*TE3 correspond to positions 1–510 of reference sequence KF843925; *tpi*TE1 and *tpi*TE2 correspond to positions 4–510 of reference sequence KT369762

substitutions at three positions each, compared with reference sequences (KT369772 and AY072723). Compared with the reference sequence (KF843925), *gdh* sequences of the determined variants exhibited 1 to 2 nucleotide substitutions at four positions. The *tpi* variants showed 1 to 2 nucleotide substitutions at two positions, compared with the reference sequence (KT369762).

Discussion

We analyzed the commonly used *bg*, *gdh*, and *tpi* genes to characterize *G. duodenalis* at the molecular level. We determined less positive rates of *G. duodenalis* in the *tpi* and *gdh* gene regions, compared with the *bg* gene. Despite many attempts and PCR optimization experiments, amplification of the *gdh* and *tpi* genes did not succeed in all *G. duodenalis* *bg*-positive samples. Several published reports on *G. duodenalis* infections in various animals revealed higher positive rates of *Giardia* for the *bg* gene than for *gdh* and *tpi* (Wang et al. 2017, 2019; Chen et al. 2019; Dan et al. 2019). Such studies on the determination of *G. duodenalis* assemblages in various hosts also suggest that *gdh* and *tpi* primers are less sensitive than *bg* primers due to the presence of nucleotide mismatches between the PCR primers and the genomic sequences (Broglia et al. 2013; Minetti et al. 2015; Bartley et al. 2019). Thus, the *bg* locus could be a good molecular marker for the determination of the parasite DNA. The *bg* locus, therefore, could be more useful as a detection marker, whereas *tpi* and *gdh* could be useful for subgenotyping (Chen et al. 2019; Wang et al. 2019; Dan et al. 2019).

We determined giardiasis with an overall prevalence of 30.2% in the research area, which is lower than the rate reported from the eastern part of Turkey (Ayan et al. 2019). This frequency is higher than in southwestern Turkey (Gultekin et al. 2017a), Iran (Malekifard and Ahmadpour 2018), India (Khan et al. 2011), Malaysia (Muhid et al. 2012), and New Zealand (Abeywardena et al. 2012). The variation in the prevalence of giardiasis in different regions could be explained by factors such as the age of cattle, feeding conditions, sampling season, farm management, husbandry system, sample size, climate, and water supply (Wegayehu et al. 2016; Xie et al. 2018; Feng et al. 2019; Kiani-Salmi et al. 2019).

Giardiasis has a high prevalence in pre-weaned calves but is rare in adult cattle older than 1 year (Trout et al. 2005; Santín et al. 2009; Zhang et al. 2012; Fan et al. 2017). Similarly, higher infections with *G. duodenalis* were determined in the age group of < 1-year-old cattle in our study. The infection was detected in young and old age groups cattle suggesting its importance as a reservoir for *G. duodenalis*. Different age groups of cattle were held together in the barns by owners due to the lack of individual shelter for calves in the research area. The water could also be contaminated with feces infected with *Giardia* cysts that are not routinely removed from shelters.

Assemblages A and B were previously reported in cattle in Turkey (Gultekin et al. 2017a; Ayan et al. 2019). Here, we determined assemblage E in cattle for the first time in Turkey as the most prevalent assemblage in the region. Assemblage E, referred to as a ruminant-specific group, is the predominant assemblage detected in livestock in several countries (Feng and Xiao 2011; Santin et al. 2012; Geurden et al. 2012; Cui et al. 2018; Wang et al. 2019). However, this assemblage has also been identified from different hosts including horse, dog, fallow deer, cat, and monkey (Cacciò and Ryan 2008; Feng and Xiao 2011; Santin et al. 2012; Ryan and Cacciò 2013), as well as humans (Soliman et al. 2011; Helmy et al. 2014; Abdel-Moein and Saeed 2016; Fantinatti et al. 2016). The *bg* sequences of the three variants (*bg*TE1-3) in assemblage E were highly similar (99.8–100%) to the sequences reported from cattle, sheep, lamb, sika deer, and alpaca in China (MH079427), India (GQ290390), Ethiopia (KT922249), Mexico (DQ116615), Spain (EU726984), and Norway (GQ337973). Similarly, the sequences of *gdh* (*gdh*TE1-3) and *tpi* (*tpi*TE1-2) also showed high identity (99.6–100%) to sequences from livestock in countries such as China (MK442908, MG602957), Egypt (MG820461), Japan (AB569406), and Ethiopia (KT922262). As this assemblage is also seen in humans, and the identified *gdh* variant (*gdh*TE1) also exhibited high identity with an isolate from a child in Bangladesh (MK982483), possible zoonotic transmission of assemblage E should not be ignored. Moreover, close contact with livestock animals increases the risk of anthropozoonotic transmission.

Assemblage A is the main group of *G. duodenalis* that includes the genotypes causing human infections with zoonotic characteristics (Li et al. 2017). It is also the second most common assemblage in livestock (Ryan and Cacciò 2013). We determined that assemblage A has a scarce distribution in cattle, compared with assemblage E. The identified *bg* variant (*bg*TA1) was highly similar (99.8%) to the sequences reported from humans in Bangladesh (MK982540), Iran (LC504268), Spain (KY499042), Brazil (KU504738), and Ethiopia (KT948085), and from a foal in Belgium (KM926505) and a dog in Croatia (JN416525). To date, several genotypes in assemblage A have also been reported from cattle in countries such as Iran, Germany, France, Italy, the Netherlands, and Austria (Huetink et al. 2001; Langkjær et al. 2007; Gillhuber et al. 2013; Malekifard and Ahmadpour 2018; Lichtmannsperger et al. 2019). All these findings provide evidence for the increase of assemblage A in cattle throughout the world and highlight the risk of zoonotic transmission of *G. duodenalis* among livestock.

Conclusion

Our study provides novel data on the distribution and genetic characterization of *G. duodenalis* assemblages among cattle in large-scale samplings from the Central Anatolia Region of Turkey. Our results reveal the wide prevalence of livestock-specific assemblage E and confirm the presence of zoonotic assemblage A in cattle. The results indicate that cattle pose a high risk for animal handlers, veterinarians, and public health professionals in the study area. Further epidemiological studies are required for revealing the zoonotic potential and genotype distribution of *G. duodenalis* in cattle in different geographical locations of Turkey.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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