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Prevalence and Molecular Characterization of *Giardia duodenalis* in Calves in Turkey

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ABSTRACT

Background: *Giardia duodenalis* (*G. duodenalis*) is an ubiquitous, flagellated intestinal protozoan with major public health significance worldwide. Limited data are available on the epidemiology of *G. duodenalis* in dairy cattle from Turkey. Determining the zoonotic potential of the *Giardia* infection requires molecular characterization. The aim of the present study was to investigate the prevalence and to molecularly characterize *G. duodenalis* in calves less than three months of age in Aydin, Aegean region of Turkey.

Materials, Methods & Results: The study was conducted on different dairy farms in the south-western part of the Turkey, Aegean Region, Aydin. A total of 198 Holstein Friesian calves less than three months of age, of both sexes were enrolled into the study. Faecal samples from each calf were collected manually from the rectum using a disposable latex glove. The consistency of collected samples was recorded as diarrhoeic or non-diarrhoeic. Diagnosis of *G. duodenalis* infection was made microscopically by detection of cysts in the faecal samples. One hundred and sixteen (58.5%) of the 198 faecal samples were diarrhoeic. *Giardia* cysts were found in 27 (23.28%) of the diarrhoeic samples and in 8 (9.76%) of non-diarrhoeic samples ($P < 0.05$). The overall prevalence of giardiasis in calves was determined as 17.67%. The prevalence of *Giardia* genotypes was identified by DNA sequence analysis of the beta-giardin gene for every PCR positive sample. The beta-giardin nested PCR assay was revealed assemblage A and sub-genotype A3 was detected in all of 35 samples (100%).

Discussion: The highest prevalence of *Giardia* infection in calves is reported at the age between 1 and 6 months, and the prevalence shows decreased rate from the age of 6 months. The present study was conducted in Aydin, a province of south-western Turkey in the Aegean Region, and the overall prevalence from a total of 198 dairy calves was 17.67%. The prevalence rate in calves with diarrhoea was higher and reached up to 23.28%, whereas it was 9.76% in non-diarrhoeic calves. A prevalence study with molecular characterization of *G. duodenalis* isolates in cattle has not yet been reported from Turkey. Molecular studies have shown that mostly assemblage E predominates in cattle, but recent studies denoted that assemblage A is increasingly being detected and might be more widespread than expected before. In the present study, *Giardia* positive samples identified with a beta-giardin nested PCR assay. The sub-genotype A3 was identified in all samples. The same sub-genotype was identified in human and dog samples from different countries. Furthermore, sub-genotype A3 was found in humans and dogs from Turkey. In this context, results of the present study suggested an important role of calves as potential reservoirs of human infections in Turkey. In conclusion, epidemiological data revealed that *G. duodenalis* infection is frequent in calves with diarrhoea in Aydin, Turkey. The presence of the potentially zoonotic sub-genotype A3 and high prevalence of *Giardia* infection in diarrhoeic calves indicated the importance of treatment and necessary preventative measures. Further studies in human and animal populations living in this region are warranted regarding the zoonotic epidemiology of *Giardia duodenalis*.

Keywords: calf, giardiasis, prevalence, molecular characterization.

INTRODUCTION

Giardia duodenalis (*G. duodenalis*) is an ubiquitous, flagellated intestinal protozoan with major public health significance worldwide [18]. Giardiasis might be transmitted by the ingestion of cysts with direct or indirect contact, including human to human, animal to human or animal, waterborne and foodborne transmission [4,40].

Livestock infection is common. In cattle, prevalence of giardiasis has been reported ranging from 9% to 73% with the farm prevalence rates even may rise up to 100% [18]. Chronic or reoccurring infections mostly observed in calves and cyst shedding may continue for months [34]. Clinical signs in calves include weight loss, lethargy, diarrhoea, dehydration and poor condition, although subclinical infections are frequent [16,38].

Determining the zoonotic potential of the *Giardia* infection requires molecular characterization [14,28,40]. Eight major genetic assemblages have been identified. Assemblages A and B infect both humans and animals, whereas the assemblages C to H are known as specific to animals [4]. Assemblage E is considered to be livestock specific and reported as most prevalent assemblage in cattle [12,29,37]. However mono or mixed infections with assemblage A [15,17,31,42,43] or assemblage B [9,26] were reported in cattle from different countries. Additionally, infection with more unexpected assemblages such as C, D and F were also detected in United Kingdom [33] and Spain [6] in cattle.

The number of studies related with the prevalence and molecular characterization of giardiasis in cattle is increasing [9,19,29,42,43]. However, only limited data is available about the epidemiology of *G. duodenalis* in dairy cattle from Turkey. Prevalence rates in calves presented between 4.1% and 14.7% from different parts of country [11,20,21] whereas none of those studies recorded molecular characterization of assemblages in calves. The aim of the present study was to investigate the prevalence and molecular characterization of *Giardia duodenalis* in calves from Aydin region of Turkey.

MATERIALS AND METHODS

Sample collection

The study was conducted on different dairy farms in the south-western part of the Turkey, Aegean Region, Aydin. A total of 198 Holstein Friesian calves less than 3 months of age, of both sexes were enrolled

into the study. The samples were withdrawn at different times of the year between March 2016 and October 2016 from several farms. Faecal samples from each calf were collected manually from the rectum using a disposable latex glove. The consistency of collected samples was recorded as diarrhoeic or non-diarrhoeic. Samples were kept on ice until laboratory examination.

Microscopic examination

Diagnosis of *G. duodenalis* infection was made microscopically by detection of cysts in the faecal samples. Faecal material was mixed through 33% ZnSO₄ solution and centrifuged at 880 x g for 5 min [44]. Fifty µL of the supernatant was emitted on a microscope slide with Lugol iodine. The slide was microscopically examined under 400x power for visualization of *Giardia* cysts.

DNA extraction

In order to extract the DNA, samples identified to be positive after microscopic examination of faeces were selected according to the procedure previously described by Mahdy *et al.* [30]. DNA extraction of the samples was achieved using QIAamp DNA Stool Mini Kit¹. The extracted DNA was preserved at -20°C until further analysis.

Nested PCR reaction

Following the procedure previously described by Caccio *et al.* [5], β-giardin gene, consisting of 753 bp fragment, was amplified using an appropriate primer. For this purpose, 10 pmol appropriate primer (G7 F5'-AAG CCC GAC GAC CTC ACC CGC AGT GC-3' forward and G759R 5'-GAG GCC GCC CTG GAT CTT CGA GAC GAC-3' reverse), 200 µM of dNTPs, 1.5 mM MgCl₂, 1U Taq Polymerase and 10X reaction buffer (500 mM Tris-HCl, pH 8.8, 160 mM (NH₄)₂SO₄ and 0.1% Tween 20)² and 100 ng DNA was diluted to final volume of 25 µL. DNA was denatured at 95°C for 15 min, followed by annealing at 60°C for 30 s and by final extension for seven min at 72°C. The DNA copies of β-giardin gene were separated using 2% agarose gel. The gel was stained with ethidium bromide and visualized with UV transillumination.

Another 511 bp fragment of the beta-giardin gene was amplified with a suitable primer (BG1F 5'-GAACGAGATCGAGGTCCG-3' forward and BG2R 5'-CTCGACGAGTTCGTGTT-3' reverse) with a modification of the method described by Lalle *et al.* [26].

DNA sequence analysis of nested PCR positive samples

The assemblage of the strains was confirmed by sequencing the 511 bp samples from both strands. For this purpose, the DNA copies obtained from PCR were purified using polyethylene glycol 4000 [39]. Sequencing was accomplished by using Big Dye-terminator chemistry³ following the instructions of the manufacturer. Briefly, the sequencing reaction was carried out in the TC-Plus⁴ thermocycler for 20 s at 96°C and annealing was conducted at 50°C for 20 s. Finally, the extension was performed for 4 min at 60°C for 30 cycles. Genome Lab Genetic Analyzer³ was used for the analysis of sequencing reactions.

Obtained sequences were compared with reference sequences (GenBank® number: M36728 for sub-genotype A1, AY072723 for sub- genotype A2, AY072724 for sub- genotype A3, AY072725 for sub- genotype B1, AY072726 sub- genotype B2, AY072727 for sub- genotype B3, AY072728 for sub-genotype B4) using by BLAST and BioEdit Sequence Alignment Editor.

Statistical analysis

Chi-square test was used to analyse the association between diarrhoea and giardiasis in calves. The 95% confidence interval was calculated for the prevalence rates. Probability values of $P < 0.05$ were considered significant.

RESULTS

One hundred and sixteen (58.5%) of the 198 faecal samples were diarrheic. *Giardia* cysts were found in 27 (23.28%) of the diarrheic samples and in 8 (9.76%) of non-diarrheic samples ($P < 0.05$). The overall prevalence of giardiasis in calves was determined as 17.67% (Table 1).

The prevalence of *Giardia* genotypes was identified by DNA sequence analysis of the beta-giardin gene for every PCR positive sample. The beta-giardin nested PCR assay was revealed assemblage A and sub-genotype A3 in all of 35 samples (100%).

DISCUSSION

Giardiasis in cattle is associated with a high morbidity, significant production loss and potential transmission to other hosts [1,4,18]. Prevalence rates may vary according to the age of the animals, design of the study, diagnostic techniques, geographical location, climatological conditions and management practices [4,18]. Additionally, intermittent nature of the cyst shedding also influences the sensitivity of diagnosis [35]. The individual prevalence ranges from 9 to 73 % and whereas the farm prevalence rates may rise up to 100 % [4,18]. The highest prevalence of *Giardia* infection is reported at the age between 1 and 6 months, and the prevalence shows decreased rate from the age of 6 months [41]. Therefore young animals are considered as the major source of infection [45]. In the present study, a total of 198 less than three months old dairy calves were enrolled.

Prevalence rates from different countries vary 2.2 to 14% in Poland [2], 9% in Portugal [32], 17.4% to 31.3% in Belgium [17], 26.6% in Spain [7], 30% in Italy [3], up to 38% in Germany [25], 49% in Norway [23], up to 40.6% in New Zealand [14], 43.6% in Denmark [27], up to 57% in Canada [9], up to 52% in the United States [24,41] and 58% in Australia [36]. The prevalence or spatial distribution of giardiasis in calves in Aegean region of Turkey is lacking.

Sivas is a city in central Turkey, where the prevalence of *Giardia* spp. in healthy asymptomatic cows and calves was determined as 1.4% and 4.1%, respectively. Highest prevalence reported in 45-90 days old calves was 14% [11]. Van is an eastern city of Turkey, where a total of 231 calves less than eight months of age with diarrhoea were evaluated, and parasitological examination revealed *Giardia* spp. cysts in 14.7% of samples [20]. In another study from eastern part of Turkey, *Giardia* spp. cysts were found 9.34 % of the dairy calves less than six months of age [21].

Table 1. Prevalence rates of giardiasis in calves from Aydin region of Turkey, 2016.

Condition	Calves (n)	<i>G. duodenalis</i> Positive (n)	Prevalence (IC 95%)	Chi-square
Diarrheic	116	27	23.28 (22.3-24.1)	$P = 0.014$
Non-diarrheic	82	8	9.76 (8.6-10.8)	
Total	198	35	17.67 (16.9-18.3)	

The present study was conducted in Aydin, a province of south-western Turkey in the Aegean Region, and the overall prevalence from a total of 198 dairy calves was 17.67%. In the present study the prevalence rate in calves with diarrhoea was higher and reached up to 23.28%, whereas it was 9.76% in non-diarrhoeic calves. There are conflicting results between the relation of giardiosis and diarrhoea [19]. However, most of the studies found a positive correlation between the occurrence of diarrhoea and *Giardia* infection in calves [38,39].

A prevalence study with molecular characterization of *G. duodenalis* isolates in cattle has not yet been reported from Turkey. Molecular studies have shown that mostly assemblage E predominates in cattle [14], but recent studies denoted that assemblage A is increasingly being detected and might be more widespread than expected before [4,15,17,31]. Distribution of assemblages according to the age has been noted in cattle. Assemblage A was mainly detected in calves whereas assemblage E was more frequent in older animals [31,41]. In a recent study from the south-eastern New York, 100% of *Giardia* positive specimens isolated from calves under 84 days of age were identified as assemblage A [31], similar to present results of our study. In the current study, overall prevalence of giardiosis in calves was %17.67 (198/35) and all 35 isolates were belong to the same sub-genotype, A3. Prevalence or assemblages of *G. duodenalis* may show significant variation according to age, breed, season or location as reported in previous studies [18,41].

Assemblages A and B have been linked with their potential zoonotic role [4,36,41]. However, the genetic structure of *Giardia* has been shown to be more complex than thought before by recent molecular studies [28,40]. Multilocus genotyping of the beta-giardin, SSU rRNA, glutamate dehydrogenase and triosephosphate isomerase loci have allowed to identify sub-assemblages [40,42]. Following this im-

provement, sub-assemblages obtained from animal and human samples were compared and results showed that the most of the infections were not sharing identical genotypes between animal and human [4,28,40]. In the present study, *Giardia* positive samples identified with a beta-giardin nested PCR assay. The sub-genotype A3 was identified in all samples. The same sub-genotype was identified in human [26] and dog [10] samples from different countries. Furthermore, sub-genotype A3 was found in humans [8,13] and dogs [22] from Turkey. In this context, results of the present study suggested an important role of calves as potential reservoirs of human infections in Turkey.

CONCLUSIONS

In conclusion, epidemiological data revealed that *Giardia duodenalis* infection is frequent in calves with diarrhoea in Aydin, Turkey. The presence of the potentially zoonotic sub-genotype A3 and high prevalence of *Giardia* infection in diarrheic calves indicated the importance of treatment and necessary preventative measures. Further studies with multilocus genotyping in human and animal populations living in this region are warranted regarding the zoonotic epidemiology of *G. duodenalis*.

MANUFACTURERS

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Ethical approval. The study protocol was approved by the institutional laboratory animals ethics committee (with no: 2016/039 and date 25.02.2016). Informed written consent was obtained from all of the owners/animal care takers prior to enrolment.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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