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Prevalence and molecular diagnosis of *Gongylonema pulchrum* in cattle and sheep in the Samsun region

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Abstract: This study was carried out between November-2017 and June-2019 to determine the distribution and molecular characterization of *Gongylonema pulchrum* in cattle and sheep slaughtered in local abattoirs and slaughtering areas during the feast of sacrifice. In this study, esophagi collected from 380 (358 young and 22 mature) cattle and 848 esophagi from sheep (816 young and 32 old) were examined. Esophagus taken after slaughtering was brought to the laboratory in plastic bag. In the laboratory, all the esophagi were cut open with scissors and all of the materials were examined using a loupe under a light source. Parasites were taken out using eye forceps and placed in 70% ethyl alcohol. Following the primary morphological examination, all the parasites cut into three parts, anterior and posterior parts were used for morphologic identification and mid part was used for molecular identification. BpCoxI-F1 and BpCoxI-R1 primers were used for molecular identification. Only two cows (older than four years) were infected and the infection rate was 0.53%. A total of 27 parasites were identified, 18 were male and 9 were female. Parasites were not found in the examined sheep. All the samples collected from the infected cattle were identified as *G. pulchrum* after morphological examinations and molecular confirmation and positive bands were detected at 400bp.
Keywords: Cattle, *Gongylonema pulchrum*, molecular, Samsun, sheep.

Samsun yöresinde sığır ve koyunlarda *Gongylonema pulchrum*'un yaygınlığı ve moleküler teşhisi

Özet: Bu araştırma, Samsun bölgesinde 2017 Kasım - 2019 Haziran tarihleri arasında, yerel mezbahalar ve kurban bayramında kesilen koyun ve sığırlarda *Gongylonema pulchrum*'un yaygınlığı ve moleküler karakterizasyonunu belirlemek amacıyla yapıldı. Çalışmada 380 sığır (22'si yaşlı, 358'i genç) ve 848 koyundan (32'si yaşlı, 816'sı genç) toplanan özefaguslar incelendi. Kesim sonrası alınan özofaguslar plastik torbalara konularak laboratuvara getirildi. Laboratuvarda tüm özefaguslar makas yardımı ile açılarak ışık altında ve büyüteç yardımı ile incelendi. Parazitler göz pensi ile çıkartılıp % 70'lik etil alkole konuldu. Parazitler üç parçaya ayrıldı, ön ve arka kısımlar morfolojik, orta kısımları ise moleküler teşhis amacıyla kullanıldı. Moleküler teşhis için Bp CoxI-F1 ve Bp CoxI-R1 primerleri kullanıldı. Yalnızca iki inek (dört yaş üstü) enfekte olmuş ve yaygınlık oranı % 0,53 bulunmuştur. Enfekte hayvanlardan 18 erkek ve 9 dişi olmak üzere 27 parazit toplandı. İncelenen koyunlarda parazite rastlanmadı. Enfekte sığırdan toplanan tüm örnekler, morfolojik incelemeler ve moleküler doğrulama sonrasında *G.pulchrum* olarak saptandı ve pozitif bantlar 400 bp'de elde edildi.
Anahtar sözcükler: *Gongylonema pulchrum*, koyun, moleküler, Samsun, sığır.

Introduction

Gongylonema pulchrum (gullet worm) is a nematode localized in the esophageal epithelium in variety of mammals and birds, particularly livestock, worldwide (31). In the genus *Gongylonema*, there are nearly 50 species identified in mammals and birds (6, 16, 32), however, *G. pulchrum* the most known and common species in Turkey (21, 23) and in the world (22, 31). The parasite had been identified for the first time in Turkey in 1922 by Tüzdil (30).

The parasite is located in shallow tunnels in the esophagus in a zig-zag pattern, and can sometimes be found in the rumen of the ruminants. The intermediate hosts are dung beetle and cockroaches (23). In Turkey, *G. pulchrum* has been detected in sheep, goats, cattle, buffalo, horses and wild boars and the prevalence rate was reported as 7.6 - 96% (4, 5, 8, 11, 28, 31).

Gongylonema pulchrum, a zoonotic parasite has been reported from many Asian, African, American and European countries, including Turkey, a large number of

human cases had been reported and have reached 200 to date (14, 33).

Morphological and genetic differences can be observed between *G. pulchrum* samples obtained from different animal species, as well as genetic differences between samples obtained from the same animal and it is suggested that there are at least two haplotypes for parasite (20, 27). The observation of genetic differences in the species has increased with the use of molecular studies, and the total mitochondrial genome of the parasite was determined in 2015 (18).

Morphological criteria are sometimes insufficient for the diagnosis of parasites and the distinction of closer species, and molecular diagnostic methods are used in such cases. For this purpose, the gene regions of the parasites such as the ribosomal DNA (ITS1, ITS2) and mitochondrial DNA (COX1) are used (20).

As a result of molecular studies, *G.nepalensis* species, which is morphologically very similar to *G.pulchrum* and separated from it only by the shorter left spiculum, has been identified in Nepal (27) and Europe (32).

Gongylonema pulchrum is considered to be insignificant when the number is low in ruminants. In severe infections, local inflammation, bloating, bleeding, mild pain, sometimes epithelial hypertrophy and acanthosis were observed in the esophagus (9), and although very rare, causes death in cattle and monkeys (15).

This study was carried out to determine the prevalence and molecular characterization of *G. pulchrum* in cattle and sheep slaughtered in the Samsun region, Turkey.

Materials and Methods

This research was carried out between November 2017 and June 2019 in Samsun, Havza (Temiz-Et Slaughterhouse) and Atakum (Florya Slaughterhouse) and sacrificial temporary slaughterhouse in Kavak district at 2017-2018. During this period, 380 (52 female, 328 male) cattle and 848 sheep (254 female, 594 male) esophagus were collected from slaughtered animals and brought to Ondokuz Mayıs University Veterinary Faculty Parasitology Laboratory.

After opening the esophagus under the light source with scissors in the laboratory, it was examined with the aid of a magnifying glass, and/or under a stereo-microscope. The parasites were removed from the esophagus with eye forceps, counted and macroscopic measurements were made and kept in 70% ethyl alcohol for each animal individually.

The parasites were morphologically divided into three equal parts and the middle parts of the body were stored in pure ethanol at -20 °C for use of molecular analysis. The front and back ends of the parasites were stored in 70% alcohol, and after making transparent with lactophenol, they were diagnosed under the microscope according to the related literature (23, 30, 31, 32). In the diagnosis, the important regions were measured and photographed.

DNA extraction: Genomic DNA was obtained separately from two worms using the commercial DNA extraction kit (Invitrogen PureLink Genomic DNA Mini Kit) according to the manufacturer's instructions and stored at -20 ºC until use.

Polymerase Chain Reaction (PCR): The COI gene region of genomic DNA was amplified using the BpCoxI-R1 (5'-ATGAAAATGTCTAACTACATAATA AGTATC-3') and BpCoxI-F1 (5'-TTTGG TCATCCTGAGGTTTATATT-3') primer pair as described in Makouloutou et al. (20) and Setsuda et al. (27).

The PCR mixture (50 µl) contained 10 mM Tris HCl, 50 mM KCl, 2.5 mM $MgCl_2$ (Thermo), 250 µM dNTP mix (Sigma), 0.5 µM each primer, 1.25 U Taq polymerase (Thermo), 2 µl template DNA, and distilled water. PCR conditions were as follows: first denaturation 94 °C for 3 min, 40 cycles at 94 °C for 45 sec, 52 °C for 1 min, 72 °C for 1 min and final cycle at 72 °C for 7 min. Amplicons were imaged and photographed under UV after electrophoresis in 2% agarose gels stained with ethidium bromide (Sigma).

Sequence and phylogenetic analysis: Purification of two PCR products obtained and sequenced for both strands with the same primers by Macrogen (Holland). The obtained sequences were verified by forward and reverse comparisons, assembled and edited with using Contig Express in Vector NTI Advance 11.5 (Invitrogen).

The blast analysis (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) of the sequences obtained in the GenBank database (1), the homologies and similarity percentages of the isolates among themselves and with other isolates in the world were determined. Multiple alignments of the nucleotide sequences of the haplotypes were performed using the Clustal W algorithm with the Mega X (17) and BioEdit (13) programs.

The genetic distances of the isolates (pairwise distance, Kimura 2 Parameter) were determined according to the Mega X program (17) and the maximum composite likelihood model (29). Nucleotide multiple alignments were converted to different file formats with the DnaSP version 6.10 program (26). In the jModelTest version 0.1 (25) program, the GTR model was found as the best DNA model using the Akaike information criterion (AIC).

The phylogenetic tree was created according to the maximum likelihood (ML) method with PhyML version 3.1 (10) program. In the bootstrap analysis, the value was set to be 100 reps, and the ≥50% bootstrap value was considered significant.

Figtree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree) program was used for tree drawing. Trees were created with the help of Inkscape 0.92 (http://inkscape.org/en/) program. The nucleotide sequence was submitted to GenBank under the accession number MK962623.

Results

Parasites were detected only in two elderly (over four years) animals from the examined 380 cattle, the prevalence rate was determined as 0.53% and gullet worms showing a typical epithelium-dwelling character (Figure 1A). Totally 27 parasites (18 male and 9 female) were collected from two infected cattle.

848 sheep (254 female and 594 male) were examined in the slaughterhouses in the Samsun region. The majority of the slaughtered sheep consisted of 816 (96.2%) young (under 1-year-old) and 32 (3.8%) old (over 2-years-old) sheep. No parasites were found in any of the sheep examined.

When female and male parasites were carefully examined on a black background, due to the difference in size and posterior ends, they can be easily differentiated with a simple magnifier (Figure 1B,C).

In the microscopic examination, there were cuticle ornaments on the one cm anterior of the parasites, which look as if they were adhered to the parasite and can spread to the outsides (Figure 2A).

It was observed that the developmental period of the parasites was different. The spicules of the three males were not yet fully developed, they were short, which were considered young (Figure 2B). The sizes of these young male nematodes were also nearly 10% smaller than the adults but remained within normal limits. All of the young and adult parasites were measured. The length of the males was, as an average, of 38 mm (29 - 47) and a thickness of 0.25 (0.21 - 0.28) mm.

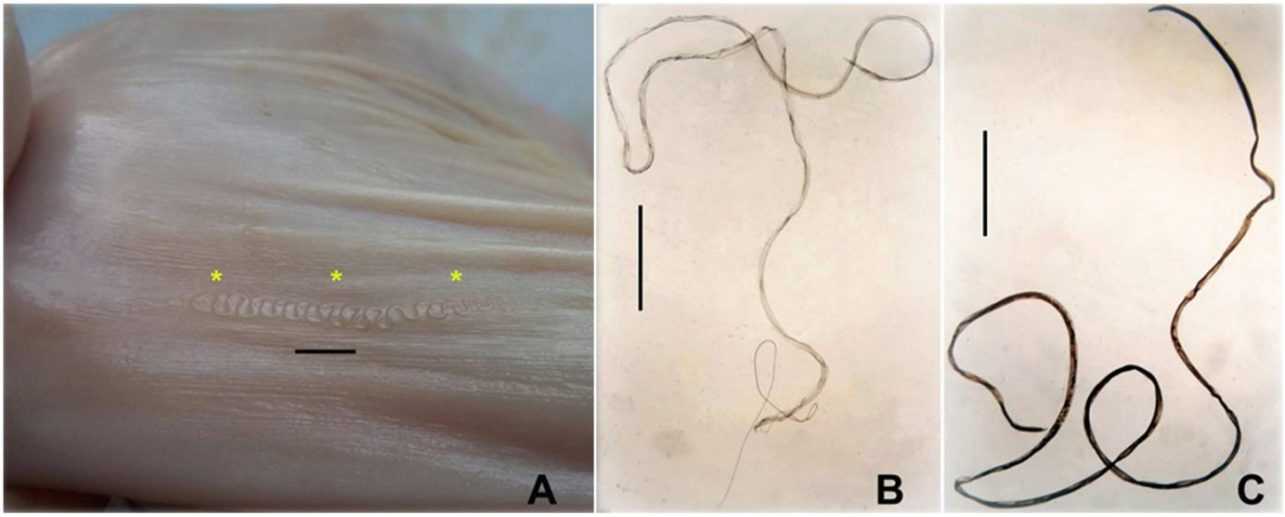

Figure 1. The natural and stereo microscopic view of *G.pulchrum*.
A. Zig-zag manner of parasite in the esophagus, B. Mature male, C. Mature female, Bar: 10 mm in all figures.

Figure 2. Microscopic view of the *G. pulchrum.*
A. Anterior end of the mature worm, B. Posterior end of the juvenile male, C. Posterior end of the mature male, D. Anal region of the female. E. Vulvar region of the female, F. Eggs in the uterus. Bar: 0.01 mm in all figures.

The spicules were asymmetrical, and the left spicule was very long and overflowing out of the bursa and it was 7.5 (5.6-12.1) mm long. The right spicule was very short and measured as 0.120 (0.100- 0.142) mm (Figure 2C).

The average length of the females was 72 (45-88) mm, and the thickness was 0.38 (0.32-0.44) mm. In females, the anus was prominent, the tail ends in a finger-like shape and the tail length was measured as 0.230 (0.190-0.270) mm (Figure 2D).

The vulva was located on the posterior half of the body and slightly protrudes outwards (Figure 2E). Thousands of eggs with a fully developed embryo were seen in the uterus of the adult female. The length of the eggs in the uterus was measured as 0.059 (0.057-0.061) mm and the width was 0.032 (0.030-0.036) mm (Figure 2F).

As a result of molecular analysis, all of the samples detected in both animals were detected as *G. pulchrum*, and positive bands were obtained at approximately 400 bp (Figure 3). The sequence result for *G. pulchrum* bovine isolate COI gene region is given in Table 1. The genetic distances of the isolates to each other (pairwise distance, Kimura 2 Parameter) are presented in Table 2 according to the maximum composite likelihood model.

The phylogenetic tree using the maximum likelihood (ML) method, which shows the phylogenetic relationships of the *G. pulchrum* bovine isolate COI gene region obtained in this study with other isolates and species in the world, is given in Figure 4. Nucleotide variations of the total alignment were shown in Figure 5.

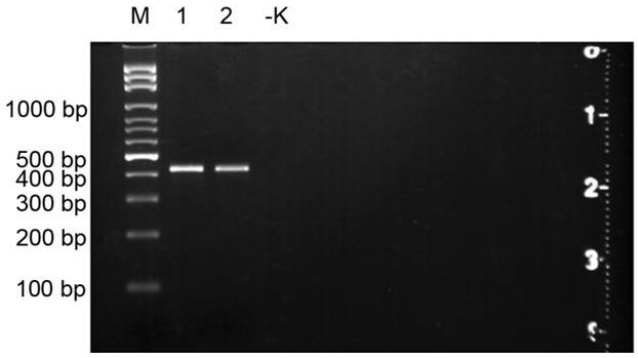

Figure 3. Electrophoresis image of the gene region of positive samples M: Marker, 1-2: Isolates, -K: Negative Control.

Table 1. The sequence result of *G. pulchrum* bovine isolate COI gene region.

>*Gongylonema pulchrum* voucher OMUPAR.32.18.01 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial
ATCCTGAGGTTTATATTATTATTTTACCTGCATTTGGTATTATTAGGGAATGTGTTTTATATCTAACTGATAAGGAAC
GATTATTTGGTCAAGCAAGTATGGTTTATGCTTCTATTTGAATTTCTGTTTTAGGTACGTCTGTTTGGGGCCATCATA
TATATACTGCAGGTCTTGATATTGACACTCGAACTTATTTTAGGGCAGCTACTGTTATTATTGCTATTCCTAGGGCGG
TTAAGGTTTTTAATTGACTTGGGACTTTATTTGGTTCTCGTCAATATTTACAACCTGTGTGATGTTGGACATATAGTT
TTATTTTTTTGTTTACTATTGGTGGTTTGAGCGGTATTATTTTGAGTACTGCTAGGTTGGATATTGTTTTACATGATAC
TTATTATGTAGT

Table 2. Genetic distances of isolates to each other.

Erişim No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
MK962623	-														
KM264298	0.00	-													
LC388895	0.00	0.00	-												
LC026040	0.00	0.00	0.00	-											
LC026035	0.00	0.00	0.00	0.00	-										
AB513728	0.00	0.01	0.00	0.00	0.00	-									
LC388914	0.02	0.03	0.02	0.03	0.02	0.02	-								
LC388905	0.02	0.03	0.02	0.03	0.03	0.02	0.00	-							
LC388909	0.03	0.03	0.03	0.03	0.03	0.03	0.01	0.01	-						
AB646115	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.01	0.02	-					
AB646122	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.00	0.01	0.01	-				
LC388893	0.10	0.11	0.10	0.11	0.10	0.10	0.10	0.10	0.10	0.10	0.10	-			
LC388892	0.10	0.11	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.00	-		
LC278393	0.10	0.11	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.00	0.00	-	
LC026046	0.13	0.13	0.13	0.13	0.13	0.13	0.14	0.14	0.14	0.13	0.14	0.13	0.13	0.13	-
LC331022	0.14	0.14	0.14	0.14	0.14	0.15	0.13	0.13	0.14	0.14	0.15	0.14	0.14	0.14	0.09

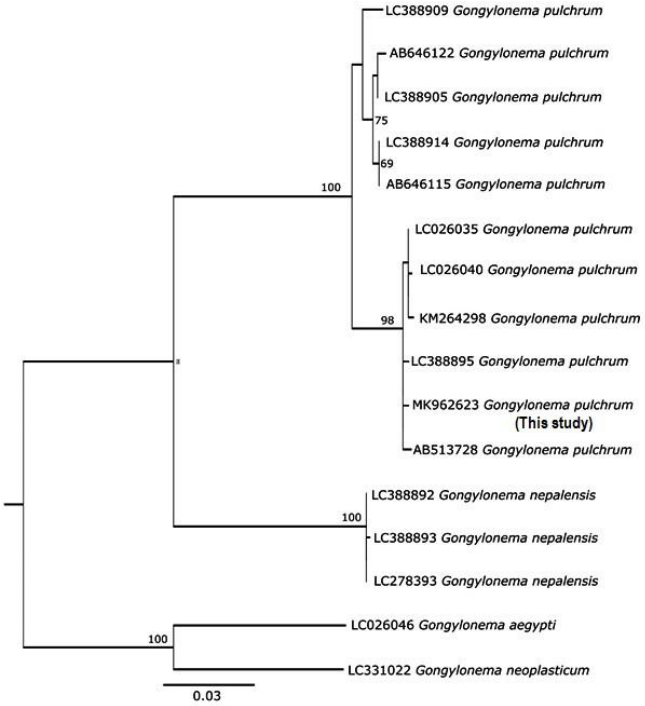

Figure 4. The phylogenetic tree made using maximum likelihood method for *G. pulchrum* cattle isolate COI gene region.

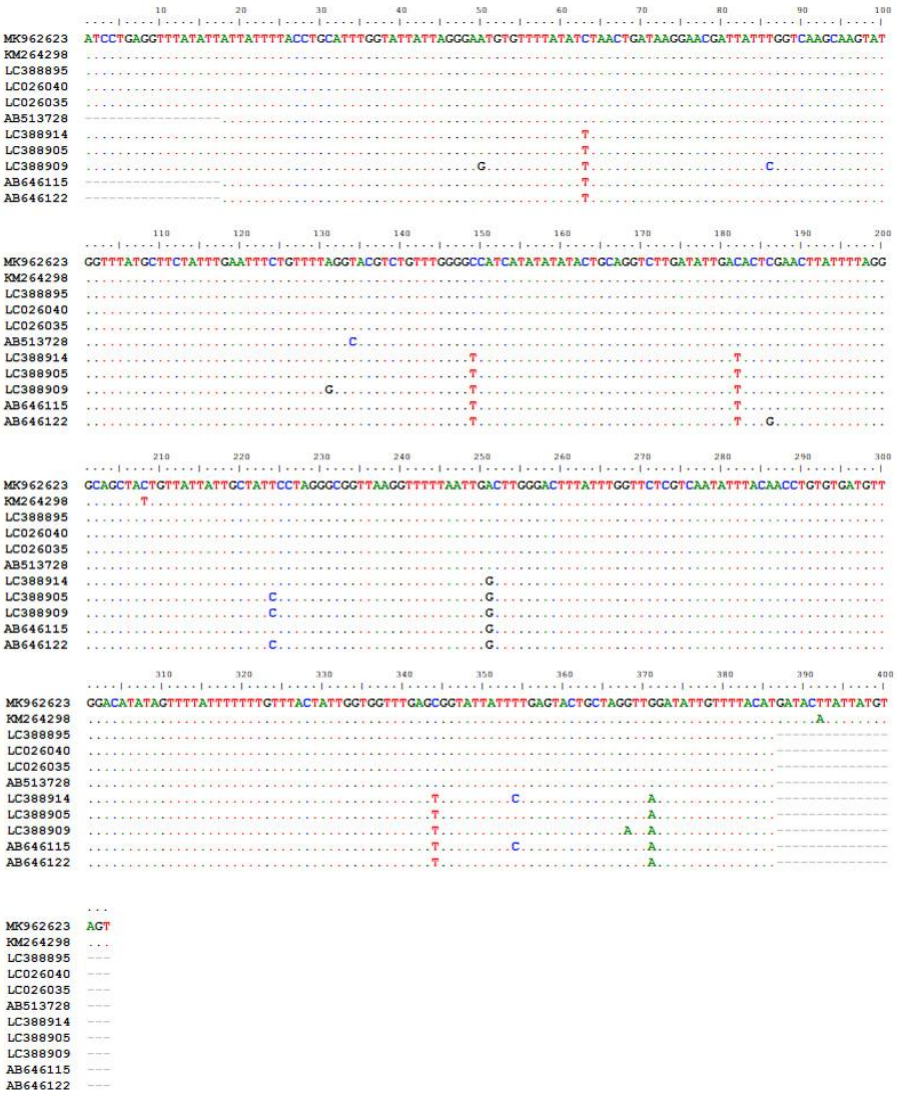

Figure 5. Nucleotide variations in mitochondrial COI gene regions of *Gongylonema pulchrum* isolate analyzed in the present study.

Discussion and Conclusion

Although the prevalence of *G. pulchrum* in cattle and sheep varies according to country and animal species recent reports (9, 15) indicated that the prevalence of the parasite was decreased. For instance, prevalence rate of the *G. pulchrum* in cattle in Iran was decreased to 16.2% in 2011 (15) compared to 49.7% in 1979 (3). Similarly, in Turkey, the prevalence was as high as 96% in Ankara region (30) while Celep et al. (5) found a significantly lower prevalence rate (6.3%) in the Samsun region close to prevalence recorded in Japan (5.3%) (20). However, in this study conducted in the Samsun region, we found the prevalence rate of the parasite as low as 0.53% in cattle.

Although there is no reliable and comparable information about the age and grazing conditions of the studies conducted, the prevalence rate of the parasite in the sheep, similar to cattle, decreases even in sheep fed more pasture. Even though Tüzdil (30), who carried out the first study on *G. pulchrum* in Turkey, reported a 60% prevalence in sheep and goats, Celep (4) found lower prevalence rate in sheep (14.7%) from Samsun region. On the other hand, the prevalence rate was recorded as 40% in sheep in Kars region (2), 42.8% (7) and 13.19% in Van region (11). In both studies performed in Van region, information about the age of the animals was not provided. This difference in the prevalence rate could be age-related. However, it should not be forgotten that the prevalence rates in the Eastern Anatolia regions of Turkey are still high and that it depends on some regional characteristics. In our opinion, the reason for this may be due to the inadequacy in veterinary services and the lack of drug use due to the widespread of nomadic livestock. Similarly, in foreign countries, in recent years, the prevalence rates in sheep are below 10%. For instance, its prevalence rate is 4.57% in Iran (9) and 5.5% in Algeria (24). In the present study, no parasites were found in 828 sheep esophagus examined.

As can be seen in the above publications, the prevalence rates are decreasing gradually both in foreign countries and in Turkey. In our opinion, the most important reason for the decrease in prevalence rates is the reduction in pasture livestock in our country, especially in the Samsun region. With the idea that it is more profitable, closed system integrated livestock farms are increasing. Since animals in such livestock farms are usually fed with commercial feeds, the probability of encountering intermediate host insects is reduced, thus the possibility of parasite occurrence is eliminated. In addition, the increase in the use of antiparasitic drugs and the restriction slaughtering of the rootstock females may also be effective.

The average length of the worms was measured 38 mm (29-47) in males, 72 (45-88) mm in females, the average thickness was 0.25 mm (0.21-0.28) in males, 0.38 mm (0.32-0.44) in females. In the adult male, the left spicula was very long and has extended beyond the bursa copulatrix and measured as 7.5 mm (5.6-12.1). The length of the eggs in the uterus of females was 0.059 mm (0.057-0.061) and the width was 0.032 mm (0.030-0.036). There was no significant difference between the body measurements in the present study as indicated in the literature (12, 23, 27).

In the present study, the number of parasites for per cattle was 13.5 (7-20). However, the parasite density in cattle in Japan was rather variable and recorded as 1-109 (20) while in Nepal the density was as low as 1-4 (19). Although no parasites were encountered in sheep in this study, the number of parasites per sheep was ranged from 10 to 100 in Iran (9, 22), and from 1 to 12 in Turkey (2).

The gullet worm which can be found all around the world has a wide selection of mammalian hosts, hence some minor morphologic (30) and genetic differences depending on the host species (20). Therefore morphological studies must be supported by molecular studies for definitive diagnosis. Although there are many studies on the morphological diagnosis of *G.pulchrum* both in our country (2, 5, 7, 11, 23, 28, 30) and in foreign countries (3, 6, 9, 14, 15), there are only a few molecular studies (18-20, 27, 32) in the world and none in our country.

As a result, *G. pulchrum* was not found in sheep in the Samsun region, and the prevalence rate was very low (0.53%) in cattle. The molecular diagnosis of the *G. pulchrum* was performed for the first time in Turkey and has been recorded in GenBank. According to the phylogenetic analysis, Turkey isolate was very close to the isolates of other countries, and the genetic distance between the isolates ranged from 0.00-0.03%. Some small nucleotide variations were observed between the nucleotides used in the study and the results of this study.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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