Kafkas Univ Vet Fak Derg
22 (1): 93-99, 2016
DOI: 10.9775/kvfd.2015.13960

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Molecular Prevalence, Phylogenetic Characterization and Benzimidazole Resistance of *Haemonchus contortus* from Sheep [1]

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[1] *This study was financially supported by Erciyes University Research Fund as a PhD thesis project with the number TSD-12-4054*
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Article Code: KVFD-2015-13960 Received: 02.07.2015 Accepted: 18.08.2015 Published Online: 18.08.2015

Abstract

This study was conducted to determine the molecular prevalence and characterization of *Haemonchus contortus* from sheep along with the benzimidazole (BZ) resistance in *H. contortus* populations. Fecal samples were collected from a total of 300 sheep in research area and analyzed by fecal flotation. qPCR assays were utilized on trichostrongylid egg positive samples in order to identify *H. contortus*. Phylogenetic analyses were performed on ribosomal ITS-2 and mt-COI gene regions. BZ sensitive and resistant allele frequencies were determined by qPCR along with sequence analyses of the β-tubulin isotype 1 gene for single nucleotide polimorphizms (SNPs). *H. contortus* was identified in 36 (24.8%) out of 145 trichostrongylid egg positive samples. *H. contortus* isolates showed 100.0% identity to each other and 0.1% difference with the isolates available in the GenBank based on phylogenetic analyses of ITS-2 gene while mt-COI analyses of the isolates exhibited a mean of 97.4±0.5% identity to each other and 5.5±0.8% difference with the isolates in GenBank. BZ sensitive and resistant allele frequencies in *H. contortus* populations were determined as 87.1%±16.2 and 12.9%±16.2, respectively. SNPs were detected only in the codon 200 of the sequenced isolates belong to resistant allele. This study provides the first data on molecular prevalence, phylogenetic characterization and BZ resistance in *H. contortus* populations from sheep in Turkey.

Keywords: *Haemonchus contortus, Sheep, Molecular characterization, Benzimidazole resistance*

Koyunlarda *Haemonchus contortus*'un Moleküler Prevalansı, Filogenetik Karakterizasyonu ve Benzimidazol Dirençliliği

Özet

Bu çalışmada koyunlarda *Haemonchus contortus*'un moleküler prevalansı ve karakterizasyonu ile benzimidazol (BZ) dirençliliğinin belirlenmesi amaçlanmıştır. Araştırma yöresinde toplam 300 koyundan dışkı örnekleri toplanmış ve fekal yüzdürme yöntemiyle incelenmiştir. Trichostrongylid yumurtalarıyla pozitif belirlenen örneklerde *H. contortus* identifikasyonu için qPCR analizleri gerçekleştirilmiştir. Filogenetik analizler *H. contortus* izolatlarının ribosomal ITS-2 ve mt-COI gen bölgeleri üzerinde yürütülmüştür. BZ duyarlı ve dirençli allel sıklıkları β-tubulin izotip 1 gen bölgesindeki tek nükleotid polimorfizmi (SNPs) temelinde qPCR ile belirlenmiş, ayrıca ilgili genin sekans analizleri gerçekleştirilmiştir. Trichostrongylid yumurtalarıyla pozitif 145 örneğin 36'sında (%24.8) qPCR ile *H. contortus* identifiye edilmiştir. ITS-2 gen bölgesi filogenetik analizine göre *H. contortus* izolatlarının %100 identik oldukları ve GenBank'ta mevcut izolatlarla %0.1 genetik farklılık gösterdikleri belirlenirken mt-COI sekans analizleri izolatların ortalama %97.4±0.5 identiklik ve GenBank'ta mevcut izolatlarla da %5.5±0.8 farklılık gösterdiklerini ortaya koymuştur. *H. contortus* populasyonlarında BZ duyarlı ve dirençli allel sıklıkları qPCR ile sırasıyla %87.1±16,2 ve %12.9±16.2 belirlenmiştir. Dirençli allele ait izolatların sekans analizlerinde yalnızca 200. kodonda SNPs saptanmıştır. Bu çalışma ile Türkiye'de koyunlarda *H. contortus*'un moleküler prevalansı ve genetik karakterizasyonu üzerine ilk veriler sağlanmış ve *H. contortus* popülasyonlarında BZ dirençliliği ortaya konmuştur.

Anahtar sözcükler: *Haemonchus contortus, Koyun, Moleküler karakterizasyon, Benzimidazol dirençliliği*

INTRODUCTION

Gastrointestinal nematodes are the most prevalent and major parasites in domestic and wild ruminants in tropical, subtropical and temperate regions worldwide [1,2]. One of the highly dangerous and economically important of these nematodes is *Haemonchus contortus* which is also known as "barber pole worm" and an abomasal pathogen of sheep [3]. It causes anemia, stunted growth, weight loss, loss in protein, disorder in fertility, decrease in milk, meat and wool production and the last death if untreated [1].

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In recent years, DNA-based techniques have been implemented to specific identification and characterization of these parasites and also to determine the level of infections. For this purpose several PCR-based methods targeting mainly the nuclear and mitochondrial genes have been applied and used in the phylogenetic relationships among trichostrongylid nematodes [1,4-6]. However there have been no molecular-based studies conducted on *H. contortus* populations in Turkey up to date. The broad spectrum anthelmintic drugs including the benzimidazole (BZ) group has been widely used for several years in the control of trichostrongylid nematodes all over the world and as a result anthelmintic resistance began in these parasites [1]. Several molecular techniques especially target the intron I of the ß-tubulin gene of trichostrongylid nematodes including *H. contortus* have been developed to specifically detect and measure the frequency of the sensitive and resistant alleles for BZ [7,8]. The β-tubulin is highly conserved gene and is found in different trichostrongylid nematodes of small and large ruminants, horses, pigs and also dogs [9]. It has been revealed that BZ resistance in trichostrongylid nematodes is particularly associated with the alterations in the β-tubulin isotype 1 gene which has an importance as genetic marker and useful for predicting BZ resistance problems [10]. A point mutations also called as single nucleotide polymorphisms (SNPs) at the codons 167, 198 and 200 of β-tubulin isotype 1 gene have been explored and linked to BZ resistance up to date [11-13].

The present study was conducted to determine the molecular prevalence and phylogenetic characterization of *H. contortus* from sheep in Kayseri region based on sequence analyses of mitochondrial cytochrome oxidase subunit 1 (mt-COI) and nuclear ribosomal internal transcribed spacer 2 (ITS-2) gene regions. Potentially problems of BZ resistance and the frequency of BZ-resistance-associated β-tubulin SNPs in *H. contortus* populations in sheep were also investigated.

MATERIAL and METHODS

Sample Collection and Parasitological Examination

Ethics committee approval was received for this study from the ethics committee of Erciyes University Local Ethics Committee for Animal Experiments (Date: 09/05/2012, Document no: 12/61). The study was conducted on a total of 300 Akkaraman sheep (≤2 age group 78, >2 age group 222; male 12, female 288) raised in various farms in Kayseri and vicinity between 2012 and 2013. Fresh fecal samples were directly collected from the rectum of each sheep into the sterile plastic bags, transferred to the laboratory, and then kept at 4°C until the parasitological examination. Zinc sulphate flotation technique was utilized to investigate trichostrongylid eggs [14]. The specimens found positive for trichostrongylid eggs were further examined by modified McMaster technique for determining the EPG values [12].

Genomic DNA Isolation

A saturated sodium nitrate flotation method was used to isolate trichostrongylid eggs from infected fecal material according to the procedures described by Bott et al.[15]. Genomic DNA was extracted from the isolated trichostrongylid eggs using a commercial kit (Axygen Biosciences, USA) according to the manufacturer's instructions. The extracted genomic DNA's were stored at -20°C until molecular analysis.

TaqMan Probe Based Real Time PCR Analyses for Identification of H. contortus

Genomic DNA from the obtained isolates were analyzed by TaqMan probe based real time PCR with the primers ITS-2F, ITS-2R and the fluorogenic probe ITS-2P labelled with FAM-BHQ1 probe that amplified ribosomal ITS-2 gene region of *H. contortus* [16].

Amplification and Phylogenetic Analyses of Ribosomal ITS-2 and mt-COI Genes of H. contortus

For specific amplification of *H. contortus* ribosomal ITS-2 gene region (248bp) the primers ERU-HconITS2F (5'GTTAACCATATACTACAATG-3') and ERU-HconITS2R (5'G AG CTCAGGTTGCATTATAC-3') were originally designed from the published sequences of the related gene of *H. contortus* isolates available in GenBank with the assistance of Primer3Plus software. Primer specificity was confirmed by primer-BLAST analyses in GenBank. PCR amplifications were utilized in a 25 µL reaction volume containing 10X PCR buffer, 2.5 mM $MgCl_2$, 100 nM each primers, 200 mM each dNTPs, 50 ng genomic DNA and 2.5 U Taq polymerase. The cycling conditions were as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 30s, 55°C for 30s and 72°C for 1 min, followed by 10 min at 72°C and 4°C to finalize. For the amplification of mt-COI region of *H. contortus* isolates, Nested PCR analyses were carried out following the described protocols [1].

The selected amplicons for both gene regions were further gel purified by a commercial kit (High Pure PCR product purification kit, Roche). Sequence analyses were performed on the obtained plasmids including the target gene regions after cloning procedures (CloneJET PCR Clonning Kit; Thermo Scientific, USA). Obtained sequences were aligned to homologues available from GenBank using the BLASTn algorithm with the default settings and edited in Geneious 6.1.6 software. Phylogenetic trees were constructed using the neighbor-joining (NJ) method based on the Kimura 2-parameter model in Mega 5.2 with 1000 boot-strap replicates for each tree [17].

Real Time PCR Amplification of β-tubulin Isotype 1 Gene for BZ Resistance and Sequence Analyzes for SNPs

TaqMan probe based qPCR assays targeting the

β-tubulin isotype 1 gene including the SNPs sites of BZ susceptible and resistant alleles of *H. contortus* were utilized as described by Walsh et al.[18]. The qPCRs were performed separately on the isolates that were determined as positive for *H. contortus* by ITS-2 real time PCR analyses. Allele frequencies were calculated by using the formula described by Germer et al.[19]. Allele frequencies of samples with no threshold cycle (Ct) value for benzimidazole resistant allele were evaluated as 100% susceptible for BZ.

β-tubulin isotype 1 gene including the SNPs sites from the isolates selected according to the determined allele frequencies in qPCR assays were further amplified by PCR with the primers HcTub-s5 and HcTub-as5 following the described protocols by Ghisi et al.[20] in order to explore the point mutations in the related gene region. The obtained amplicons were gel purified, cloned and plasmid isolations were performed. The plasmids were sequenced and the point mutations were analyzed in the obtained nucleotide sequences.

RESULTS

Fecal Examination and Real Time PCR Results

145 of the examined sheep were positive for trichostrongylid type eggs with a prevalence of 48.3%. The EPG values for trichostrongylid type are shown in *Table 1*. The mean EPG value in the trichostrongylid type positive samples was determined as 120.3±53.5. EPG values were determined as approximately two times higher in over 2 years age group than under ones and this difference was found statistically significant (P<0.05).

Of the 145 sheep with positive for trichostrongyle eggs, 36 (24.8%) were found to be infected with *H. contortus* by TaqMan qPCR assay. Parasitemia based on the mean Ct values *(Table 1)* were found to be higher in >2 year age group than ≤2 year age group and this difference was found statistically significant (P<0.001).

Sequence and Phylogenetic Analyses of Ribosomal ITS-2 and mt-COI Gene Regions

The ITS-2 gene regions of totally four *H. contortus* isolates (TrERUHcon04, TrERUHcon05, TrERUHcon06 and TrERUHcon07) were deposited into the GenBank database under accession numbers KJ188203 to KJ188206. The *H. contortus* Kayseri isolates showed 100% identity to each other and 0.1% genetic diversity with the available ITS-2 sequences of *H. contortus* in GenBank. Our isolates were also found 100.0% identical with the isolates obtained from the sheep in Brazil (JN128898, JQ342247), China (HQ844231), Iranian (HQ389229), Uzbekistan (KC503915), from the goat in USA (EU084691) and from the human stool in Australia (KC632567) and clustered together *(Fig. 1)*.

Mt-COI gene region of three *H. contortus* isolates (TrERUHcon01 to TrERUHcon03) were also deposited into the GenBank, with the accession numbers KJ188200 to KJ188202, respectively. *H. contortus* Kayseri isolates showed a mean of 97.4±0.5% identity to each other and a mean genetic diversity of 5.5±0.8% with the isolates available in GenBank. TrERUHcon01-03 isolates clustered together *(Fig. 2)* with the mt-COI sequences from the *H. contortus* isolates from sheep in Pakistan with a mean pairwise identity of 95.9±0.7% and showed a mean 7.7±1.6%, 7.8±1.4%, 8.4±1.6% genetic differences with the isolates from USA, Brazil and Australia, respectively which constituted the other major cluster.

BZ Resistance Allele Frequencies and Sequence Analyzes for SNPs

The qPCR analyses targeting the SNPs in β-tubulin isotype 1 gene region for BZ resistance in totally 145 *H. contortus* positive samples revealed a frequencies of 87.1±16.2% and 12.9±16.2% for sensitive and resistant alleles which were calculated by using the cycle threshold (Ct) values, respectively *(Table 2)*. Susceptible allele frequency was found as sevenfold higher than resistant allele frequency and this difference was found statistically

Table 1. *Distribution of EPG and Ct values over the age groups and gender of trichostrongyle type and H. contortus positive sheep*
Tablo 1. *EPG ve Ct değerlerinin trichostronhylid tip ve H. contortus pozitif koyunlarda yaş grupları ve cinsiyete göre dağılımı*

Factor	Number of Trichostrongylid Type Positive Sheep	EPG					Ct (dR)					
		Min-Max (95% Confidence)	Mean	St. Dev	F	P	Number of *H. contortus* Positive Sheep	Min-Max (95% Confidence)	Mean	St. Dev	F	P
Age Group												
≤2	22	51.6-80.2	65.9	32.3	32.744	0.000	10	25.3-33.5	29.2	1.96	86.93	0.000
>2	123	121.0-139.1	130.1	50.7			26	33.7-37.8	35.5	1.37		
Gender												
Male	8	69.1-168.4	118.7	59.4	0.007	0.931	3	25.3-36.8	31.0	5.78	0.000	0.998
Female	137	11.4-129.5	120.4	53.4			33	27.0-37.8	30.9	3.23		
Total	145	111.5-129.1	120.3	53.5			36	25.3-37.8	30.9	3.38		

F: *Anova Test;* ***EPG:*** *Eggs per gram of feces;* ***Ct:*** *Threshold cycle;* ***(dR):*** *Fluorescence;* ***St. Dev:*** *Standard deviation*

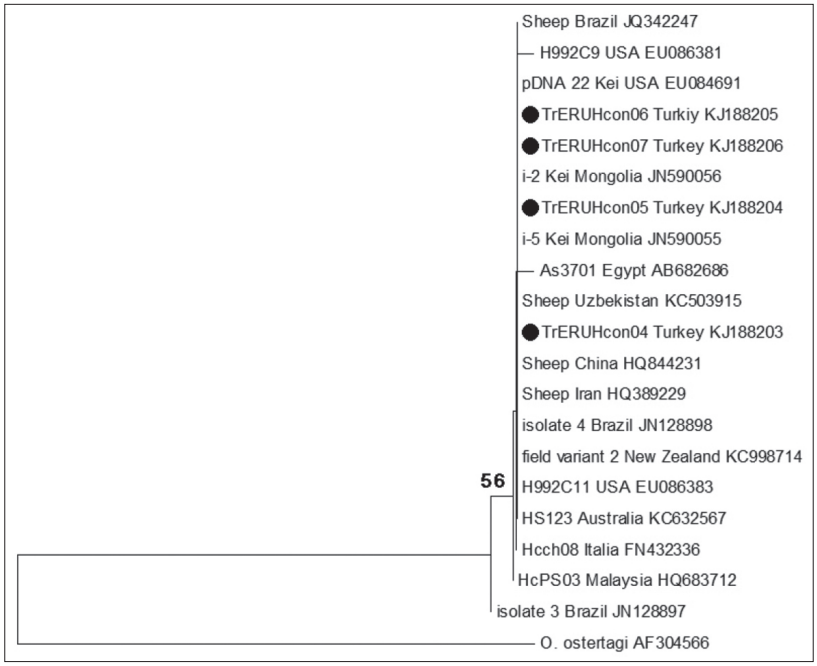

Fig 1. Phylogenetic relationship among the obtained *H. contortus* isolates (with the symbol ●) and some other *H. contortus* isolates from different countries with in the same or different genotypes as inferred from sequences of the ribosomal ITS-2 gene. The sequences were given as isolate name, country and GenBank accession number. The numbers above branches indicate neighbor-joining bootstrap supports (1000 replicates). Scale bar indicates number of nucleotide substitutions per site. *Ostertagia ostertagia* (AF304566) was used as an out group

Şekil 1. Ribosomal ITS-2 gen bölgesi sekanslarına göre elde edilen *H. contortus* izolatları (● sembolü ile işaretli) ile Dünyada çeşitli bölgelerden aynı veya farklı genotipteki diğer bazı *H. contortus* izolatları arasındaki filogenetik ilişki. Sekanslar izolat ismi, ülke ve GenBank aksesyon numarası olarak verilmiştir. Branşların önündeki rakamlar neighbor-joining bootstrap desteğini (1000 tekrar), ölçek çizgisi bölgeye göre nükleotid değişimini göstermektedir. Dış dal olarak *Ostertagia ostertagia* (AF304566) kullanılmıştır

Fig 2. Phylogenetic relationship among the obtained *H. contortus* isolates (with the symbol ●) and some other *H. contortus* isolates from different countries with in the same or different genotypes as inferred from sequences of the partial mt-COI gene. The sequences were given as isolate name, country and GenBank accession number. The numbers above branches indicate neighbor-joining bootstrap supports (1000 replicates). Scale bar indicates number of nucleotide substitutions per site. *Ostertagia ostertagia* (AB246112) was used as an out group

Şekil 2. Mt-COI gen bölgesi sekanslarına göre elde edilen *H. contortus* izolatları (● sembolü ile işaretli) ile Dünyada çeşitli bölgelerden aynı veya farklı genotipteki diğer bazı *H. contortus* izolatları arasındaki filogenetik ilişki. Sekanslar izolat ismi, ülke ve GenBank aksesyon numarası olarak verilmiştir. Branşların önündeki rakamlar neighbor-joining bootstrap desteğini (1000 tekrar), ölçek çizgisi bölgeye göre nükleotid değişimini göstermektedir. Dış dal olarak *Ostertagia ostertagia* (AB246112) kullanılmıştır

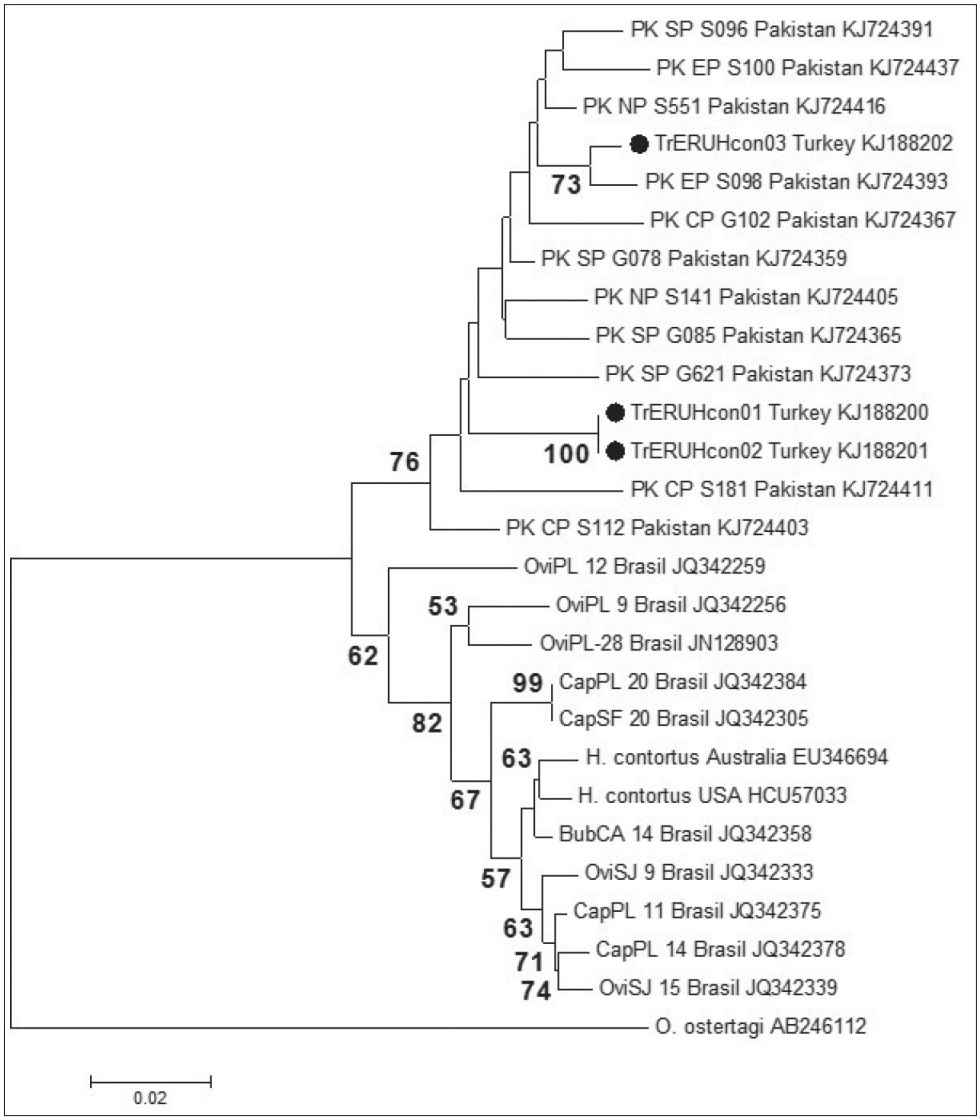

Table 2. *BZ sensitive and resistant allele frequencies in H. contortus populations according to age groups and gender of infected sheep*
Tablo 2. *Enfekte koyunların yaş grupları ve cinsiyetine göre H. contortus populasyonlarında BZ duyarlı ve dirençli allel sıklıkları*

Factor		Allele Frequency (%)					
		Sensitive		Resistant		F	P
		Min-Max (95% Confidence)	Mean+St.dev	Min-Max (95% Confidence)	Mean+St.dev		
Age group	≤2	55.2-100.0	83.2±18.5	0.0-44.8	16.8±18.5	0.820	0.372
	>2	56.9-100.0	88.6±15.3	0.0-43.1	11.4±15.3		
Gender	Male	55.2-100.0	81.8±23.6	0.0-43.1	18.2±23.6	0.349	0.559
	Female	56.9-100.0	87.6±15.8	0.0-44.8	12.4±15.8		
Total		55.2-100.0	87.1±16.2	0.0-44.8	12.9±16.2	378.404	0.000

F: *Anova Test;* ***St.dev:*** *Standard deviation*

significant (p<0.001). No statistically significant difference (P>0.05) was found in the susceptible and resistant allele frequencies over age groups and gender of sheep.

Totally 14 isolates in which 9 had a susceptible allele frequency of 100.0% and 5 had a resistant allele frequency of over 39.0% were subjected to the sequence analyses in order to screen the point mutations in β-tubulin isotype 1 gene. Sequence analyses of the 9 isolates with 100.0% susceptible allele frequency and 3 of 5 isolates with over 39.0% resistant allele frequencies revealed that phenylalanine (TTC), phenylalanine (TTC) and glutamine (GAA) amino acids were present in 167, 200 and 198 codons as in BZ susceptible populations. Whereas a point mutation [transformation of phenylalanine (TTC) to tyrosine (TAC)] only at the codon 200 was detected in both remaining two isolates from over 39.0% resistant allele frequencies. Two isolates belong to susceptible (TrERUHcon08) and resistant (TrERUHcon09) alleles were deposited to the GenBank under accession numbers KJ410522 and KJ410523, respectively.

DISCUSSION

The prevalence and intensity of gastrointestinal nematodes in sheep may vary in different regions. The present study reports the first molecular prevalence of *H. contortus* in Turkey with a rate of 24.8% in sheep, which indicates high prevalence of the parasite and insufficient control measurements in the study area. The number of nematode eggs in a fecal sample varies according to such factors as host and parasite species. Generally less than 500, 500-1500 and over 1500 EPG are considered as low, moderate and high infection levels, respectively [21]. In this study a mean of 120.3±53.5 EPG was determined in infected sheep in the study area which indicates a low infection level. This low level might be attributed to several factors such as host immunity, time of sampling and pasture status depending on climatic conditions and anthelmintic treatment process. However, age depending differences in EPG values was found statistically significant in the study and the sheep over 2 age group had higher EPG values than under ones. This result is also in accordance and supported with the *H. contortus* parasitemia levels which were indicated by Ct values in qPCR assays in our study. This could be related to the development of hypobiotic larvae (arrested at the early L4 stage), triggered by periparturient relaxation of immunity which is also indicated by several researchers [21-23].

Phylogenetic characterization and genome sequencing techniques on several gastrointestinal parasites have been contributed to improve knowledge of the biology and physiology of these parasites [1]. Genetic characterization of these parasites is also an important indicator for the diagnosis of drug resistance and formation of the control strategies for the parasite infections [6]. In this perspective, the current study evaluated the genetic diversity in *H. contortus* from sheep in Kayseri region for the first time in Turkey based on the sequences of a mitochondrial DNA marker (mt-COI) and a nuclear ribosomal marker (ITS-2) which have been widely used in phylogenetic characterization of trichostrongylid nematode species [1,4,5]. Genetic diversity among the Kayseri isolates based on the mt-COI gene was determined as higher than ITS-2 gene region which indicates the usefulness of mt-COI gene in the investigation of intraspecific variations and genetic variability in *H. contortus* populations as also indicated by Hussain et al.[5]. However the regional mt-COI sequences in GenBank are restricted with only the isolates from sheep, cattle and goats in USA, Australia, Brazil and Pakistan. As it has been seen in several nematodes, *H. contortus* has also a high biotic potential along with a high infection rate and direct life cycle which does not need an intermediate host. These feature gains this parasite a large effective population size with wide genetic variability [5,24]. In accordance with this situation on the characteristics of *H. contortus*, genetic variability based on mt-COI phylogeny among the Kayseri isolates and the other isolates from Pakistan, USA, Brazil and Australia was also found high with a mean rate of 5.5%. The mt-COI sequence analyses also indicates that *H. contortus* Kayseri isolates are genetically

more close to the Pakistan isolates than the isolates from USA, Brazil and Australia which might be attributed to geographical proximity of the Pakistan than the others.

BZs have been widely used as main drugs for the last five decades to control the trichostrongylid nematodes in different hosts and showed anthelmintic activity over β-tubulin, by leading to interference in microtubule polymerization dynamics. The wide usage of BZs has leaded to co-evolution of resistant parasite alleles across worldwide [1,25]. BZ resistance in *H. contortus* populations has been well explored and is a model for the studies on population genetics of anthelmintic resistance due to the availability of molecular tools and increasing knowledge of its genetics and population biology [26,27]. In general *in vivo* and *in vitro* methods such as fecal egg count reduction test (FECRT) and the egg hatch assay (EHA) has been widely used for BZ resistance in trichostrongylids [28]. However these techniques are insufficient for measuring the level of resistant and sensitive alleles within the population and also *in vivo* tests are rather slow and expensive. Thus, in recent years several rapid, reliable and sensitive molecular tools including real time PCR in order to explore resistant alleles have been developed [8,18,29]. The current study provides the first molecular data and allele frequencies on the BZ resistance in *H. contortus* populations from sheep in Turkey by utilizing highly sensitive qPCR assay targeting the β-tubulin isotype 1 gene as also indicated by several researchers [8,18,29]. A mean resistant allele frequency of over 12.0% determined with this study indicates an increasing risk for sustainably control of the parasites in the region in which the commercial deworming agents have been only choice for struggling with trichostrongylid nematodes.

Three non-synonymous SNPs in the isotype-1 β-tubulin gene have been described associated with benzimidazole resistance in *H. contortus* until today. A point mutation in codon 200 (TTC to TAC), causing a phenylalanine to tyrosine substitution, is the most common SNP and often at high frequency in several countries [1,26,27,30]. While the SNPs at codons 167 (TTC to TAC) and 198 (GAA to GCA) are less common and have been reported in a number of different countries [1,11,20,27,31]. In the present study sequence analyses of the isolates which had an over 39.0% resistant allele frequency revealed the existence of only the widest SNP at codon 200 in *H. contortus* populations in the study area which is in agreement with above studies [1,26,27,30]. However further studies should be conducted on large scale isolates from different regions in Turkey in order to explore true picture of the benzimidazole resistance in *H. contortus* populations from livestock which is essential for guiding and establishing effective control strategies.

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