



PCR-RFLP STUDY ON DRUG RESISTANCE OF *HAEMONCHUS CONTORTUS* TO BENZIMIDAZOLE IN SHEEP, NORTH REGION OF IRAN

M. HAMZEHALI TEHRANI¹, B. SHEMSHADI¹, P. SHAYAN²,
S. SHIRALI³ & N. PANAHI⁴

¹Department of Pathobiology, Science and Research Branch, Islamic Azad University, Tehran, Iran; ²Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Iran; ³Department of Biotechnology, Ahvaz Branch, Islamic Azad University, Ahvaz, Iran; ⁴Department of Basic Sciences, Faculty of Veterinary Medicine, Sciences and Research Branch, Islamic Azad University, Tehran, Iran

Summary

Hamzehali Tehrani, M., B. Shemshadi, P. Shayan, S. Shirali & N. Panahi, 2024. PCR-RFLP study on drug resistance of *Haemonchus contortus* to benzimidazole in sheep, North region of Iran. *Bulg. J. Vet. Med.*, 27, No 1, 47–56.

Haemonchosis is one of the most important parasitic diseases of the gastrointestinal tract of small ruminants in different parts of Iran and worldwide. While the disease poses major economic problems in the livestock industry, there are many reports on parasites' resistance to benzimidazoles necessitating to study of the level of this drug resistance in pathogens. The aim of this study was to evaluate the drug resistance of *Haemonchus contortus* to benzimidazole using molecular method in sheep from the North region of Iran. In this study, the resistance to benzimidazole was investigated using the PCR-RFLP method in the nematode *Haemonchus contortus* from sheep slaughtered in the northern region of Iran (Gilan and Mazandaran provinces). The samples examined in this study were evaluated between April 2020 and September 2021 from a total of 2400 sheep by determining the age groups of <2 years, 2–4 years, and >4 years. Three hundred *Haemonchus contortus* nematodes were randomly selected and investigated by means of PCR-RFLP and using Taal endonuclease enzyme (SNP) in the beta-tubulin gene, responsible for drug resistance of *Haemonchus contortus* to benzimidazole. *Haemonchus contortus* was found in 66.8% and 60.5% of sheep in Gilan and Mazandaran. The results of the study showed that the drug sensitivity rate was 24% (72/300), the drug resistance rate was 54% (162/300) and 22% (66/300) of the samples had both resistant and sensitive alleles. Presented data showed a high level of prevalence of drug resistance in the nematode *Haemonchus contortus* in sheep in the northern region of Iran, which requires special attention to control the development of this resistance and the epidemic of haemonchosis in ruminants.

Key words: benzimidazole, drug resistance, haemonchosis, *Haemonchus contortus*, PCR-RFLP

INTRODUCTION

The use of benzimidazole compounds is very common to control the digestive nematodes of small ruminants in Iran as in many other countries. Resistance to benzimidazoles is one of the major problems in sheep production and breeding worldwide, and there are many reports on resistance of this parasite to benzimidazoles. In some regions, such as South Africa, the South Pacific, and South America, resistance to benzimidazoles presents a major threat to livestock production. Past studies have shown that the presence of point mutations (SNP) in codons 167, 198, and 200 of the nematode beta-tubulin gene is associated with resistance to benzimidazoles (Samson-Himmelstjerna, 2006; Rufener *et al.*, 2009; Gary Ian *et al.*, 2013). In *H. contortus*, a point mutation (SNP) in the codon 200 of the isotype 1 beta-tubulin gene leads to the replacement of phenylalanine (TTC) with tyrosine (TAC), which is the most important mutation associated with resistance to this class of drugs (Ghisi *et al.*, 2007). Due to the highly pathogenic effect of *H. contortus* on animal health and significant economic losses to the country's livestock production system, the need for such a genetic analysis has become apparent. PCR-RFLP method for detection of spot mutation (SNP) in codon 200 of beta-tubulin 1 gene of this nematode was first used by Tiwari *et al.* (2006) at the Taal endonuclease (ACT/GT) cleavage site and showed that the mutation A point (TTC to TAC) causes a cut site for the enzyme. In the present study, the *Haemonchus contortus* beta-tubulin gene was amplified using specific primers and evaluated by the PCR-RFLP method.

The aim of this study was to evaluate the drug resistance to benzimidazole of *Haemonchus contortus* infecting sheep

from the North region of Iran using molecular method.

MATERIALS AND METHODS

Areas under study

The climatic zone in the North Iran geographical areas is the coastal region of the Caspian Sea, with an annual rainfall of 40 to 150 cm. There is rainfall in most months of the year. The relative humidity is high, and the average annual temperature ranges from 8 to 26. Rasht city (Gilan province) is located in region 1 of the climate classification of Iran. Rasht has an area of about 180 square kilometers. Its altitude is 8 m above sea level, its average annual temperature is 15.9 °C, and its annual rainfall is 1,359 mm. Its annual temperature ranges from 37 to -5 °C. The second city Sari (Mazandaran province), is located in region 1 of the climate classification of Iran. Sari has an area of about 54 square kilometers. Its altitude is 54 m above sea level, its average annual temperature is 15 °C, and its annual rainfall is 789.2 mm. Its annual temperature is 22.4 to -5.2 °C.

Sample collection

This cross-sectional descriptive study was performed on 2,400 slaughtered sheep (1,200 heads from each province of Gilan and Mazandaran) in industrial slaughterhouses by simple random sampling. The population of sheep in each province is divided into two groups of 600 males and females, each subdivided into three age groups: less than two years, two to four years, and over four years. Samples were collected after obtaining permission from the relevant authorities and coordination with the technical officials of slaughterhouses from April 2020 to September

2021. After dissection and examination of the abomasum, the abomasum of the slaughtered sheep was completely shaved using a plastic file, kept in separate sampling containers, and transferred to the laboratory for separation. Then the sample was pour into a stainless-steel sieve (100 mesh) and rinsed with tap water. The samples were examined with lactophenol (clarifying solution) under a light microscope (Nikon YS2-Japan), and 500 isolated *H. contortus* (males) were randomly selected from the approved samples. They were isolated and washed three times with saline phosphate buffer solution (PBS) and kept in 1.5 mL microtubes with 96° ethanol (fixing solution) in the freezer at -20°C until DNA extraction (Mohammedsalih *et al.*, 2020).

DNA extraction and polymerase chain reaction

For the DNA extraction, the worms were removed from ethanol, dried, washed in PBS (phosphate-buffered saline), and stored for 1–2 days without any buffer in 1.5 mL microtubes at -20 °C. The extraction of DNA from a single worm was performed using a DNA extraction kit (MBST, Iran) according to the manufacturer's instructions. All extracted DNA was stored at -20 °C until used. The PCR primers were designed from the beta-tubulin isotype 1 gene sequence of *Haemonchus contortus*, registered under ac-

cession number X674890 in Gene Bank. The DNA was amplified using the primers P1 (forward) and P2 (reverse) to obtain a PCR product of 403 bp length containing codon 200. For the semi-nested PCR reaction, primers P3 (forward) and P2 (reverse) with accession number X67489 (World Gene Bank) was used with 225 bp PCR product (Table 1).

The PCR was performed in a total volume of 50 µL including 4 µL genomic DNA, 1× PCR buffer, 1.5 U Taq polymerase (Ampliqon, Denmark), 30 pmol of each primer (Metabion, Korea), 100 µM of each dATP, dTTP, dCTP, and dGTP (Ampliqon, Denmark), and 1.5 mM MgCl₂ in an automated thermocycler (SimpliAmp, USA) with the following program: 5 min incubation at 95 °C to denature double-strand DNA, 35 cycles of 45 s at 94 °C (denaturing step), 45 s at 60 °C (annealing step), 45 s at 72 °C (extension step). Finally, PCR was completed with the additional extension step for 10 min. Samples without genomic DNA were used as negative controls. The PCR products were analysed on 1.5% agarose gel in 0.5× TBE buffer and visualised using ethidium bromide and UV illuminator.

PCR-RFLP

Restriction enzyme Taal was used for restriction fragment lengths polymorphism (RFLP) analysis. Before PCR-

Table 1. Nucleotide sequences of primers for the detection of drug resistance of *Haemonchus contortus* based on the PCR-RFLP method

Reactions	Name of primer (accession number of the corresponding gene)	Nucleotide sequence (5'– 3')	PCR product
First PCR for all samples	P1 (X674890)	GTTCTCCGTTGTTCCATCACCC	403 bp
	P2 (X674890)	CGTGACACCAGACATTGTGACAG	
Semi-nested PCR	P3 (X67489)	CTACCCTTTCCGTCCATCAA	225bp
	P2 (X67489)	CGTGACACCAGACATTGTGACAG	

(RSC-PCR-RFLP)
TaaI ACNGT

A

```

TAGAGAACACCGATGAAACATCTGTATTGACAACGAAGCTCTGTATGATATCTGCTTCGCGACTTTGAA
ACTCACAAATCCAACCTATGGAGATCTCAACCACCTTGGTAAATGTTGTTACACTCGCCAAAGTATATTT
AGACTCTTTTACATGCTGAAAATGTGCAATTGAAGTGTCTGTCTCACAATGTCTGGTGTCTCAGACCTGCCT
TCGATTCCCTGGACAACCTGAATGCTGATCTTCGCAAGTTAGCCGTAAACATGGTTCCATTCCCTCGTCTT
CACTTCTTCATGCCCGGTTTGTCTCCACTGTCTGCAAAGGGTGTCTCAAGCATATCGCGCTTCGACAGTTG
CTGAGCTTACACAGCAAGTACGCCATTACTCTTTATAGCATAGTGATTGCGAATAACAGCCACCCATAT
TCTTAGATGTTTCGATGCAAAGACATGATGGCTGCCTGTGATC
    
```

Fig. 1. Sequence of beta-tubulin isotype-1 gene of *Haemonchus contortus* and point mutation site (SNP).

RFLP analysis, the first PCR product was purified using a PCR purification kit (MBST, Iran), according to the manufacturer's instructions. Briefly, 200 µL binding buffer was added to 100 µL PCR product solution. After adding 150 µL ethanol (96%) to the sample, the mixture was poured into the column. The column was washed twice with washing buffer, and the PCR product was eluted from the column using 100 µL elution buffer. The purified PCR product was amplified twice, and semi-nested PCR was used for the application of TaaI.

Semi-nested PCR and application of restriction enzyme (TaaI)

Enzyme digestion method using TaaI endonuclease enzyme (HpyCH4III) from Thermo Scientific (a 5'... ACN ↓ GT ... 3' sequence cutter), was used to investigate the point mutation (SNP) in the beta-tubulin isotype 1 gene (Fig. 1). PCR-RFLP reaction ingredients included semi-nested PCR at 10 µL, Tango 10* at 2 µL, TaaI endonuclease enzyme (HpyCH4III) at 1 µL (Thermo scientific, 10 U/µL), and distilled water at 7 µL. After adding the enzyme, the sample was incubated for 2 hours at 37 °C and at the same time, a sample of semi-nested PCR product without adding the enzyme was placed in the incubator at 37 °C as a control. Then, to observe the cuts and the weight of the

bands, the samples were placed on 1.5% agarose gel. Using a UV Illuminator, DNA bands (PCR products) were examined on the gel and photographed.

The point mutation (single nuclear polymorphism) created in the sequence of isotype number 1 of the beta tubulin gene associated with drug resistance to benzimidazole is associated with a change in the amino acid phenylalanine with the code (TTC) to the amino acid tyrosine with the code (TAC). As a result, this mutation will create a cutting site for the mentioned endonuclease enzyme in the DNA sequence amplified by the PCR method. The length of the drug-sensitive nematode *Haemonchus contortus* (SS) DNA strand is 225 bp, which is due to the lack of a cut point in the mentioned position, while if there is a cut point due to drug resistance in this strand, the length of the fragment is 48 bp. and it will be 177 bp, which is known as homozygous resistant (RR). On the other hand, samples with a resistant allele and a sensitive allele have a fragment length of 225, 48 and 177 bp, which are known as heterozygous (RS) samples. It should be noted that 48 bp bands were not visible in gel electrophoresis due to the short length of the part and the low intensity of the staining.

To confirm the function of the restriction enzyme and the presence of point

mutation (SNP) from sensitive homozygous (SS), resistant homozygous (RR), and heterozygous (RS) samples of each province, the nucleotide sequence of PCR product was determined through Kowsar Company (Iran).

Data analysis

Statistical analysis was performed by using the software package SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Paired sample t-test test was done and P-value of less than 0.05 was considered statistically significant.

RESULTS

The adult *Haemonchus contortus* are easily identified because of their specific location in the abomasum and their large size (2.0–3.0 cm). Microscopically, the male has an asymmetrical dorsal lobe and barbed spicules (Fig. 2A); the female usually has a vulval flap. In both sexes, there are cervical papillae and a tiny lancet inside the buccal capsule. Infective larvae have 16 gut cells, the head is narrow and rounded and the tail of the sheath is offset. In fresh specimens, the white ovaries winding spirally around the blood-filled intestine produce a 'barber's pole' appearance (Fig. 2B). The egg is medium-

sized (74×44 µm), resembling a regular broad ellipse with barrel-shaped sidewalls and numerous blastomeres, which nearly fill the entire egg (Fig. 2C).

The amplified fragment size was 403 bp after the initial PCR reaction with primers P1, P2, and 225 bp in a semi-nested PCR reaction with primers P2 and P3 to investigate the presence of point mutation (SNP) in the amplified sequence (Fig. 3). All selected samples could be amplified with the mentioned primers.

From the examination of 300 DNA samples extracted from *H. contortus* using the PCR-RFLP method, 72 samples (24%) had two alleles encoding phenylalanine and were sensitive homozygous (SS), 162 samples (54%) contained two alleles encoding tyrosine (resistant homozygous, RR) and 66 samples (22%) had a healthy allele and a resistant allele (heterozygous, RS) (Table 2).

The prevalence of infection with *Haemonchus contortus* in Gilan province was 66.8% (802/1200) and 60.5% (726/1200) in Mazandaran province. In terms of host sex, rates were 32.75% and 34% in Mazandaran province and 30.3% and 30% in Gilan province for male and female sheep respectively. According to the age, the infection with this parasite in both provinces was the highest in sheep

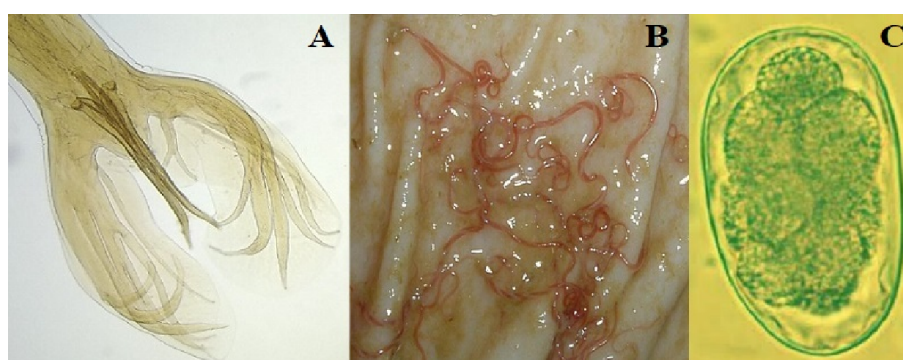


Fig. 2. A. *Haemonchus contortus* male; B. *Haemonchus contortus* macroscopic appearance; C. *Haemonchus contortus* egg.

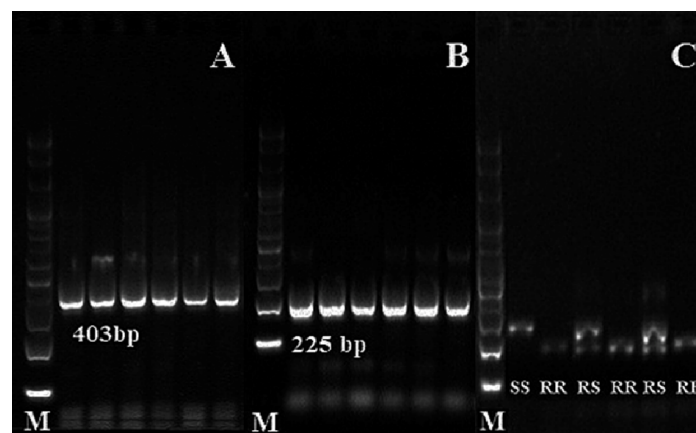


Fig. 3. **A.** Genomic DNA from some adult male *H. contortus* was amplified to obtain PCR products of 403 bp; **B.** Semi-nested PCR was performed on the first 403 bp PCR products with *TaaI* restriction enzyme; **C.** Semi-nested PCR products digested by *TaaI*. The first lane with uncut product 225 bp was identified as sensitive homozygous (SS); lanes showing the formation of 48 bp and 177 bp bands, correspond to resistant homozygous (RR) pattern, and lanes showing 48, 177 and 225 bp bands are heterozygous (RS) specimens. The 48 bp bands were not visible in gel electrophoresis due to the short length and the low intensity of the staining. M= 100 bp marker.

Table 2. Results of enzymatic cleavage on the population of selected *Haemonchus contortus*

Province	Number of samples	Homozygous sensitive (SS)	Homozygous resistant (RR)	Heterozygous (RS)
Gilan	150	30	78	48
Mazandaran	150	42	84	18
Total	300	72	162	66

less than two years old (25.5% and 25.75%). These rates were 23.6% and 22.25% for the age group of 2 to 4 years and lower in sheep above 4 years (17.9% and 12.5%) (Table 3).

DISCUSSION

In recent years, an increase in the prevalence of resistance to benzimidazoles has been reported in many parasitic nematodes, while the incorrect use of anthelmintics as benzimidazoles causes an increase in drug-resistant species (homozygous resistant and heterozygous) and elimination of susceptible species. Over

time, this issue causes a change in the parasite population and a much higher prevalence of drug-resistant species. Scientists believe that early detection of drug resistance to worms is a critical point in farm management, and the use of prevention strategies at this stage can effectively reduce the speed of action of resistance (Dong dong *et al.*, 2019; Arsenopoulos *et al.*, 2020). In Iran, sheep and goats are very important in the livestock industry. However, due to the dry climate in most parts of the country and the severe cold in winter, the abomasum infectious burden with nematodes is lower than in many other countries around the world. Hence, the observation of clinical symp-

Table 3. *Haemonchus contortus* prevalence and rate of infection in sheep from Gilan and Mazandaran provinces by host age and sex

	Gilan province (n=802)		Mazandaran province (n=726)	
	Number	Infected (%)	Number	Infected (%)
Gender				
Male	393	32.75	364	30.30
Female	409	34.00	362	30.00
Age				
<2 years	303	25.50	309	25.75
2–4 years	284	23.60	267	22.25
>4 years	215	17.90	150	12.50

the observation of clinical symptoms due to high infection rarely occur. Since the growth of larvae of *H. contortus* occurs optimally at relatively high temperatures, haemonchosis is primarily a sheep disease in warm climates. The frequency and severity of the disease outbreak largely depend on the amount of rainfall in each particular area (Shayan *et al.*, 2007). Due to the unfavourable environmental situation in Iran, infection with *Haemonchus contortus* is less than among other nematodes, especially *Teladorsagia circumcincta* and *Marshallagia marshalli* (Shokrani *et al.*, 2012).

One of the major disadvantages of the stool egg count reduction test (FECRT), which is a widely used method for evaluating the effect of anthelmintic drugs, is the low sensitivity for detecting drug resistance of worms in one species to the invasion of nematodes of several species (Coles *et al.*, 2006). However, the high sensitivity of molecular methods in detecting such resistance has been outlined (Palcy *et al.*, 2010). On the other hand, in *H. contortus*, single nucleotide polymorphism (SNP), which is more associated with resistance, has been reported in the codon 200 of isotype 1 beta-tubulin

(Walsh *et al.*, 2007). Therefore, using effective molecular methods such as PCR-RFLP, the detection of SNP in codon 200 is considered one of the best options for evaluating the resistance to benzimidazoles in *H. contortus*. Field analysis based on faecal egg count reduction test for albendazole resistance in sheep gastrointestinal nematodes in Khuzestan province showed resistance to *Teladorsagia circumcincta* and *Marshallagia marshalli*, while no resistance was observed in *Haemonchus contortus* (Nabavi, 2017). Some scientists believe that the rate of mutation can increase under environmental stress, and this theory has been proven for bacteria (Kaushik *et al.*, 2016; Ramünke *et al.*, 2016; Zongze *et al.*, 2016; Baltrušis *et al.*, 2018). However, the present study shows quite definite results. Resistance to benzimidazole was predicted to increase further under high dry environmental stress. The most important factor that has been confirmed in increasing anti-worm resistance is the repeated use of the same anti-worm drug (Zuccherato *et al.*, 2018). In Iran, due to the harsh climate, the infectious load of sheep abomasum nematodes is low. Therefore, even though anti-worm com-

pounds are significantly less frequently used in therapeutic periods compared to other parts of the world, the issue is using an amount that is lower than the effective dose. Although the use of benzimidazole in the country has a long history, the rate of induction of resistance is slower than in many parts of the world. Many factors other than the genetics of worms are involved in the dynamic process of resistance selection, including the biology and epidemiology of the parasite, the dynamics of the host-parasite relationship, the frequency of treatments, and treatment strategies (Kaplan, 2004). It has been shown that environmental conditions have a great impact on resistance behaviour. The results of this study showed that the resistance of *H. contortus* to benzimidazole in Iran, contrary to what was predicted in the past, is a very serious problem, and today is the best time to apply preventive measures against this great danger to the livestock production system. In the study of Nabavi *et al.* (2011), 102 adult male *H. contortus* worms were tested. No resistant allele (homozygous or heterozygous) in codon 200 of isotype 1 was provable, and it was shown that the worm population of Khuzestan, Isfahan, and Mazandaran provinces all have two benzimidazole sensitive alleles. In the study of Shokrani *et al.* (2012), all 138 *H. contortus* worms obtained from three geographical locations of Iran including Babol (north), Chadegan (center), and Shushtar (south), and tested by PCR-RFLP contained two alleles encoding phenylalanine in codons 167 and 200 of the beta-tubulin gene. Moreover, no resistant allele (resistant homozygous or resistant heterozygous) was observed in the obtained samples. To control ruminant diseases, especially parasitic diseases, paying special attention to this issue is more important than ever.

The results of the present study showed that haemonchosis and drug resistance due to genetic mutation is a crucial and fundamental issue for the livestock industry economy. In the process of drug resistance detection, sensitive diagnostic methods such as PCR-RFLP are very important because if benzimidazole resistant nematodes reach moderate levels in the general population, there is no possibility of reversal even if another anthelmintic is used without any cross-resistance. This can lead to the spread of drug resistance within the nematode population.

REFERENCES

- Arsenopoulos, K., S. Minoudi, I. Symeonidou, A. Triantafyllidis, A. Katsafadou, D. T. Lianou, G. C. Fthenakis & E. Papadopoulos, 2020. Frequency of resistance to benzimidazoles of *Haemonchus contortus* helminthes from dairy sheep, goats, cattle, and buffaloes in Greece. *Pathogens*, **9**, 347.
- Baltrušis, P., P. Halvarsson & J. Höglund, 2018. Exploring benzimidazole resistance in *Haemonchus contortus* by next-generation sequencing and droplet digital PCR. *The International Journal for Parasitology*, **8**, 411–419.
- Changizi, E. & M. Salimi Bajestani, 2011. Guide Laboratory Procedures and Identification, University of Semnan Press.
- Coles, G. C., F. Jackson & W. E. Pomroy, 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology*, **136**, 167–185.
- Dong-Dong, S., P. Zhi-Wei, M. Hu, Z. Zhang, Z. Hou & Z. Sheng Liu, 2019. A detection of benzimidazole resistance-associated SNPs in the isotype-1 β -tubulin gene in *Haemonchus contortus* from wild blue sheep (*Pseudois nayaur*) sympatric with sheep in Helan Mountains China. *Biomed central Veterinary Research*, **15**, 89.

- Gary Ian, S., J. D. Wasmuth, R. Beech, R. Laing, M. Hunt, H. Naghra, J. A. Cotton, M. Berriman, C. Britton & J. S. Gilleard, 2013. Characterization and comparative analysis of the complete *Haemonchus contortus* β -tubulin gene family and implications for benzimidazole resistance in strongylid nematodes. *The International Journal for Parasitology*, **43**, 465–475.
- Ghisi, M., R. Kaminsky & P. Maser, 2007. Phenotyping and genotyping of *H. contortus* isolates reveals a new putative candidate mutation for benzimidazole resistance in nematodes. *Veterinary Parasitology*, **144**, 313–320.
- Hosseini, H. & B. Meshgi, 2010. *Veterinary Parasitology and Helminthology*, University of Tehran Press.
- Kaplan, R. M., 2004. Drug resistance in nematodes of veterinary importance a status report. *Trends in Parasitology*, **20**, 477–481.
- Kaushik, R., P. K. Rout, D. K. Sharma & S. Paul, 2016. Molecular characterization of beta-tubulin isotype-1 gene of *Haemonchus contortus* in goats with focus on benzimidazole resistance. *Animal Science Reporter*, **10**, 48–54.
- Mohammedsalih K. M., M. Salih, J. Krücken, A. Khalafalla, A. Bashir, F. R. Juma, A. Abakar, A. Abdalhakaim, H. Abdalmalaik, G. Coles & G. Von Samson-Himmelstjerna, 2020. New codon 198 β tubulin polymorphisms in highly Benzimidazole resistant *Haemonchus contortus* from goats in three different states in Sudan. *Parasites & Vectors*, **13**, 114.
- Nabavi, R., 2017. Comparison of morphologic and morphometric parameters in *Haemonchus* nematodes separated from domestic ruminants in the southeast of Iran. *Journal of Veterinary Research*, **72**, 15–21.
- Nabavi, R., P. Shayan, H.R. Shokrani, A. Eslami & S. Bokaie, 2011. Evaluation of benzimidazole resistance in *Haemonchus contortus* using comparative PCR-RFLP methods. *Iranian Journal of Parasitology*, **6**, 45–53.
- Palcy, C., A. Silvestre, C. Sauve, J. Cortet & J. Cabaret, 2010. Benzimidazole resistance in *Trichostrongylus axei* in sheep: Long-term monitoring of affected sheep and genotypic evaluation of the parasite. *The Veterinary Journal*, **183**, 68–74.
- Ramünke, S., L. Melville, L. Rinaldi, H. Hertzberg, T. de Waal, G. N. Samson-Himmelstjerna, G. Cringoli, F. Mavrot, P. Skuce, J. Krücken & J. Demeler, 2016. Benzimidazole resistance survey for *Haemonchus*, *Teladorsagia*, and *Trichostrongylus* in three European countries using pyrosequencing including the development of new assays for *Trichostrongylus*. *The International Journal for Parasitology*, **6**, 230–240.
- Rufener, L., R. Kaminsky & P. Maser, 2009. *In vitro* selection of *H. contortus* for benzimidazole resistance reveals a mutation at amino acid 198 of beta-tubulin. *Molecular and Biochemical Parasitology*, **168**, 120–122.
- Samson-Himmelstjerna, G. N., 2006. Molecular diagnosis of anthelmintic resistance. *Veterinary Parasitology*, **136**, 99–107.
- Shayan, P., A. Eslami & H. Borji, 2007. Innovative restriction site created PCR-RFLP for detection of Benzimidazole resistance in *Teladorsagia circumcincta*. *Parasitology Research*, **100**, 1063–1068.
- Shokrani, H., P. Shayan, A. Eslami & R. Nabavi, 2012. Benzimidazole-resistance in *Haemonchus contortus*: new PCR-RFLP method for the detection of a point mutation at codon 167 of isotype 1 β -tubulin gene. *Iranian Journal of Parasitology*, **7**, 41–48.
- Tiwari, J., S. Kumar, A. P. Kolte, C. P. Swarnkar, D. Singh & K. M. Pathak, 2006. Detection of benzimidazole resistance in *H. contortus* using RFLP-PCR technique. *Veterinary Parasitology*, **138**, 301–307.
- Walsh, T. K., A. A. Donnan, F. Jackson, P. Skuce & A. J. Wolstenholme, 2007. Detection and measurement of benzimidazole resistance alleles in *H. contortus* using real-time PCR with locked nucleic acid Taqman probes. *Veterinary Parasitology*, **144**, 304–312.

- Zongze, Z., R. Gasser, X. Yang, F. Yin, G. Zhao, M. Bao, B. Pan, W. Huang, C. Wang, F. Zou, Y. Zhou, J. Zhao, R. Fang & M. Hu, 2016. Two benzimidazole resistance-associated SNPs in the isotype-1 β -tubulin gene predominate in *Haemonchus contortus* populations from eight regions in China. *The International Journal for Parasitology*, **6**, 199–206.
- Zuccherato, L. W., L. F. Furtado, C. D. Medeiros, C. S. Pinheiro & E. M. Rabelo, 2018. PCR-RFLP screening of polymorphisms associated with benzimidazole resistance in *Necator americanus* and *Ascaris lumbricoides* from different geographical regions in Brazil. *PLOS Neglected Tropical Diseases*, **9**, 66–67.

Paper received 12.04.2022; accepted for publication 30.06.2022

Correspondence:

Bahar Shemshadi
Department of Pathobiology,
Faculty of Veterinary Medicine,
2nd floor, block A west, ibn Sina Bld,
Islamic Azad University, Science and
Research Branch, Martyr Hesarak Blvd,
Sattari Highway, Tehran, Iran
phone: 00989125082497
fax: 00982144865154-8
e-mail: bshemshadi@yahoo.com
ORCID: 0000-0001-8996-3062