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Original Article

Evaluation of Benzimidazole Resistance in *Haemonchus contortus* Using Comparative PCR-RFLP Methods

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ABSTRACT

Background: In order to deworm the ruminants especially of sheep in Iran, consumption of benzimidazoles has more than 2 decades history and today farmers are using imidazothiazoles, macrocyclic lactones and mostly benzimidazole compounds (BZs) to treat infected farm animals. It has been demonstrated that the most common molecular mechanism leading to BZsresistance in *Haemonchus contortus* is a single mutation at amino acid 200 (phenylalanine to tyrosine) of the isotype 1 of beta tubulin gene. According to the report of such mutations in Iranian *Teladorsagia circumcincta* isolates with Restriction Site Created PCR-RFLP, we decided to evaluate the frequency of such mutations in *H. contortus* in three different geographical areas of Iran.
Methodes: A total of 102 collected adult male *H. contortus* were evaluated with PCR-RFLP (using PSP1406I as restriction enzyme). By means of a second step to compare function of different methods and to increase sensitivity of detection mechanism, a third of samples were examined by another PCR-RFLP method (using TaaI as restriction enzyme) and finally beta tubulin gene of two samples was sequenced.
Results: All of samples were detected as BZss homozygote. Finally, beta tubulin gene sequencing of two samples showed no point mutation at codon 200.
Conclusion: It seems that BZresistance of *H. contortus* in Iran is not a serious problem as anticipated before.
Keyword: *Haemonchus contortus*, Benzimidazole, Drug Resistance, PCR-RFLP, Beta tubulin gene

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Introduction

In order to control small ruminant's helminthes in Iran such as many other countries in the world, using benzimidazole compounds are very common and there are more than 2 decades passing from first usage of albendazole in Iranian sheep flocks (1).
BZs-resistances are of major problems in sheep production world wide and there are plenty of reports indicated that BZs-resistances are so prevalent. In some areas such South Africa, South Pacific and South America, the BZs-resistance is a very great threat to livestock production system (2- 4).
It was discovered that three Single Nucleotide Polymorphism (SNP) at codones 200,167 and 198 (was recently shown) of veterinary nematodes beta tubulin gene are considered to be closely linked with BZ-resistance (5-9).
In *Haemonchus contortus*, the SNP at codon 200, which leads to the substitution of phenylalanine to tyrosine, is most important mutation linked with BZ-resistance (2, 10, 6). First report of such mutation in Iranian sheep nematodes was illustrated in *Teladorsagia circumcincta* (11). Because of high pathogenic effect of *H. contortus* in animal health and substantial economic losses to livestock production system and because of very poor data about BZ-resistance in Iranian *H. contortus*, especially molecular information, the necessity of such genetic analysis was apparent. There are two available PCR-RFLP methods for the detection of SNP at codon 200 of *H. contortus*. Tiwari et al. (12) found that point mutation (TTC to TAC) at codon 200 created recognition site for TaaI enzyme (ACT/GT). They amplified the DNA fragment containing codon 200 triplet and the resulting PCR product was subsequently cut with the above mentioned restriction endonuclease TaaI to detect the point mutation (12). Shayan et al. (11) illustrated a new innovative PCR-RFLP method for detecting point mutation at codon 200 of beta tubulin. In their procedure a modified forward primer (University of Tehran, Faculty of Veterinary Medicine, modified forward primer, UTvet MF-primer) was used. Accordingly nucleotide A at the position 637 (Two nucleotide beside mutation site of beta tubulin of *Teladorsagia circumcincta*) was substituted by nucleotide G to create a restriction site for the restriction endonuclease PSP1406I.
In the present study, the beta tubulin gene of *H. contortus* was analyzed using both methods described above. In order to compare benzimidazole resistance situations of different environmental areas, a sampling procedure was done in three different geographical provinces of Iran.

Materials and Methods

Sample collection

Parasite samples were collected from native sheep Abomasa of Khuzestan, Isfahan (mountain area) and Mazandaran provinces. Thirty-four adult male *H. contortus* from each area (total number=102) identified morphologically and then all were stored in 70% ethanol until used.

DNA extraction and Polymerase Chain Reaction

For the DNA extraction, the worms were removed from ethanol, dried and washed in PBS (phosphate-buffered saline) and stored for 1–2 days without any buffer in 1.5 ml tube at −20°C. The extraction of DNA from single worm was performed using DNA extraction kit (MBST, Iran) according to the manufacturer's instructions. All extracted DNA were stored in −20°C until used. The PCR primers were designed from beta tubu-

lin isotype 1 gene sequence of *H. contortus*; registered under accession number x67489 in Gene Bank. The DNA was amplified using primers; P1 (forward) and P2 (reverse) to obtain a PCR product of 403 bp length containing codon 200 triplet (Table 1).

The PCR was performed in total volume of 50 µl including, 4µl genomic DNA, one time PCR buffer, 1.5U Taq polymerase (Cina Gene, Iran) , 30 pmol of each primer (Cina Gene, Iran), 100 µM of each dATP, dTTP, dCTP, and dGTP (Fermenta), and 1.5 mM MgCl2 in automated thermocycler (MWG, Germany) with the following program: 5 min incubation at 95°C to denature double-strand DNA, 35 cycles of 45s at 94°C (denaturing step), 45s 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 analyzed on 1.5% agarose gel in 0.5× TBE buffer and visualized using ethidium bromide and UV illuminator.

PCR-RFLP

Two different restriction enzymes PSP1406I and TaaI were used to restriction fragment lengths polymorphism analysis. Before PCR-RFLP analysis, the first PCR product was purified using 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 applied into the column. The column was washed twice with washing buffer, and PCR product was eluted from the column using 100 µl elution buffer. The purified PCR product was amplified twice. At first one semi nested PCR performed in order to complete PCR-RFLP with PSP1406I. Secondly another different semi nested PCR were used in order to application of TaaI.

Semi nested PCR and application of restriction enzyme (PSP1406I)

In order to create recognition site for PSP1406I, semi nested PCR technique was performed, using P3 primer (based on beta tubulin sequences of *H. contortus*) as a forward primer and P4 reverse primer (Table 1). We designed a modified forward primer from nucleotide in positions 2668 to 2709 (42-mer), in which, at the position 2708, the nucleotide A was substituted through the nucleotide G (purine/purine: P3-primer) to create a restriction site for the restriction endonucleases PSP1406I (Table 1). 0.01 microliter of each first purified PCR product used to perform semi nested PCR. Amplified fragment (222bp), was purified with PCR purification kit (MBST, Iran). Ten µl of each resulting semi nested PCR product was digested by 2 µl PSP1406I (Fermenta,10 U/µl) in 2 µl 10× buffer Tango and 18 µl nuclease-free water for 16 h at 37°C.

Semi nested PCR and application of restriction enzyme (TaaI)

From all specimens, 34 numbers were selected in order to examine with PCR-RFLP method was described by Tiwari et al. (12).We decided to complete this method on first PCR products (403 bp). Because of presence of another restriction site for TaaI (nucleotide 2643-2647, ACT/GT) rather than recognition target site (nucleotide 2710 2714, ACT/GT), straight application of TaaI on the first PCR products was inaccurate. For elimination of first restriction site another semi nested PCR was performed using P5 as a forward and P6 as a reverse primer (Table1). Subsequently amplified fragment (225bp) was digested with TaaI. Briefly the PCR product was first purified using PCR purification kit (MBST,Iran). After purification the 10 µl of each purified PCR product was digested with 2 µl TaaI(Fermenta,10U/µl) in 2 µl 10× buffer Tango and 16 µl nuclease-free water for 16h at 65°C. As enzyme

functional control, first PCR product (403bp) was digested with the restriction enzyme. In some cases, the nucleotide sequence of PCR product was determined through Kowsar Company (Iran).

Results

DNA was extracted from 102 single adult male *H. contortus* (34 numbers from each geographical zone). The isolated genomic DNA of all worms was separately used to amplify the isotype 1 beta tubulin gene. The isotype 1 beta tubulin specific PCR product showed an expected fragment of 403 bp in length (Fig. 1A). To create a restriction site for PSP1406I at codon 200, the PCR product was amplified using modified forward primer (P3) and P4 to obtain a PCR product of 222 bp in length (Fig. 1B1). This PCR product was then cut with restriction endonucleasis PSP1406 I to detect the SNP at codon 200. The normal allele (TTC) can be cut into two fragments (183bp and 39 bp), whereas the mutated allele (TAC) cannot be cut with PSP1406 I (Fig. 1B2). The DNA solutions extracted from 102 single worms were analyzed for the SNP at codon 200. The RFLP analysis showed that all 102 worms had two susceptible alleles (BZss homozygote). No resistant allele (BZrs heterozygote or BZrr homozygote) was demonstrable at this codon (Fig. 1B2).

The semi-nested PCR products were also used for the SNP at codon 200 using restriction endonuclease TaaI. The first PCR products were amplified using primers P5/P6 to obtain a PCR product of 225 bp in length (Fig. 1B3). This PCR product was the cut with restriction endonucleasis TaaI. The restriction endonucleasis TaaI recognizes the nucleotides ACTGT. Interestingly, the mutated allele shows nucleotide A at the first position of restriction site (ACTGT), whereas the normal allele has nucleotide T at this position. Consequently, the mutated allele can be cut with TaaI, whereas normal allele cannot be cut. The PCR-RFLP analysis showed that the Semi-nested PCR products of all 34 (Third of all samples) single worms could not be cut with the mentioned enzyme (Fig. 1B4). These results confirmed the results of PCR-RFLP prepared with restriction endonucleasis PSP1406I and showed that all worms had two susceptible alleles (BZss homozygote) at codon 200 and no resistant allele (BZrs heterozygote or BZrr homozygote) was demonstrable in this codon. For final confirmation of BZss homozygote, beta tubulin sequences (222bp) of two specimens were sequenced. Sequenced data indicated no point mutation at codon 200 (Fig. 2).

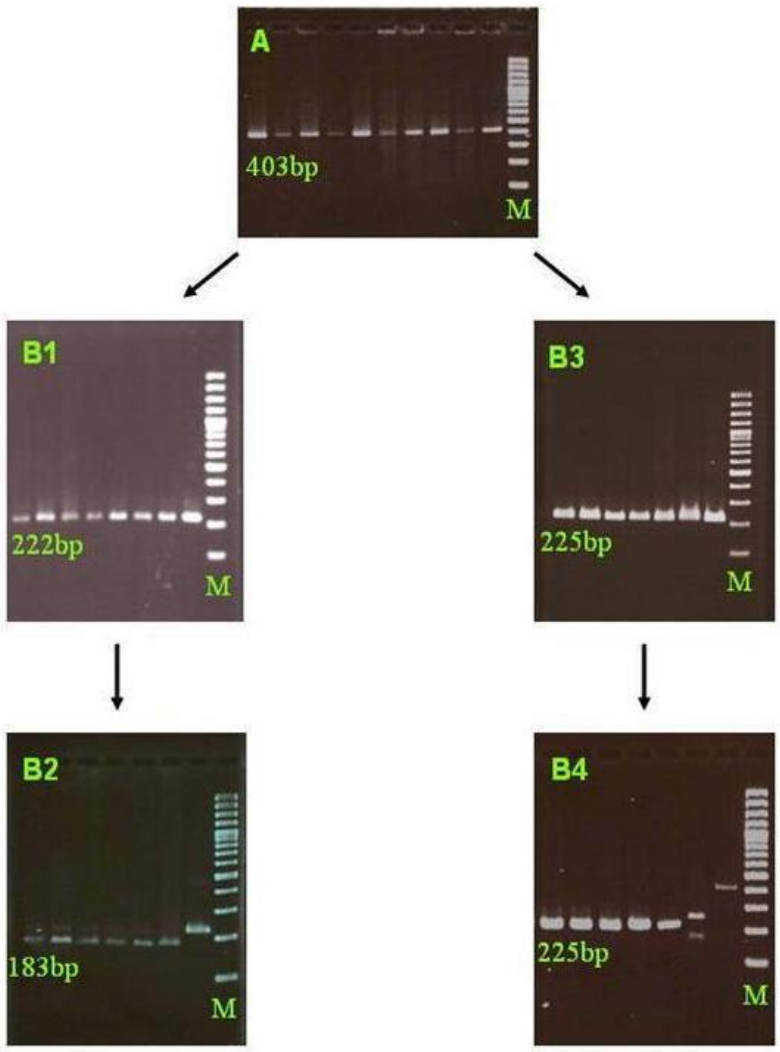

Fig.1: A, Genomic DNA from some adult male *H. contortus* were amplified to obtain PCR products of 403bp. **B1,** In order to create recognition site for PSP1406 I, semi nested PCR technique was performed, using UT vet MF-primer and reverse primer. All lanes are the representative PCR products with 222bp in length. **B2,** 222bp PCR products were cut with the PSP1406I. First lane is uncut control sample and all lanes showed BZss homozygote. **B3,** A semi nested PCR was performed on first 403bp PCR products in order to use another restriction enzyme TaaI. **B4,** 225bp PCR products were digested by TaaI. First lane is first 403bp uncut control and second lane is 403bp product cut with TaaI and two fragments with 245 and 158 bp are detectable. All lanes didn't cut with enzyme and showed BZss homozygote. In all **A, B1, B2, B3, B4** M is 100bp marker

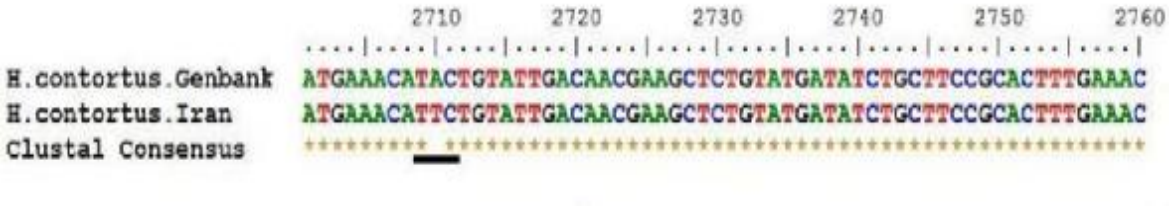

Fig.2: Alignment of beta tubulin sequences (position of codon 200) of Iranian isolate of *H. contortus* with a resistance isolate from GenBank (Accession number, X67489). Underline reveals the position of codon 200, indicating no point mutation at nucleotide no 2710 in Iranian specimen

Table 1: The sequence of primers used in PCR from beta tubulin isotype 1 gene sequence of *H. contortus*

Name of reactions	Name of primer& accession no of corresponding gene	Nucleotide sequence (5´- 3´)	PCR product
First PCR for all samples	P1, X67489	GTTCTCCGTTGTTCCATCACCC	403 bp
	P2, X67489	CGTGACACCAGACATTGTGACAG	
Semi nested PCR(PSP1406I)	P3, Shayan et al(2007)	CCCTTTCCGTCCATCAACTGGTAGAGAACAC CGATGAAACGT	222bp
	P4, X67489	CGTGACACCAGACATTGTGACAG	
Semi nested PCR(TaaI)	P5, X67489	CTACCCTTTCCGTCCATCAA	225bp
	P6, X67489	CGTGACACCAGACATTGTGACAG	

Discussion

Today BZ-resistance in some of sheep bursate nematodes is so prevalent and has been noted as a great threat to small ruminants' production system in many countries in the world (2, 4, 6). *H. contortus* has been considered as one of the most important parasites developing resistance against all of anthelminthic compounds, especially benzimidazoles. In addition, multi drug resistant *H. contortus* isolates threaten the viability of

small ruminant industries in South America, South Africa, Malaysia, India, and Southeast USA (2, 6, 13, 14).

Scientists believe that early detection of anthelminthic resistance is a critical point in farms management and application of prevention strategies at this step could reduce speed of resistance procedure efficiently (3, 15).

In Iran, small ruminants (sheep & goats) constitute the major basis of livestock production. Consequently, there are 52 million sheep and 26 million goats grazing in Iranian pastures (16). However, due to the high dry climate of most parts of the country and severe cold in the winter, infectious burden of bursate nematodes is lower than many other countries in the world. Hence the observation of clinical signs due to high infestation has rarely occurred. Because larval development of *H. contortus* occurs optimally at relatively high temperatures, haemonchosis is primarily a disease of sheep in warm climates. The frequency and severity of outbreaks of disease is largely dependent on the rainfall in any particular area (17). Concerning the unfavorable environmental status in Iran, infestation with *H. contortus* is lower than other nematodes especially *Teladorsagia circumcincta* and *Marshallagia marshalli*.

One of the greatest disadvantages of Faecal Egg Count Reduction Test (FECRT), a widely used method of assessing anthelmintic efficacy, is a low sensitivity for the detection of anthelmintic resistance in one species in the context of multi-species nematode infestations (4, 10, 18-20). However, high sensitivity of molecular methods in detecting such resistance has been presented in many publications (19). On the other hand in *H. contortus*, the single nucleotide polymorphism (SNP) most commonly associated with resistance is at codon 200 of beta tubulin isotype1 (21). Hence, using easy effective molecular methods such as PCR-RFLP, detecting SNP at codon 200, were considered as one of the best choice for BZ-resistance evaluation of *H. contortus* in Iran.

A field survey (based on FECRT) on resistance to albendazole in gastrointestinal nematodes of sheep in Khuzestan province of Iran showed resistance in *T. circumcincta* and *M. marshalli* and no resistance was detected in *H. contortus* (22). The results of the present study confirmed the results of the field survey and it contrasts sharply with the findings of studies carried out on the same parasite in other countries. No resistance alleles were detected. Some scientists believe that the rate of mutation could be increased under environmental stress and this theory has been demonstrated for bacteria (9).

Our present study reveals completely antithetical results. It was anticipated that under high dry environmental stress BZ-resistance should be high. The most important factor, which has been incriminated in promoting anthelmintic resistance, is the frequent and repeated use of the same anthelmintic (19). In Iran due to the harsh climate, infectious burden of sheep bursate nematodes is low. So the consumption of anthelmintic compounds especially in treatment frequencies is significantly lower than warm rainy areas in the world. However the consumtion of benzimidazoles has a long history in the country but speed of resistance induction was slower than many parts of the world.

Previously our group showed BZ-resistance in *T. circumcincta* from Khuzestan province, by PCR-RFLP (PSP1406I), (11). It has been demonstrated that many factors other than the genetics of the worms are involved in the dynamic process of resistance selection, including the biology and epidemiology of the parasite, the dynamics of host-parasite relationship, treatment frequency, and treatment strategies (2). We speculate that environmental status has a strong influence on resistance procedure. Considering poor available

information about this subject, it could be advisable to study this effect in varietal geographical and environmental areas.
The results of our study showed that BZ-resistance of *H. contortus* in Iran is not a serious problem as was anticipated before and today is the best time for application of prevention strategies against this great danger for livestock production system.

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