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Study of β-tubulin gene polymorphisms in *Haemonchus contortus* isolated from sheep populations in Khouzestan, southwestern Iran

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Summary

In this report, β-tubulin gene polymorphism was investigated in *Haemonchus contortus* populations isolated from sheep flocks in different regions of Khouzestan province, southwestern Iran. The samples were chosen on the basis of regional variation in benzimidazole treatment background of sheep flocks. Our objective was to study the relationship between treatment background and β-tubulin gene polymorphism of *H. contortus* isolated from the selected regions. *H. contortus* isolated from Shooshtar revealed reduction in β-tubulin gene polymorphism restriction fragment length polymorphism (RFLP) in respect to other isolates indicating that benzimidazole-resistant isolates of *H. contortus* developed in some sheep flock of Shooshtar region.

Key words: *Haemonchus contortus*, Drug resistance, β-tubulin, RFLP-PCR

Introduction

Control of internal parasite of sheep is mostly undertaken using anthelmintics including benzimidazole. Frequent use of these drugs results in emergence of resistant strains of the parasites (Sangster, 2001). Various studies have clearly indicated that molecular techniques are powerful tools to analyse genetic changes caused by drug resistance in nematode parasites. β-tubulin polymorphism plays a critical role in the molecular mechanisms of benzimidazole resistance in worms and reduction in the polymorphism effects positively on the benzimidazole resistance in *Haemonchus contortus*.

Since benzimidazoles were used for more than 30 years to control sheep nematodes in Iran especially in Khouzestan province, this study was conducted to investigate the probable changes in β-tubulin gene polymorphism in *H. contortus* due to benzimidazole treatment in different regions of Khouzestan province.

Materials and Methods

Using restriction fragment length polymorphism-polymerase chain reaction (RFLP PCR), *H. contortus* was isolated from sheep slaughtered in the regional slaughterhouse with different benzimidazole treatment background and polymorphism patterns in *H. contortus* of different geographic regions were compared with each other.

Sample collection

Nematodes were isolated from seventy (ten from each region) sheep which were slaughtered in Shooshtar, Ahvaz, Shadegan, Hamidieh and Sosangerd slaughterhouses of Khouzestan province, southwestern Iran. From the nematodes of each region *H. contortus* were collected and used for the next step.

DNA extraction

To extract the genomic DNA, 20–30 adult *H. contortus* worms from each region were pooled, crushed and then incubated at

41°C in a lysis buffer including 0.1 M sodium dodecyl sulphate (SDS), 0.1 M Tris-50 mM EDTA, 10 mg/ml proteinase K and 20 units RNase overnight. Finally, the DNA was extracted by salting out method. Five µl of extracted DNA was then used as template for PCR.

Estimation of restriction profile

We have analysed the theoretical restriction profile of the enzymes *EcoRI*, *EcoRV*, *DraI*, *RsaI* and *HinfI* for the PCR product of β-tubulin gene with length of 3055 bp (gene bank accession No. X67489) using the software "webcutter" and estimate the number of putative produced fragments for each enzyme (Fig. 1). Accordingly, the enzymes *EcoRI*, *EcoRV* and *DraI* have one cut site for the PCR product and the enzyme *RsaI* would cut the 3055 bp fragment seven times. Because of putative frequent restriction sites of PCR product (β-tubulin gene) for enzyme *HinfI* which produced very small and overlapped fragments, this enzyme was excluded for the next digestion step (Fig. 1). It should be noted that electrophoretic separation of fragments with less size difference is very difficult.

Polymerase chain reaction

The β-tubulin gene of interested samples was amplified in two steps (Fig. 2). A 4.5 kb fragment was first pre-amplified with 50 pmol forward primer TUBF: 5'-TTTC-TCATCCAAACTTCCGC-3' and 50 pmol reverse primer TUBR: 5'-AACCTCAT-CACACAGCGGAT-3' by the high Expand TM Long Template PCR System (Roche, Swiss) according to the supplier's instructions. The nested-PCR was performed with two internal primers (TUBNF: 5'-AAATCGTTCATGTGCAAGCC-3' as forward and TUBNR: 5'-CCTACCCG-TTTCGCTTAAAA-3' as reverse primers) and five µl of PCR product from the first step as template, generating a three kb fragment (Fig. 3). All primers were designed from gene bank-reference sequence with the accession number X67489. Amplification was performed with 35 cycles and following time and temperature profile: 95°C for 15 sec, 55°C for 60 sec and 72°C for 120 sec. The final extension step was done at 72°C for 10 min.

Agarose gel electrophoresis

PCR products were analysed by 1%

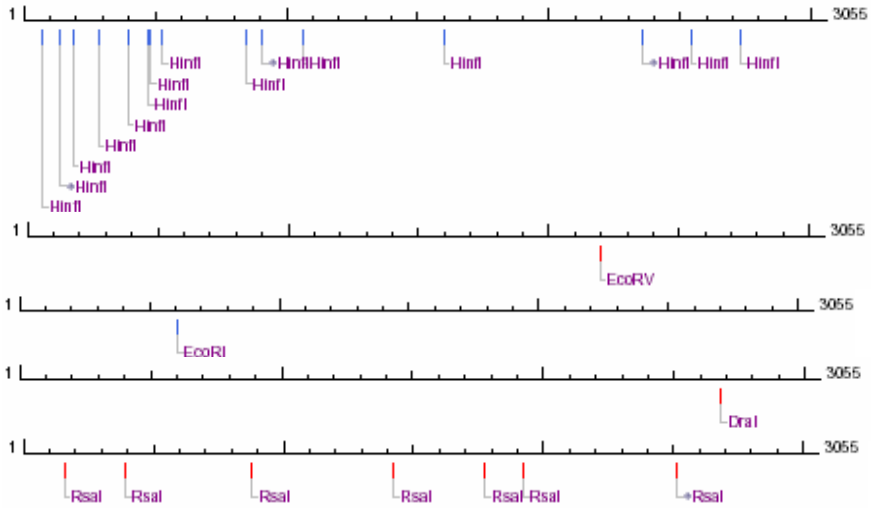

Fig. 1: Expected restriction profile of five enzymes for the PCR product (3055 bp)

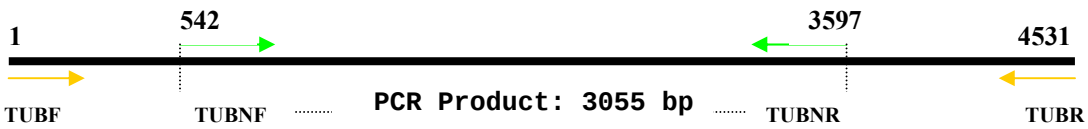

Fig. 2: Schematic diagram of primer binding sites on β-tubulin gene

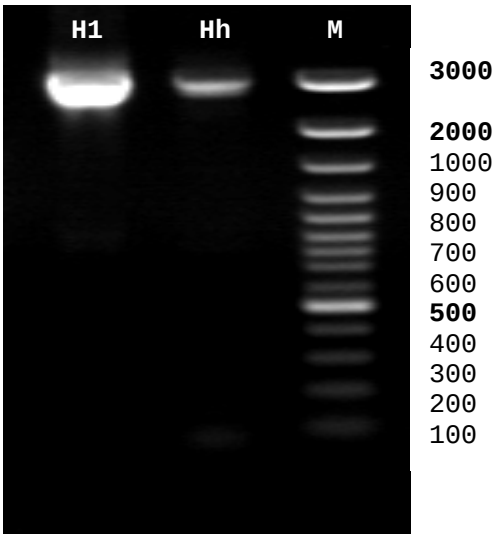

Fig. 3: Nested-PCR product with a length of 3055 bp was electrophoresed on 2% agarose gel for 75 min at 80 V. H1 = *H. contortus* of Shooshtar; Hh = *H. contortus* of Hamidieh city; M = DNA size marker (lambda 100-bp ladder from Fermentase)

agarose gel electrophoresis and the products with good intensity were used for RFLP.

Restriction fragment length polymorphism

PCR products were digested using restriction enzymes (*EcoRI*, *EcoRV*, *DraI*, *RsaI*) separately. Ten µl of PCR product from nested-PCR and 10 units of each enzyme with 2 µl of 10X buffer (*Roche*, Swiss) were used overnight at 37°C in a 20 µl final reaction volume for digestion and fragments were electrophoresed on 2% agarose gel.

Results

For each sample, a fragment of β-tubulin gene with the length of 3055 bp was successfully amplified and compared with DNA size marker (Fig. 3). Because of relatively long fragment size, we used a special *Taq* polymerase with high processivity (see Materials and Methods). Then PCR products were digested separately with restriction enzymes *EcoRI*, *EcoRV*, *DraI* and *RsaI*. Digestion products were electrophoresed to separate the fragments, subsequently. For example as shown in Fig. 4a, *EcoRV* digested samples from different geographic regions. The β-tubulin gene of isolates from Shooshtar (H1) reveals fewer fragments after digestion with *EcoRV*, in comparison with other isolates. All isolates with exception of H1 had more than one restriction sites, which confirm increased

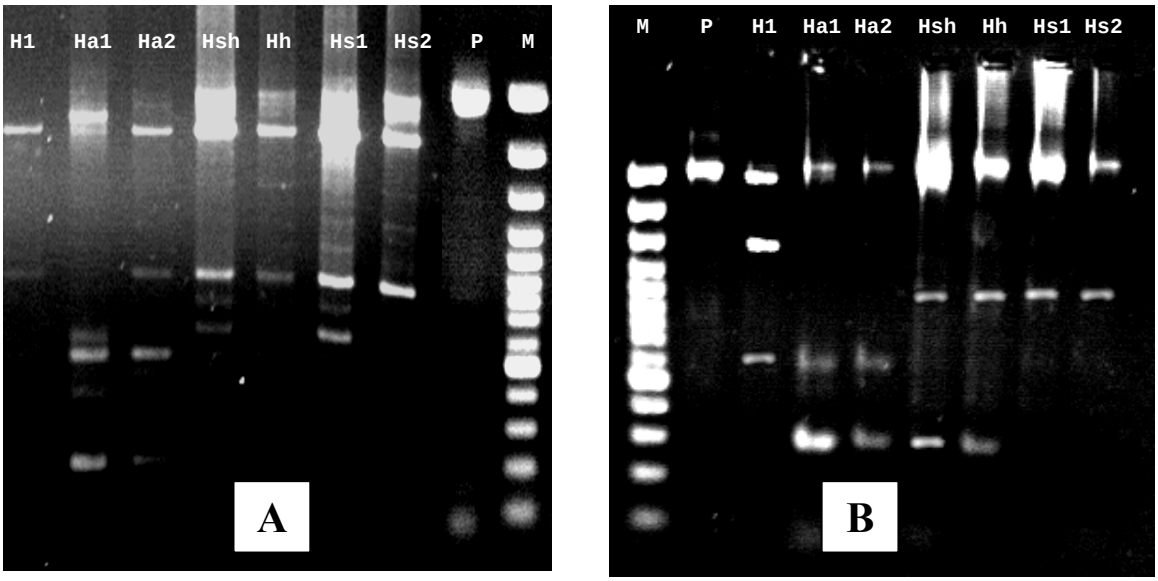

Fig. 4: Restriction pattern of a) *EcoRV* and b) *DraI* for amplified β-tubulin gene of nematodes from different regions of Khouzestan province. It showed that restriction profile from Shooshtar isolate (H1) was different from Ahvaz (Ha1, Ha2), Shadegan (Hsh), Hamidieh (Hh) and Sosangerd (Hs1, Hs2) isolates and it indicates that polymorphism in β-tubulin gene of *H. contortus* of Shooshtar is lower than *H. contortus* of other regions. P: PCR product before digestion, M: the same DNA size marker used in agarose gel in Fig. 3

Table 1: Number of RFLP fragments after digestion with four enzymes

Location	Sample code	*EcoRI*	*EcoRV*	*DraI*	*RsaI*	Treatment background
Shooshtar	H1	2	2	3	2	25 years
Ahvaz	Ha1	6	6	3	4	15 years
Ahvaz	Ha2	4	5	3	4	15 years
Shadegan	Hsh	5	7	4	5	10 years
Hamidieh	Hh	7	5	4	4	15 years
Sosangerd	Hs1	5	7	3	4	13 years
Sosangerd	Hs2	7	5	2	2	13 years

polymorphisms (Fig. 4a). Also, restriction profile of other enzyme (*DraI*) showed more differences in the length fragments (Fig. 4b). However, the effect of all enzymes on different isolates demonstrated similar pattern like *EcoRV*. As we can see in Table 1, digestion results of four restriction enzymes were compared with treatment background of albendazole in each region.

Discussion

In this study, using RFLP-PCR and RFLP, we investigated β-tubulin gene polymorphism in *H. contortus* isolated from sheep with longer benzimidazole treatment background (Shooshtar) and compared the results with that of isolates taken from other regions of the province with shorter benzimidazole treatment time. Results, as summarized in Table 1, indicate that restriction profiles of β-tubulin gene in *H. contortus* vary with treatment background. The restriction enzymes used in this study, *EcoRV* showed the clearly recognizable profile according to the length and size of fragments.

The digestion reaction of all four enzymes revealed reduced number of RFLP fragments in Shooshtar isolates in comparison to isolates taken from other regions. The number of fragments is inversely related to treatment background (Table 1). Shooshtar isolates with the highest treatment has the least number of fragments. The isolates from two regions of Ahvaz (Ha1 and Ha2) like those taken from two regions of Sosangerd (Hs1 and Hs2) showed different restriction profiles for the enzyme *EcoRV* (Fig. 4a). It seems that difference between polymorphism in Ahvaz isolates Ha1 and Ha2 with the same treatment background is due to the treatment frequency per year. Therefore, we can conclude that Shooshtar isolates with higher frequent benzimidazole treatment have fewer fragments in β-tubulin gene of *H. contortus* populations in comparison to those from other regions with less frequent use. This indicates the development of benzimidazole resistance in one isolate of *H. contortus*.

Genetic studies suggest that change of polymorphism pattern in β-tubulin gene can be responsible for drug resistance (Elard and Humbert, 2000; Osten *et al*., 2001; Prichard and Tait, 2001). Two main types of genetic change occur in the benzimidazole resistance trichostrongylid nematodes. One is a loss of allelic diversity of β-tubulin gene in *H. contortus* and *Trichostrongylus colubriformis* (Lubega *et al*., 1994; Grant and Mascord, 1996). The reduction in β-tubulin polymorphism has been interpreted as the result of selection, and is considered to be a major method of acquiring benzimidazole resistance (Kwa *et al*., 1994; Lubega *et al*., 1994; Elard *et al*., 1999). The second is the occurrence of mutation at the amino acid 200 (Phe→Tyr) of the isotype I from β-tubulin gene which leads to benzimidazole resistance in three dominant species in temperate zone, *H. contortus*, *T. colubriformis* and *Teladorsagia circumcincta* (Kwa *et al*., 1994; Elard *et al*., 1999).

Benzimidazole-resistant populations were characterized by a low diversity of the restriction profile in comparison with the susceptible populations (Taylor *et al*., 2002). Roos *et al*. (1990) investigated DNA polymorphism of benzimidazole susceptible and resistant populations of larval and adult *H. contortus* using RFLP. They indicated that there was specific difference between benzimidazole susceptible and resistant populations of *H. contortus*. Southern blot analysis of susceptible strains using a homologous β-tubulin clone as a probe,

showed five to eight hybridizing fragments with different sizes in susceptible worm populations. In resistant population, however, a single fragment was observed. The gene that showed the reduction in RFLP fragments encodes a β-tubulin referred to by Roos *et al*. (1990). Similar observation was made for benzimidazole resistance in *T. colubriformis* (Sangster *et al*., 2002).

Benzimidazoles were used for more than 30 years to control sheep nematodes in Iran and result of this study showed that frequent use of these drugs resulted in emergence of resistant strains of the parasites. Although, recent field survey (Gholamian *et al*., in print) in Khouzestan province using faecal egg count reduction test and efficacy test showed that benzimidazoles-resistant isolates of *Ostertagia circumcincta* and *Marshallagia marshalli* have been developed in some sheep flocks, and that *H. contortus* detected to be susceptible to the drug, we could detect benzimidazole-resistant *H. contortus*. Many study showed that genetic tests are very sensitive and can identify resistance when the frequency of resistant alleles is as low as 1% (Sangster and Dobson, 2002).

Anthelmintic resistance has been recognized as a great threat to the grazing animal industry development and *H. contortus* is a prototype of an important group of parasites infesting sheep (Waller, 1997). Spread of resistant isolates of this nematode can cause high economic loss to sheep farms in Iran. Therefore, we prefer molecular techniques to identify the prevalence of drug resistance and use more effective drugs to prevent spread of drug resistance and reduce the management cost.

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