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ORIGINAL ARTICLE

Molecular evidence on the emergence of benzimidazole resistance SNPs in field isolates of *Marshallagia marshalli* (Nematoda: Trichostrongylidae) in sheep

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Abstract The infection with members of the Trichostrongylid nematodes has been frequently reported from sheep and goats. Because of the widespread use of Benzimidazoles (BZs), the resistance suspected to occur in some worms populations. In this study, we focused on the prevalent nematode, *Marshallagia marshalli*, from the abomasa of sheep. Samples were obtained from at least 10 infected farms and diagnosed with morphological and molecular methods. For resistance analysis, genomic DNA from pooled adult samples of all farms were analysed for the beta tubulin gene to detect any polymorphisms at codon positions of F167Y, E198A and F200Y. According to the results, seven farms (70%) revealed resistance (R) allele at F200Y with relatively high frequency. No other mutations were identified at the other two positions. Also, except for one homozygous (RR) occasion, the isolates with R allele had heterozygous (RS) genotype. This finding indicates that the worm populations are still affected by drugs of the BZ class. However, the genetic data also notes on developing resistance mechanisms in *M. marshalli* populations in sheep.

Keywords *Marshallagia marshalli* · Beta tubulin isotype 1 · Sheep · Internal transcribed spacer-2 (ITS-2)

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Introduction

The increasing selection of drug-resistant nematodes in small ruminants have affected the economics of farming through decreases in productivity and reproduction (Waller 1997). Among the anthelmintic classes, the Benzimidazoles (BZs) have successfully used worldwide against nematode infections in sheep and goats (Falzon et al. 2013). In contrast, many reports have documented the development of resistance to BZ derivatives in prevalent gastrointestinal nematodes (GINs) in the trichostrongylid group (Kaplan, 2004; von Samson-Himmelstjerna et al. 2007). Therefore, a bulk of investigations have focused on the mechanisms and functional diagnosis of the resistance to BZs in those nematodes (Alvarez-Sánchez et al. 2005; Ghisi et al. 2007; Esteban-Ballesteros et al. 2017).

The molecular techniques have demonstrated a close relation between the occurrence of single nucleotide polymorphisms (SNPs) in the beta tubulin isotype 1 gene and the resistance to BZ. The SNPs related to BZ-resistance were first found in codon position of F200Y (TTC to TAC) in *Haemonchus contortus* (Kwa et al. 1994). Then, mutations in two additional codons, F167Y (TTC to TAC) (Elard and Humbert 1999; Silvestre and Cabaret 2002) and E198A (GAA to GCA) (Ghisi et al. 2007) were also attributed to BZ-resistance; however, F200Y appears to be the most commonly associated genetic marker (von Samson-Himmelstjerna et al. 2007). In the past two decades, the resistance related SNPs in the beta tubulin gene have been explained for different trichostrongylids such as *H. contortus* (Silvestre and Humbert 2000; Alvarez-Sánchez et al. 2005; Walsh et al. 2007; von Samson-Himmelstjerna et al. 2009), *Teladorsagia circumcincta*, *Trichostrongylys colubriformis* (Humbert et al. 2001; Skuce et al. 2010), *Cooperia oncophora* (Winterrowd et al. 2003) and *H.*

placei (Chaudhry et al. 2014). These studies indicate that molecular investigation on beta tubulin gene could be a suitable tool for the early detection and characterization of BZ-resistant GINs.

Marshallagia marshalli (Orloff 1933) is a member of tricostrongylid nematodes which infects the abomasum of small ruminants. The worm is belonging to the Ostertagiine helminths and has been described from different countries (Lichtenfels and Pilitt 1989), particularly in the Middle East area (Altaş et al. 2009). In Iran, the infection with *Marshallagia* sp. has been frequently reported from a long time ago (Eslami et al. 1979). Because the BZs are used routinely for the elimination of helminth infections, there is a possibility of resistance in some areas. According to the literature, there is no information on the beta tubulin gene and the resistance-related SNPs in *Marshallagia* populations. Therefore, this study was undertaken to discover any potential signs of BZ resistance in this species using the genotypic approach.

Materials and methods

Collection of field samples and diagnosis

During several inspections, the abomasa with relatively high nematode infections were collected randomly from a total of 10 farms of sheep referred to local abattoirs in Shiraz (29.5926° N, 52.5836° E), Fars province, South of Iran. The sampled animals were all male and under 1 year of age. The adult (male and female) worms were separated, washed in normal saline and stored in 70% ethanol. In order to morphologic characterization, male and female nematodes were mounted and identified according to keys described by Lichtenfels and Pilitt (1989), Lichtenfels and Hoberg (1993) and Hoberg et al. (2012).

Molecular diagnosis

In order to species confirmation, worms were randomly selected from at least 3 farms and subjected to genomic extraction using DNA extraction Kit (MBST, Iran) according to the manufacturer's recommendation. A primer set, F (5′-GCAGACGCTTAGAGTGGTGA-3′) and R (5′-TCCTTGTTAGTTTCTTTTCCTCCG-3′), was designed to identify the complete internal transcribed spacer-2 (and partial 5.8S and 28S subunits) in ostertagiine nematodes. The PCR reaction mix included 12.5 µl of PCR premix (Ampliqon, Denmark, Cat. No. A180301), 1 µl of each primer, 6.5 µl H_2O and 4 µl of DNA as template. The cycling program consisted of an initial denaturation at 95 °C for 5 min, 94 °C for 30 s, followed by 35 cycles of 60 °C for 30 s, the extension at 72 °C for 30 s and the final extension at 72 °C for 10 min. The amplicons were assessed by electrophoresis—in 1% (*w/v*) Tris–acetate/EDTA agarose gel and visualized under ultraviolet illumination. Products were sequenced (ABI 3730 DNA analyzer; Bioneer, Korea). The comparison of the sequences was made with other available sequences in NCBI using BLAST search. The sequence data was aligned with homologous sequences existing in the GenBank using Clustal W program by MEGA 6 software (Tamura et al. 2013). The phylogenetic tree was constructed by maximum likelihood (ML) method and analyses was carried out using the Kimura 2-parameter distance estimate (Kimura 1980).

The beta tubulin gene analysis

For each farm, the DNA material was extracted from pools containing at least 7 adult male and female worms. Depending on the number of worms recovered, 2 or 3 replicates were done for each case. There was no available GenBank record for the beta tubulin gene sequence of *Marshallagia* species. So, the existing reports for other trichostrongylid nematodes were focused including *H. contortus* and *T. circumcincta*. A set of primers (Forward: 5′-CCAGTCAGGAGCGGGTAAC-3′ and Reverse: 5′-CAGGGAATCGRAGGCARGT-3′) were designed to cover codon positions of 167, 198 and 200 of the beta tubulin isotype 1 gene. The PCR reaction involved an initial denaturation at 95 °C for 5 min, 94 °C for 30 s, followed by 35 cycles of 62 °C for 30 s, the extension at 72 °C for 30 s and the final extension at 72 °C for 10 min.

Determination of SNP frequencies

Sequence traces were investigated using Chromas 2.6.6 software (Technelysium Co.), regarding F167Y (TTC/TAC), E198A (GAA/GCA) and F200Y (TTC/TAC) SNPs. The resistance-related SNP frequencies were estimated by dividing the known nucleotide peak height by the sum of the two (relative sensitive and resistant) peak heights as previously described (Von Samson-Himmelstjerna et al. 2007).

Results

Species confirmation

Round worms in the abomasa (Fig. 1) had small to medium dimensions. The morphometric investigation on male and female worms (Table 1) were in consistent with available descriptions for *M. marshalli*.

In this study, the expected sequences of the ITS-2 ribosomal gene (of about 400 bp) were detected and

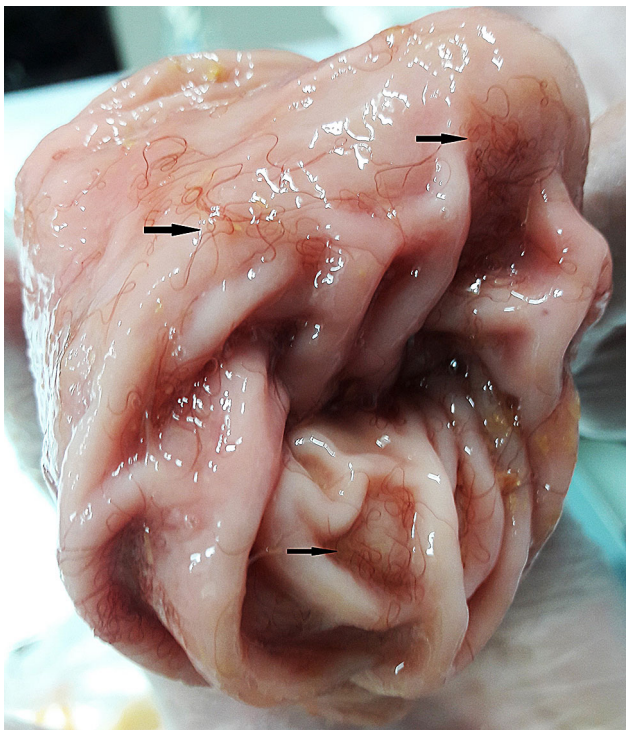

Fig. 1 The abomasum of sheep showing the high infection with thread like, red to brown *M. marshalli* nematodes

successfully amplified in samples (GenBank accession no. MN888760). The phylogenetic analysis showed that our isolates grouped with *M. marshalli* and *Ostertagia occidentalis* (Fig. 2a) and separated well from a closely related species, *T. circumcincta*, and the other *Ostertagia* species. Sequence analysis revealed about 99.0% identity between our specimens and one record of *M. marshalli* (KT428384) and 92.4 to 97.8% with other records of *M. marshalli* and *O. occidentalis*.

Resistance analysis

Fragments of about 700 bp were achieved for the beta tubulin gene. One of the sequence reads was recorded in the GenBank as MN974474. As expected, the isolates had high percent identity to *T. circumcincta* (96.6 (92–97.8)), compared to *H. contortus* (73 (72.8–73.1)) and *Cooperia* species (72.8 (72.3–74.2)). The phylogenetic analysis (Fig. 2b) also grouped our isolates with *T. circumcincta* and separated *Marshallagia* specimens from other Trichostrongylid nematodes. This data confirms the close relationship between the genera *Teladorsagia* and *Marshallagia*.

The present sequences did not show any SNPs at codons 167 and 198. But mutations detected as T**T**C to T**A**C substitution at the position of F200Y (Fig. 3). The resistance allele (TAC) was found in 7 out of 10 sampled farms with different percent frequencies ranged between 13.2 and 100 (Table 2). In addition, except for one farm with homozygous (RR) genotype worms, all the others were heterozygous (RS) types.

Discussion

The present study investigated, for the first time, the occurrence of BZ resistance-associated beta tubulin SNPs in a prevalent trichostrongylid member, *M. marshalli*. Since a long time ago, this nematode have reported as a common GIN parasite in small ruminants in areas like the Middle East including Turkey (Atlaş et al. 2009) and Iran (Eslami et al. 1979). Despite detailed descriptions on *M. marshalli*, previous studies has shown that the morphologic characterizations may not easily distinguish between this species and some other members of the subfamily Ostertaginae. For example, Lichtenfels and Pilitt (1989)

Table 1 Morphologic characterizations of *Marshallagia* specimens separated from sheep abomasa in southern Iran

Character	Male (n = 26)	Female (n = 35)
Body length	8.8 ± 0.6 (7.56–10.04)	11.49 ± 1.44 (8.76–14.65)
Body width	217 ± 26.3 (165–271)	205.23 ± 44.6 (108.5–277.5)
Esophagus length	894.11 ± 111.2 (638.9–1107)	938.67 ± 115.6 (731.7–1246)
Dorsal ray length	334.77 ± 55.6 (231.3–451.23)	–
Spicule length (average of right and left spicules)	341.24 ± 31.48 (268–429.6)	–
Copulatory bursa, ray pattern	Elongate, 2-1-2	–
Egg length	–	170.42 ± 34.6 (107–225)
Egg width	–	73.95 ± 17.7 (44–122)
Vulvar flap width	–	8.2 ± 1.4 (5.62–10.82)

Data are given as mean ± SD and the range in parenthesis. All measurements are expressed as Micrometer, except for body length which is presented as Millimeter

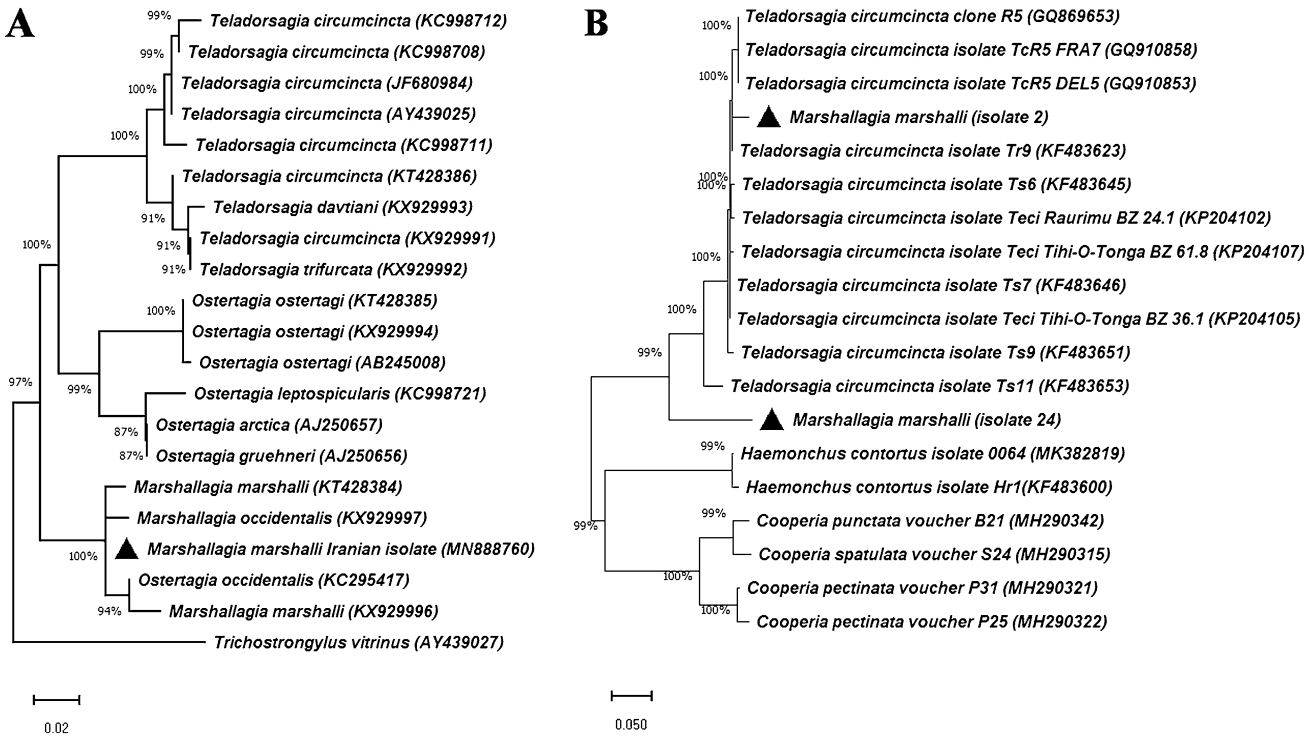

Fig. 2 Phylogenetic position of *M. marshalli* isolated from Iranian sheep among some trichostrongylid species inferred from the ITS2 rDNA (**a**) and the beta tubulin isotype 1 (**b**) genes using the maximum-likelihood method. The numbers associated with nodes represent the percentage of 2000 bootstrap reps and the horizontal distance is proportional to hypothesized evolutionary change as indicated (scale bar)

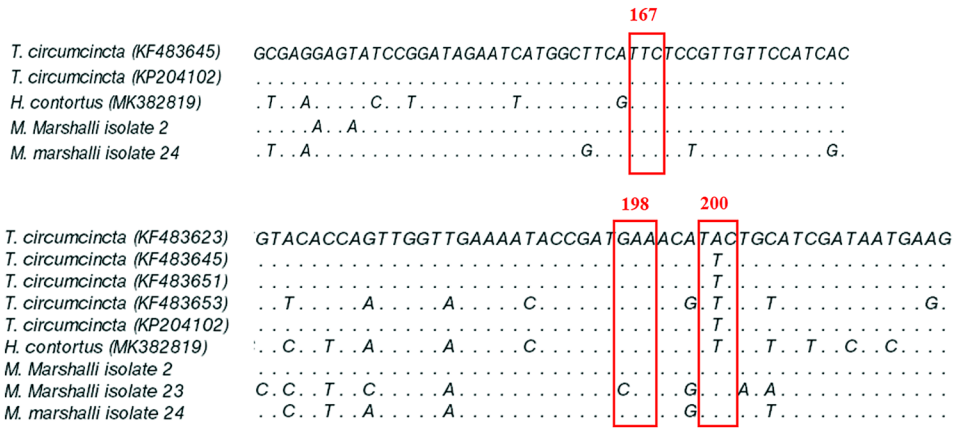

Fig. 3 Alignment of the sequences containing the beta tubulin isotype 1 gene in three codon positions of 197, 198 and 200 (showed by boxes) in trichostrongylid nematodes, *T. circumcincta*, *H. contortus* and *M. marshalli* (the present study). Note that there is not any resistance in the first two positions, but the expected resistance allele (TAC) is seen in the sequence of *T. circumcincta* (KF483623) and also our three isolates

provided evidence that *O. occidentalis* may be only a polymorphic form of the *M. marshalli*. So, the former was also named as *M. occidentalis*. Our molecular data on the ITS-2 region confirmed the close relationship between both species and a significant interspecific divergence in sequence with others. Moreover, the phylogenetic analysis separated these two species from *Teladorsagia* and *Ostertagia* with most similar morphological and biological traits. This result is in line with some previous studies (Heise et al. 1999; Dallas et al. 2000) indicating that the ITS-2 region is a valid marker for identifying the abomasal nematode community in ruminants.

In spite of using deworming agents in the past three decades in Iran, the inspections in abattoirs shows a relatively high rates of the infection with *M. Marshalli* in sheep and goats. This observation may be imply that resistant type worms have selected during therapies with BZs and the problem is expected to increase if worm control is not optimized. In this study, the presence of the TAC allele in 70% of the examined farms confirmed the development of

Table 2 Results of the PCR-based technique to detect resistant (R) or sensitive (S) status of *M. marshalli* adult worms

Farm number	Genotyping status*	Resistance allele frequency (%)
1	SS	
2	RS	65
10	RS, SS	55
15	SS	
20	RR	100
21	SS	
22	RS, SS	32
23	RS, SS	72
24	RS	24.6
18	RS	13.2

**SS* Homozygous susceptible, *RS* Heterozygous resistant, *RR* Homozygous resistant

resistance mechanism to BZs. However a high proportion of worms bearing the R allele were corresponded to RS genotype. The RS and Homozygous susceptible (SS) genotypes are expected to be effectively eliminated by the recommended dose (Elard et al. 1998). So, one conclusion is that the present specimens are still sensitive to correct doses of the BZs. The advantage of the RS genotype worms is to withstand the under dosed treatments. In sheep experimentally infected with *T. circumcincta*, the survival of RS worms was 4.5 fold greater than that of SS genotypes at 1/4 of the recommended dose (Silvestre et al. 2001). Because farmers in the studied region usually treat small ruminants with BZ members, it can also be postulated that the observed RS genotypes is related to under dosing or drench failure with unknown reasons.

In the past, genotyping and other in vitro and in vivo diagnostic tests have quantified the level of resistance in GIN nematodes. Of the recommended routines (Coles et al. 2006), the genetic assay was claimed to be more accurate, reliable and sensitive method (von Samson-Himmelstjerna et al. 2009). Nevertheless, comparisons on the results demonstrates the complexity of interpretation for the achieved data. One reason is that highly variable R allele frequencies have been reported for both susceptible and clinically resistant trichostrongylids. In sheep infected with *H. contortus*, a relatively high rate of TAC allele frequency (> 81%) was observed in farms with no clinical resistance using the FECRT method. Disagreement between molecular and the in vivo routine were also found in some other flocks with lower allele frequencies (Höglund et al. 2009). A wide range of RR (0 to 66.7%), RS (5.9 to 77.3%) and SS (4 to 83.3%) genotype frequencies in resistant field isolates of *H. contortus* in Brazil (Niciura et al. 2012), proportions of RR and RS alleles in BZ sensitive *H. contortus* (Cudeková et al. 2010) and the high rates of F200Y polymorphism in *Trichostrongylus colubriformis* follow the treatment with Albendazole (Esteban-Ballesteros et al. 2017) indicate that the value of resistance is likely more than that of estimated by the other standard methods. In addition, some researchers claimed that the level of resistance correlates with the percentage of resistant alleles (Esteban-Ballesteros et al. 2017). It was also predicted that the resistance to BZs will develop more rapidly in susceptible populations having degrees of RS genotype (Silvestre et al. 2001). Therefore, the high rate of RS genotypes in some present farms may have also led to degrees of BZ resistance similar to that occur by RR types. So, regular diagnostic measures and more in-field assays should be concerned when farmers are using this class of drugs.

Conclusions

Molecular analysis on the ITS-2 region of morphologically identified *M. marshalli* confirmed its close phylogenetic relation with other reports for this nematode and *O. occidentalis*. So, it seems that the ITS-2 could assigned as a good biomarker for distinguishing between ostertagiine nematodes. *Marshallagia* specimens were also examined for the resistance to BZs. The main idea for this examination were the prevalence of *M. marshalli* in small ruminants in Iran and the wide use of Albendazole for the prevention of helminth diseases. Single mutations at codon 200 of the beta tubulin gene in 70% of the examined farms supports that the resistance in the area is developing in worm populations. In addition, domination of the heterozygous worms reveals they still sensitive to BZs, but cautions exists for under dosing treatments. Although we could not predict the exact behavior of worms when treated with the anthelmintic drugs, the molecular investigation could be of great value for the emergence of resistance in this type of nematodes.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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