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Original paper

Molecular evidence of potential albendazole-resistance in *Teladorsagia circumcincta* in Iraqi sheep

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ABSTRACT. Gastrointestinal nematodes infection is one of the major health problems in the livestock industry in many parts of the world and Iraq. Anthelmintic drugs are heavily relied upon to control them so that repeated use lead to development of anthelmintic resistance. The present study was carried out to detect the albendazole-resistance in *Teladorsagia circumcincta* from Iraqi sheep in Soran district, Kurdistan Region. A total of 140 sheep abomasa were randomly collected. Allele-specific PCR (ASP) was used to investigate the frequency of single nucleotide polymorphisms (SNPs) in codon 200 of isotype I β-tubulin gene. The frequency of the homozygous susceptible (SS), homozygous resistant (rr) and heterozygous susceptible (Sr) were 60.9%, 0.0% and 39.1% in the examined *T. circumcincta* isolates, respectively. From the results of the present study, it was concluded that *T. circumcincta* was sensitive to albendazole however; the presence of resistance allele in some populations may lead to the emergence of resistant individuals in the region.

Keywords: *Teladorsagia circumcincta*, albendazole, sheep, Soran, Iraq

Introduction

Gastrointestinal nematodes have the most economically impact on small ruminant's husbandry [1–3]. These parasites cause gastroenteritis and lead to productivity reduction, decreased reproductive efficiency, low growth, weight loss and increased food conversion [4].

Teladorsagia circumcincta or brown stomach worm is a pathogenic abomasal nematode, which infect small ruminants [5,6]. Like other abomasal nematodes, third-stage larvae (L_3) migrate through the lumen, however; inhibit in parietal glands until it migrates back into the lumen and becomes mature [7]. Unlike *H. contortus*, the adult forms of *T. circumcincta* do not feed on blood of definitive host. In addition, larval stages of these parasites are causes of pathogenic effects in small ruminant hosts. During the development of larval stages in gastric glands of abomasum, infectious nodules develop in mucosa, leading to considerable lesions in parietal cells and reducing the hydrochloric acid production. This finally led to clinical signs and economic loses [2]. This parasite is therefore an omnipresent pathogen that causes seasonal outbreaks of parasitic gastroenteritis in spring, especially in weaned lambs [4,8,9].

For decades, despite of different strategies for controlling of nematodes, control of these helminthic parasites and treatment of infected host animals relied on anthelmintic drugs. This led to develop drug resistance [10,11]. It is determined that three non-synonymous single nucleotide polymorphisms (SNP) in the isotype 1 β-tubulin gene were responsible for benzimidazoles (BZs) resistance in some ovine gastrointestinal nematodes (OGIN) species such as *T. circumcincta* and *Haemonchus contortus* [12]. These polymorphisms lead to substitution of phenylalanine to tyrosine at position 200 (F200Y), phenylalanine to tyrosine substitution at position 167 (F167Y) and glutamic acid to alanine substitution at position 198 (E198A). There are also some resistant nematodes without any of the three known mutations, which indicated

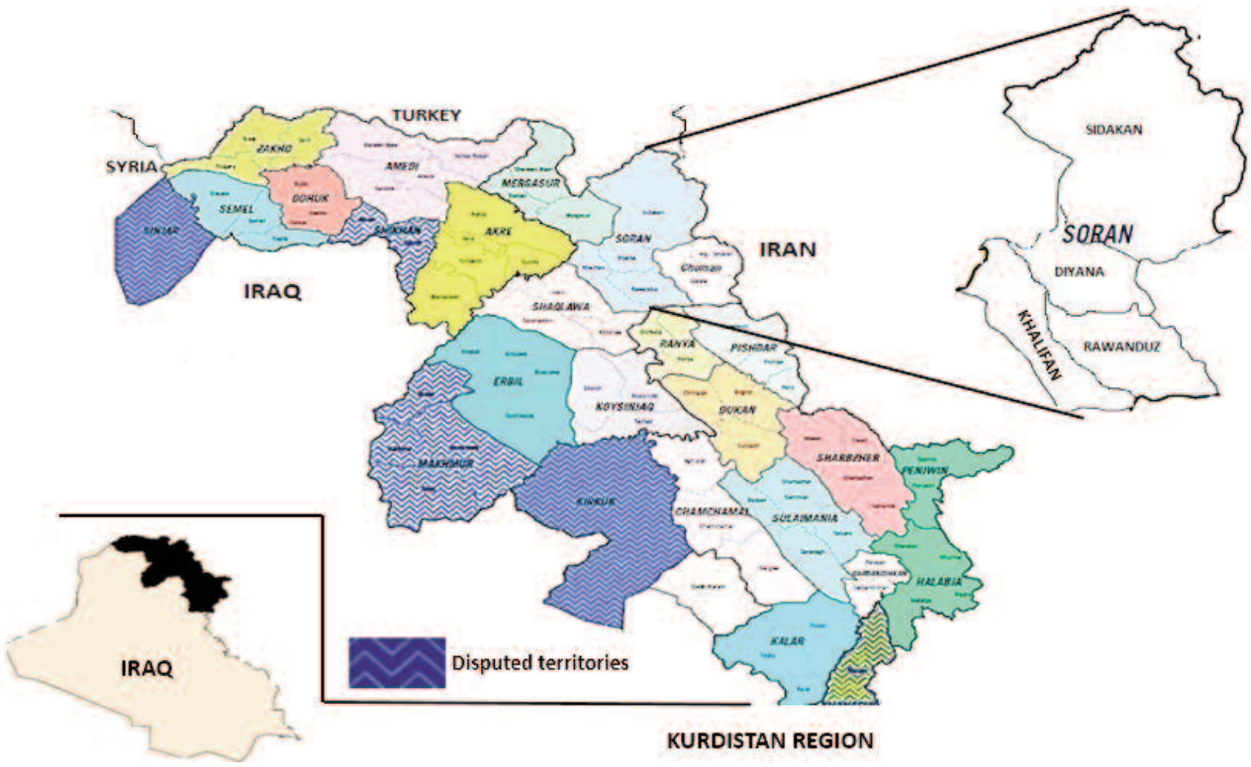

Figure 1. Map of sampling region in Soran district, North East of Iraq

existence of additional determinants of resistance [11]. Different types of SNP techniques like allele-specific PCR (AS-PCR) were developed to detect single nucleotide polymorphisms in the genome. The most benefits of this technique are low cost and rapid detection without genes' sequencing [13].

The main factor in developing drug resistance is yearly frequency and repetition of the same anthelmintic drugs applying [14]. According to veterinary pharmacies in Soran city, albendazole has been employed for several years. There were no any published data on *T. circumcincta* drug resistance to albendazole, in Soran, Iraq. Thus, this study was aimed to investigate the presence of resistant alleles to albendazole in *T. circumcincta* in sheep in the region.

Materials and Methods

Area of study

The current study was conducted on slaughtered sheep at central slaughterhouse of Soran district, Kurdistan region, Iraq (N 36º.39' and E 44º.32'). Soran or Diyana city is a district with four sub-districts in Erbil province of Kurdistan region, North East of Iraq in neighboring countries of Turkey and Iran (Fig.1). More than 20% (6–8 million) of Iraqi sheep population exist in the region [15]. According to the Agriculture and Veterinary administration, of those, about one million sheep and goats are raised in this district.

Helminth collection and identification

A total number of 140 abomasa of Iraqi sheep of

Table 1. The primers' sequences employed to identify albendazole resistance in *Teladorsagia circumcincta* isolated from Iraqi sheep

Primer	Primer sequence
P1	5′-GTCCCACGTGCTGTTCTTGT-3′
P2S	5′-TACAGAGCTTCATTAATCGATGCAGA-3′
P3R	5′-TTGGTAGAAAACACCGATGAAACATA-3′
P4	5′-GATCAGCATTCAGCTGTCCA-3′

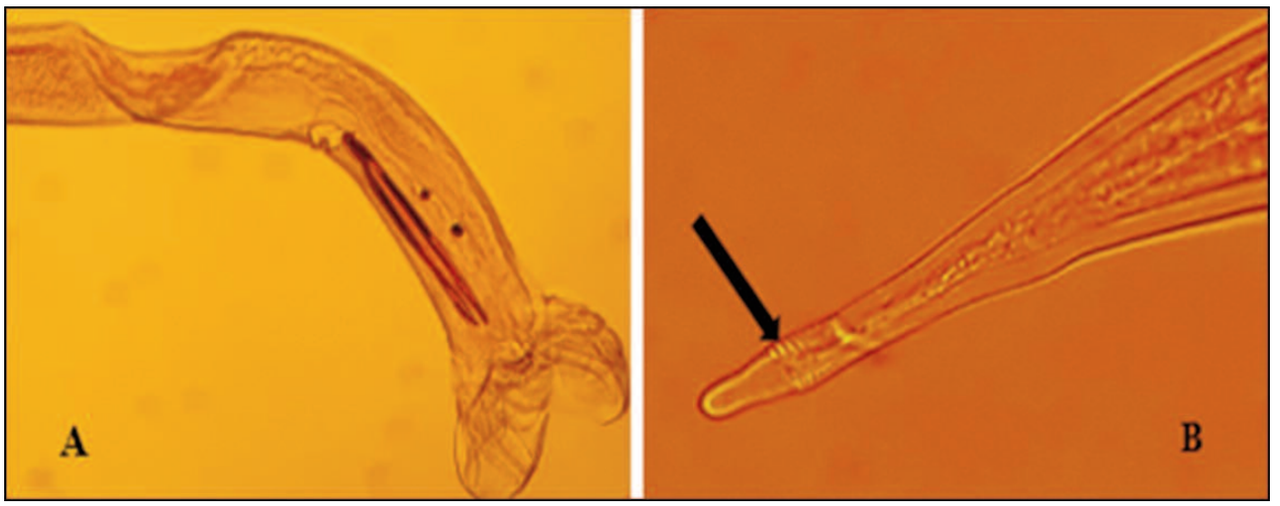

Figure 2. (A) The posterior end and copulatory bursa in the adult male of *Teladorsagia circumcincta* containing three lops, a little dorsal lop and well expanded lateral lops. The long and thin spicules and the posterior end of spicules with two divided branches of equal length (100×); (B) In females, the thin and rounded tip of tail ends with four to five transversal striations (black arrowhead, 100×)

both sex were randomly collected. They were kept in plastic bags and transferred immediately to the Lab of Biology, Soran Faculty of Sciences, Iraq. Abomasa content and mucosa were washed and then collected nematodes were identified based on morphological characteristics described by Taylor [4]. Identified male and female *T. circumcincta* were fixed in 70% ethanol (Merck, Germany) and washed several times in phosphate-buffered saline (0.01M, pH 7.2) until molecular analyses.

DNA extraction and allele specific PCR (*ASP*)

Genomic DNA extraction was performed on adult nematodes of *T. circumcincta* using commercial DNA extraction kit DNP (SinaClon, Iran) according to the manufacturer's instruction. The allele specific PCR (ASP) was employed to detect resistance to albendazole in identified nematodes as *T. circumcincta*. ASP was performed using specific primers' sets including P2S and P3R for sensitive and resistance allele, and P1 and P4 for allele-nonspecific [16,17] (Tab. 1). PCR reaction was prepared in 40 µl reaction mixture containing

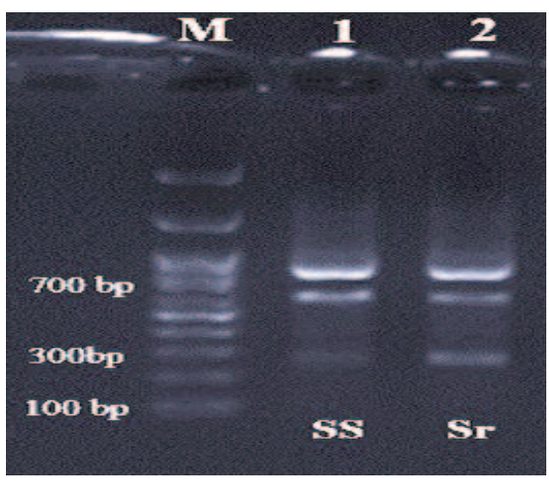

Figure 3. The allele specific PCR (ASP) result represents 250bp fragment length of albendazole resistance from *Teladorsagia circumcincta* (white arrowhead). M: Marker, 1: Susceptible homozygous (SS), 2: Susceptible heterozygous (Sr), M: 100 bp marker.

20 µl 2× Master Mix (Pishgaman, Iran), 4 µl (70 ng) of genomic DNA, 1 µl (10pm/µl) of each allele-nonspecific primer, 2 µl (20pm/µl) of each allele-

Table 2. The frequency of genotypes of *Teladorsagia circumcincta* isolated from Iraqi sheep abomasa after allele-specific PCR (ASP) for detecting resistance to albendazole in Soran, Kurdistan district, Iraq

Genotype	No. of examined sheep	Allele frequency (%)
Susceptible homozygous (SS)	45	100
Resistance homozygous (rr)	0	0
Susceptible homozygous (Sr)	9	39.1

specific primer and 10 µl of distilled water. ASP reaction was run at 95°C and 30s for denaturing, 55°C and 30s for annealing, 72°C and 1 min for extension on a PeqStar 96 Universal gradient thermal cycler (Peqlab, Germany). ASP products were run on 1.5% agarose gel (Merck, Germany) for about 45 min at 120V. The gels were stained with safe stain (SinaClon, Iran) and visualized under UV light.

Results

A total number of 1531 adult nematodes of *T. circumcincta* from 45(32.1%) infected examined sheep abomasa were collected and identified (Fig. 2).

Molecularly, resistant allele to albendazole uncovered. The amplified products of 250 bp were resistance alleles (rr) while the 550 bp fragments were identified as susceptible (SS) alleles. Products of 750 bp were also amplified and indicated the presence of *T. circumcincta* as internal control (Fig. 3).

Both susceptible and resistance (heterozygous) were detected in all infected sheep abomasa to subpopulation of *T. circumcincta* in Soran, Kurdistan district, Iraq. Susceptible heterozygous (Sr) detected in 9(39.1%) infected sheep abomasa with subpopulation of *T. circumcincta*. Susceptible homozygous (SS) was also detected in all infected sheep abomasa with *T. circumcincta* subpopulation. While resistance homozygous (rr) did not detect in all examined sheep abomasa (Tab. 2).

Discussion

BZs are the most important class of anthelmintic drugs, which used for prevention and treatment of gastrointestinal nematodes for a long time in many parts of the world. For instance, in Iran, this class has been prescribed for more than 4 decades in order to treat infected small ruminant to nematodes [5]. According to the declaration of veterinary pharmacies in Soran city, Iraq, BZs has been also applied for several years. In a few studies, resistance to albendazole reported in infected sheep (66.6%) using faecal egg count reduction technique (FECRT) in South and North East of Iraq [18].

Although some previous studies noted that drug resistance in sheep nematodes in Iraq, however, the researchers mainly applied the traditional techniques, and the studies on molecular detection of resistance alleles in sheep nematodes, particularly on commonly used benzimidazoles, have not been performed and the available data regarding this issue is insufficient. Whereas most traditional assays are insensitive and difficult to standardize [19]. It has also reported that the presence of resistance in helminth population, is more widespread than that of estimated by standard methods such as faecal egg reduction test [20]. Moreover, the species of drug-resistant parasites in such studies in many cases has not been specified. In the current study, albendazole resistance-associated beta tubulin SNPs was detected for first time in *T. circumcincta* nematodes in this region.

In the world, drug resistance in nematodes is increasingly report from ruminants [21–23]. Worldwide, Sweden in Europe, Argentina, Brazil, Uruguay and Paraguay in South America, Kenya and South Africa in Africa, Malaysia in Asia, Australia and New Zealand, drug resistance reported to one and/or several drugs from helminths parasites with multi-resistances to all other major drug classes [25–27].

A substitution of phenylalanine to tyrosine at codon 200 (F200Y) of the isotype1 β-tubulin gene is the most commonly reported indicator of BZs resistance in parasitic nematodes of small ruminants [11]. In the present study, F200Y substitution was confirmed in infected Iraqi sheep with *T. circumcincta*. Additionally, there was also found susceptible and resistance alleles (heterozygote) to albendazole. It was in agreement with previous studies in Ireland and Iran [5,11]. High level of *T. circumcincta* isolates were also reported resistant to BZs at codon 200 (F200Y) in Scotland and Ireland [11]. In contrast, Martínez-Valladares et al. [28] noted that all examined *T. circumcincta* isolated from Spanish sheep were sensitive to BZs and there was no SNP at codon 200 and other codons (167 and 198). Development of anthelmintic resistance and differences among obtained results may be due to management systems, anthelmintic underdosing, mass treatment, climatic and environmental factors, methods of resistance detection, single-drug regimen, keeping the sheep and goats together, animals' movement, regularly treatment, and treatment frequency [29].

The homozygote resistance genotype (rr) was not detected in examined helminths subpopulation, however; a high rate of investigated helminths (39.1%) bearing the resistant allele were corresponded to susceptible heterozygote (Sr)

genotype. It has been indicated that the resistance allele (r) was inherited recessively and only (rr) helminths can be recovered after treatment. Furthermore, the installation of BZs resistance in a *T. circumcincta* subpopulation was irreversible [30]. The existence of resistance allele in susceptible heterozygote (Sr) genotype gives a selective advantage to the parasitic helminth compared to homozygote susceptible (SS) helminths. Comparisons on the results of experiments demonstrated that in experimentally infected sheep with *T. circumcincta,* the survival of (Sr) helminths was 4.5 fold greater than that of (SS) genotypes at 1/4 of the treatment dose. In other words, the selective advantage is 4.5 times higher for the (Sr) genotype. This can lead to spread and develop of resistance more rapidly in susceptible populations of helminths in the next generations [31]. In conclusion, albendazole-resistant allele in subpopulation of *T. circumcincta* exist and uncovered for the first in this region. It is therefore useful towards a better knowledge of parasitic drug resistance issue among small ruminants in Iraq.

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