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Molecular evaluation of E198A SNP in the iso-type 1 β – tubulin gene of *Haemonchus contortus* isolated from sheep in Al-Diwanyiah, Iraq

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Abstract

This study investigated the benzimidazole resistance in *Haemonchus contortus* parasitic nematodes from sheep from October 2021 to December 2022 in Al-Diwanyiah city/Iraq, and samples were processed at the laboratory of Parasitology in Veterinary Medicine College of Al-Qadissyiah University through the detection of E198A mutation and SNP polymorphism of the β-tubulin gene at this codon position. Ninety adult *H. contortus* samples were collected from the abomasum of sheep (n = 400) and then tested by qPCR and tetra-primer ARMS-PCR. Of these, three different genotypes have been found for E198A SNP: heterozygous (RS), homozygous (SS), and homozygous resistant genotype (RR). The frequencies for these genotypes were 31.11% heterozygous, 57.77% homozygous, and 11.11% homozygous resistant. The current study indicated the spread of benzimidazole resistance for *H. contortus* of sheep in Iraq, Al-Diwnayiah by utilizing qPCR and tetra-primer ARMS-PCR for the first time. It is speculated that the BZ-resistance is due to excessive and irregular *H. contortus* drug abuse and inter-species transfer between ruminants at the commonly grazing pastures and from imported sheep.

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Introduction

Small ruminants (sheep and goats) constitute an essential source of the human protein diet (1-4). However, parasitic infections with gastrointestinal nematodes (GINs) in all animals is a complicated matter, among which the highly pathogenic *Haemonchus contortus* (belongs to the order Strongylida) that leads to economic losses for the livestock production sector worldwide, especially in the tropical and subtropical regions (5-7). Therefore, various anthelmintic drugs have been adapted to control its prevalence among small ruminants. Because of these drugs' prolonged and excessive use, anthelmintic drug resistance emerged against three main anthelmintics classes: benzimidazoles, imidazothiazoles, and macrocyclic lactones (8,9). Surprisingly, drug resistance for each class emerged within a decade from when they were introduced. Given that, BZ-resistance was discovered for the first time in 1964 (10) and spread globally (11). It was reported that benzimidazole binds specifically and selectively to the β-tubulin gene (on chromosome 1) in BZ-resistant *H. contortus* nematodes, which subsequently interferes with the microtubule formation process (12-14). Molecular studies indicated that different β-tubulin gene mutations had been associated with the BZ-resistance in *H. contortus*, and namely, these Single Nucleotide Polymorphisms, SNPs are F167Y (TTC to TAC), E198A (GAA to GCA), and F200Y (TTC to TAC) (10,11,15). Moreover, mutations at 198 codons (16) are independent of SNPs mentioned above and showed variable alleles; E198L, E198V, E198K, and E198I (17). Interestingly, and based on *H. contotus* β-tubulin genetic studies, it is speculated that two SNPs or more within the same β-tubulin allele are catastrophic. Furthermore, these mutations confer resistance by changing the protein's physical structure and reducing the BZs binding affinity (18-20). Traditionally, *H. contortus* used to be diagnosed by time-consuming techniques such as faecal egg counts (FEC) and larvae recovering from faecal culture (LC), which lacks

both sensitivity and specificity (21,22). In addition, these techniques require professionally trained staff and precisely identified parasitic species to conduct such tests. Given that, eggs and larvae of these parasites are complicated sometimes when it comes to diagnosis and identification based on the morphological features (20,23,24). The development and utilization of Polymerase chain reaction (PCR) made it feasible to adopt this technique as a diagnostic tool for *H. contortus* and its associated drug resistance SNPs (7). Therefore, in this study, we utilized the amplification refractory mutation system (tetra-primer ARMS-PCR) to investigate for isotype-1 β-tubulin SNPs within this gene that utilizes four PCR primers to distinguish between the three genotypes at each SNP position (homozygous sensitive, SS; homozygous resistant, RS; and heterozygous resistant; RR) (20,25). Even though those SNPs have been investigated in countries like the UK, USA, Canada, France, Brazil, India, and China, our knowledge is still lacking regarding the BZ-resistance and its associated SNPs in Iraq. In this study, we targeted these SNPs to monitor their presence and prevalence in Iraq and to establish the grounds for more future investigations into *H. contortus* resistance.

Materials and methods

Ethical approve

Study and samples collection were conducted upon approval by the in Veterinary Medicine College of Al-Qadissyiah University ethics and animals' welfare committee under the number 2710 and dated by 01-10-2021.

Sample collection

Samples were collected from the slaughterhouses in AL-Diwanyiah city, where 400 sheep were examined. After slaughtering sheep and removing alimentary tract from the abdomen, abomasa were ligated at both terminals. To transport, it was dissected and sent to the laboratory for analysis at the veterinary medicine College of University AL-Qadisiyah for microscopic examination. The abomasa removed from omasum and duodenum. Afterward, abomasa opened along the greater curvature, and their contents were thoroughly washed under tap water using a mesh sieve. The appeared nematodes were differentiated based on red and white appearance due to their white ovaries wrapping around their blood-filled intestine. Then, the collected worms were counted and preserved in 70% alcohol containing 5% glycerin (26).

DNA extraction and PCR

A single adult worm was lysed, and total genomic DNA was extracted according to the protocol of (Addbio, Korea). Afterward, the purity and integrity of extracted DNA were assessed on 1% agarose gel, stained with ethidium bromide, and visualized using the gel documentation system. Subsequently, species-specific primers for the mt-COI conserved region were utilized to detect *H. contortus* species, as shown in Table 1. This test was used to confirm the positive specific *H. contortus* species. Thus, it can be further analyzed by ARMS-PCR (20,27,28). This technique was utilized by employing primers targeting 198 codons within the β-tubulin gene to detect SNPs responsible for BZ resistance in *H. contortus*. According to the previously described protocol by (Addbio, Korea), these primers were used (F-Outer, R-Inner, F-inner198s, R-inner198s) all in a single PCR tube as shown in table 1. Then, tetra-primer ARMS-PCR was carried out in a final volume of 20 µl comprised of 10 µl of master mix (AddBio), 1 µl (0.5 pmol/20 µl) of each primer (forward outer and inner, reverse outer and inner), 2 µl (100 ng) of DNA template, and 4 µl of PCR molecular grade water. Cycling conditions included an initial denaturation step of 95 °C for 5 minutes followed by a denaturation step of 39 cycles of 95 °C for 35 s and annealing of 55.5 °C for 30 s, extension of 72 °C for 40 s, and 1 cycle final extension of 72 °C for 5 min, by using (BioRad, USA). Thermocycling conditions: PCR thermal conditions were conducted using a conventional PCR thermocycler system. PCR products were analyzed on 2 % agarose and visualized by a gel documentation system (29).

Table 1: Oligoes used for qPCR and tetra-primer ARMS-PCR targeting the mt-COI and β-tubulin gene of *H. controtus*

Oligos name	5'-------3'	Amplicon size/ bp	Representing genotype
HconF-Sybr	GGCGGGAACAAGTTGAACAGT	150bp	mt-COI
HconR-Sybr	CCCTAAAATTGATCTTAAACCC	150bp	mt-COI
F-inner198s	ATCAACTGGTAGAGAACACCGACGA	200 + 433	Susceptible homozygous genotype (SS)
R-inner198r	AGCTTCGTTGTCAATACAGAATGCTG	433+284+200	Heterozygous genotype (RS)
F-Outer	TCAAAAATTCGTGAAGAGTACCCTGA	433+ 284	Resistant homozygous genotype (RR)
R-Inner	ACATTGTGACAGACACTTCAATTGCA	433+ 284	Resistant homozygous genotype (RR)

Results

To optimize for the exact annealing temperature for the mt-COI gene and 198 β-tubulin genes, we ran a gradient-PCR on 8 samples of *H. contorus* for both conventional and qPCR, and we found that 55.5 °C is the optimum annealing temperature for both as indicated in figure 1A. Thus, the protocol can be used for qPCR or PCR as a species-specific assay.

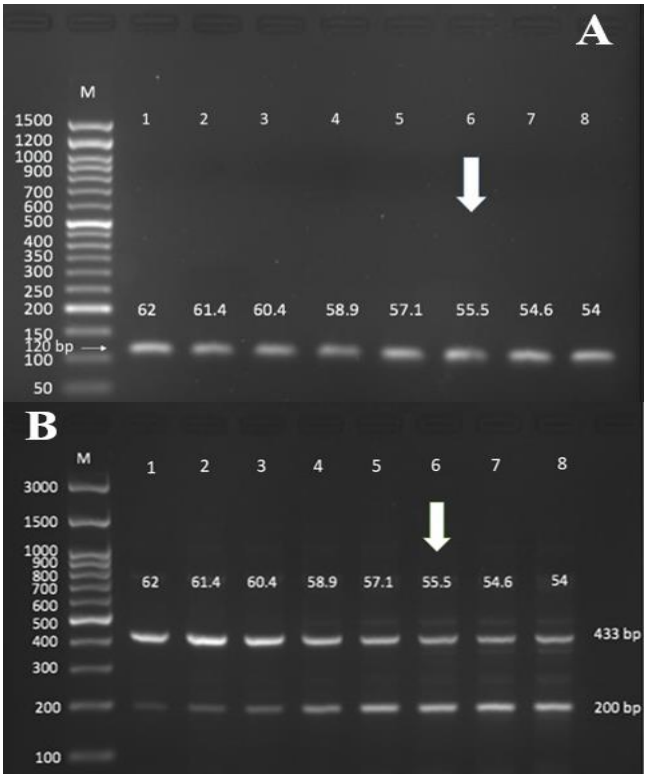

Figure 1: Representative 2% gel electrophoresis results of the serial gradient-PCR for *H. contortous* isolated from sheep indicated that 55.5 is the optimum annealing temperature for mt-COI and β-tubulin genes. A) Lane 1-8 indicates 55.5 is the optimum temperature for the mt-COI gene required for qPCR. B) Lane 1-8, the optimum temperature for theβ-tubulin gene using the outer and inner primers for tetra-primer ARMS-PCR; all samples were susceptible to homozygous genotypes (SS). M: Molecular DNA marker. Real-time qPCR was performed for all adult *H. contortus*, and the test results.

Our results showed a single peak for the mt-COI gene of adult *H. contortus*, indicating a precise amplification of the gene as shown in figure 2. All samples were confirmed to be *H. contortus* by real-time qPCR and subsequently subjected to the tetra-primer ARMS-PCR to investigate BZ resistance due to β- tubulin gene polymorphism.

First, we performed ARMS-PCR to prove the optimum annealing temperature using the outer and inner primers, which was 55.5 °C, as shown in the figure 1B. Then this ideal degree was used under the tetra- ARMS PCR reaction, and all confirmed samples examined were *H. contortus.* Of the 90 *H. contortus* parasites, 57.77% were homozygous BZ-susceptible genotype (SS) and produced two bands with 433 bp, and 200 bp in size on the 2% agarose gel see figure 3. However, 31.11% of the samples were heterogenous BZ-resistant genotype (RS) (Indicated by targeting the 198 codon position within β-tubulin) and produced triple bands with 433 bp, 284 bp, and 200 bp in size. Finally, 11.11% were homozygous BZ-resistant genotype (RR) and produced two bands with 433 bp and 284 bp in size.

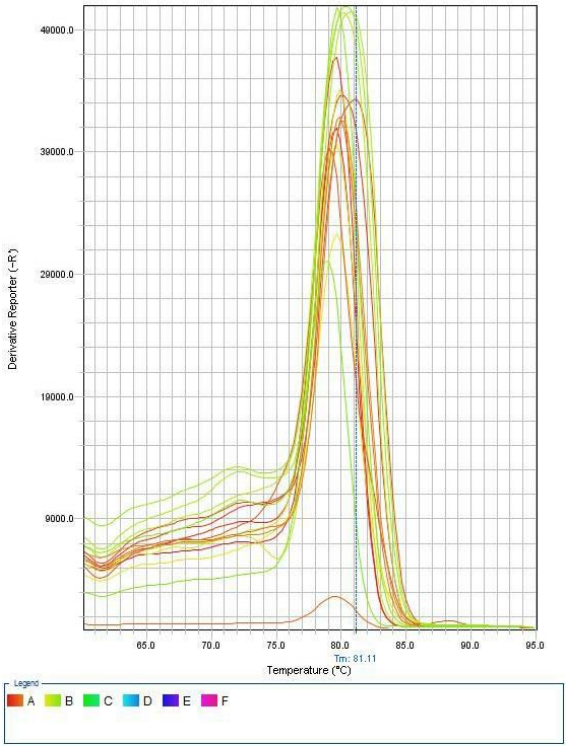

Figure 2: Melting curves analysis generated by real-time qPCR assay by testing mt-COI gene of *H. contortus*. This shows a amplification (one peak) without a non-specific reaction or primer dimmer.

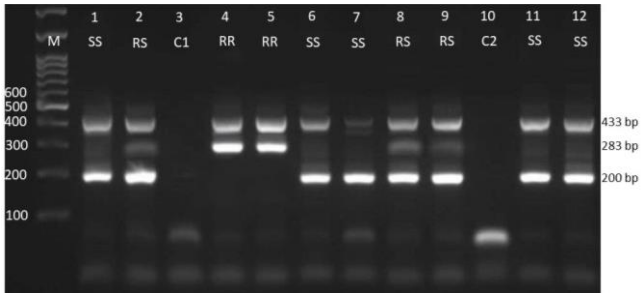

Figure 3: Tetra-primer ARMS-PCR demonstrated different genotypes of the *H. contortus* β-tubulin gene. Lane 1,6-7,11, and 12; show the susceptible homozygous genotypes (SS; 433 bp and 200 bps). Lanes 2,8, and 9; show the resistant heterozygous genotypes (RS; 433, 284, and 200 bps). Lanes 4 and 5 show the resistant homozygous genotypes (RR; 433 and 284 bps). Lanes 3 and 10, indicate experimental controls. M is the molecular DNA marker.

Discussion

Broad-spectrum benzimidazoles have been widely associated with drug resistance in nematodes due to their irregular use. Therefore, resistance against this class of drugs has been developed in *H. contortus* and other gastrointestinal nematodes (GINs) (30). In *H. contortus*, previous studies detected an SNP in the amino acid residue at position 198 of the gene encoding β-tubulin protein (31). Even though sheep are economically significant small ruminants in Iraq, benzimidazole resistance from these ruminants has not been investigated yet, and this investigation is to shed light for the first time on BZ resistance in sheep from Iraq.

The main goal of using the real-time qPCR technique is to prove the efficiency of this technique in the confirmed diagnosis of worm *H.* (32, 33), where it was possible to rely on the macroscopic examination of adult worms. However, due to the presence of significant similarity in the larval stages of order Strongylida and the difficulty of distinguishing through eggs, it was necessary to rely on rapid diagnostic methods. Through this study, it has become possible to rely on this technique in the confirmed diagnosis of any order of Strongylida.

Previously, (34) used the real-time qPCR technique to diagnose *H. contortus* where the (mt-COI) gene was targeted (34,35). Real-time qPCR assays showed excellent specificity to target nematodes. This is perfectly consistent with Reslova's group finding (36). Other researchers who have used this technique concluded that the real-time PCR assay saves time and requires no post-PCR gel electrophoresis, which is consistent with the current study's findings (37-39). Moreover, mt-COI is considered a diagnosis in *Hypoderma spp* by adapting the PCR-RFLP technique (31,40,41).

The detection of the presence of the benzimidazole resistance gene in *H. contortus* became critical due to the absence of previous studies in Iraq related to this field, where this gene was evaluated based on a technique tetra-primer ARMS-PCR and after the gel electrophoreses procedure, three genotypes were observed in the codon E198 and as follows (SS 57.77%, RS 31.11% and RR 11.11%) and this corresponds to what found him Zongze and his group (20)

After reviewing previous studies and given the association between the three SNPs (E198A, F167Y, and F200Y) in the β-tubulin gene associated with BZ-resistance (31,42), the aim here was to discover these mutations in the β-tubulin gene of *H. contortus* where 70% of mutations are found within E198A SNP (43). Therefore, we chose E198A SNP without other SNPS for our investigation in this study.

Conclusions

BZ-resistance in sheep infected with *H. contortus* parasite in Diwaniyah is due to the excessive and irregular use of antihelminthic, especially benzimidazole. However, the resistant strains may have come with the sheep by importing from neighboring countries.

Acknowledgments

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Conflicts of interest

The authors declare that no conflicts of interest have been associated with this work.

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التقييم الجزيئي للمورثة E198A متعددة الأشكال لجين التيوبيولين بيتا في الدودة السلكية والمعزولة من الأغنام في الديوانية، العراق

أكرم مدلول أمانه و منصور جدعان علي

فرع الأحياء المجهرية البيطرية، كلية الطب البيطري، جامعة القادسية، العراق

الخلاصة

تناولت هذه الدراسة مقاومة الديدان السلكية للبنزاميدازول في الديدان المعزولة من الأغنام في مدينة الديوانية / العراق وتمت معالجة العينات في مختبر الطفيليات في كلية الطب البيطري في جامعة القادسية من خلال تشخيص الطفرات الوراثية عند الموقع ١٩٨ متعددة الأشكال ضمن مورثة التيوبيولين بيتا تم جمع تسعون عينة من طفيلي الدودة السلكية البالغة من المعدة الرابعة من الأغنام (العدد = ٤٠٠) وبعدها تم اختبارها بتقنية تفاعل سلسلة البلمرة الكمي و تفاعل سلسلة البلمرة الرباعي لقد وجدت الدراسة الحالية ثلاث أنماط وراثية مختلفة للمتغاير الوراثي E198Aمتعدد الأشكال وهيود متغاير الزيجة و متماثل الزيجة وأخيرا نمط الزيجة المتماثل والمقاوم . ظهرت هذه الأنماط الوراثية بنسب مختلفة ٣١% لمتغايرة الزيجة، ٥٧% متماثلة الزيجة، و ١١,١١% متماثلة الزيجة المقاومة. أشارت الدراسة الحالية ولأول مرة في مدينة الديوانية الى انتشار مقاومة مضادات البنزيميدازول بين الأغنام في العراق وفي مدينة الديوانية خصوصا باستخدام تقنيتي تفاعل سلسلة البلمرة الكمي وتفاعل سلسلة البلمرة الرباعي. يعتقد أن سبب للمقاومة لهذه المضادات هو الاستخدام الخاطئ والمفرط، وكذلك انتقال الطفيلي بين أنواع المضائف بالإضافة الى الأنواع التي تأتي مع الأجناس المستوردة.