

MOLECULAR STUDIES OF *HAEMONCHUS CONTORTUS* FOR DETECTION OF BENZIMIDAZOLE RESISTANCE

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ABSTRACT

Benzimidazole (BZ) resistance in *Haemonchus contortus* is linked primarily with the mutation in the β -tubulin isotype-1 gene that substitutes phenylalanine (Phe) to tyrosine (Tyr) at 200 codon of the gene. In the present study, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used for detection of BZ resistance in the β -tubulin isotype-1 gene of *H. contortus*. Following PCR amplification of the region containing mutation in the β -tubulin gene and subsequent RE digestion with *Taal*, the 'rr' individuals (homozygous resistant) revealed 257 and 48 bp bands, the 'rS' individuals (heterozygous) showed 305, 257 and 48 bp bands, while 'SS' individuals (homozygous susceptible) revealed uncut 305 bp band. A total of 30 L3 larvae of *H. contortus* were collected from the faecal samples of sheep from Central Sheep Breeding Farm (CSBF), Hisar and were genotyped for analyzing BZ resistance. The results revealed that 27 larvae were 'rS' type, 3 'rr' type and 1 'SS' type. Overall, the prevalence of BZ resistant allele (r) was higher (86%) as compared to BZ susceptible allele (S) (14%). The study indicated that RFLP-PCR is an easy, reproducible and less expensive technique which would be helpful in establishing the prevalence of BZ resistance in *H. contortus* and can also be utilized for evaluating the existing worm control programme.

Key words: Anthelmintic resistance, *Haemonchus contortus*, benzimidazole, RFLP-PCR, sheep.

Of the various diseases affecting sheep, parasitic gastroenteritis caused by gastrointestinal nematodes, mainly *Haemonchus contortus*, is an important disease and is responsible for high mortality and morbidity. In the past 25 years, no new classes of anthelmintics have been developed for use in animals. Anthelmintic resistance has been documented to all classes of anthelmintics and multi-class resistance exists in many flocks. The prevalence of anthelmintic resistance is highest for *H. contortus*, making it the most economically important gastrointestinal nematodes of sheep (Fleming *et al.*, 2006). Detection of the anthelmintic resistance by conventional methods like faecal egg count reduction test (FECRT), egg hatch assay (EHA) and larval development assay (LDA) have been shown to be the most suitable tests (Coles *et al.*, 1988). But these methods have some limitations like low sensitivity, more expensive and more laborious. On the other hand PCR based molecular assays are more sensitive, less expensive, less laborious and rapid Prichard, 1997; Elard *et al.*, 1999; Von Samson-Himmelstjerna, 2006). Moreover, the conventional methods can detect the anthelmintic resistance in a worm population when more than 25% of the parasites are resistant. In contrast, molecular techniques discriminates as little as 1% resistant individual in samples of susceptible population (Pape *et al.*, 2003). Chandra *et al.* (2014) detected benzimidazole resistance in *H. contortus* using allele-specific PCR

(AS-PCR) and found it quick and reliable method for detection of early stage of development of resistance. Alvarez-Sanchez *et al.* (2005) found that real time PCR is more sensitive, rapid and less expensive for detection of benzimidazole resistance in trichostrongylids of sheep. Tiwari *et al.* (2006, 2007) detected benzimidazole resistance in *H. contortus* using PCR-restriction fragment length polymorphism (PCR-RFLP) technique and found PCR-RFLP to be more convenient and reproducible as compared to AS-PCR. Therefore, in this study PCR-RFLP technique was used to study the polymorphism in β -tubulin gene of *H. contortus*.

MATERIALS AND METHODS

Collection of L₃ Infective Larvae of *H. contortus*: Infective larvae (L₃) were collected from the faecal samples of Central Sheep Breeding Farm, Hisar. The faecal samples were mixed with activated charcoal and incubated at 27±2°C for 7 days in petri dishes. The faecal material was kept moist by sprinkling water on every alternative day. The infective larvae, L₃, were collected from the water of large petridish, cleaned by repeated washing and identified as per technique of Keith (1953).

Extraction of Genomic DNA: Genomic DNA from L₃ infective larvae was collected by the method of Silvestre and Humbert (2000) after minor modification. Larvae were exsheathed by incubation for 5 min. in a petri dish containing 10 ml larvae suspension and 450 μ l of sodium

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hypochlorite (aqueous solution, 4% active chloride; Fisher Scientific). Larvae were recovered in 10 µl suspension and killed by placing them in a microtube at -20°C for 30 min. 25 µl extraction buffer (1mM Tris HCl, 0.1mM EDTA, 5mg/ml Proteinase-K (20 mg/ml, Thermo Scientific) was added to each microtube and incubated at 55°C for 3-4 h. Proteinase-K was deactivated by incubating the microtubes at 95°C for 30 min. 4 µl suspension was used for PCR amplification.

Primers: One forward primer, Para 2F (5'-CTACCCTTTCGGTCCATCAA -3'), and one reverse primer, Para 2R (5' -TGAAGACGAGGGAATGGAAC -3') as reported by Tiwari *et al.* (2006) were used to flank the 200 codon of the β-tubulin isotype 1 gene sequence of *H. contortus*. The primers were reconstituted in nuclease free water (NFW) as instructed in the information brochure of the manufacturer (Sigma Life Science).

Amplification of β-tubulin Gene by PCR: The PCR reaction mixture (25 µl) consisted of 5.00 µl of 5X Phusion buffer (Bio Labs), 200nM each of Para 2F and Para 2R primers, 0.50 µl of 10mM dNTPs, 0.25 µl of Phusion DNA polymerase (0.02 U/µl) and 4.00 µl of DNA template. The reaction mixture was made up to 25 µl with NFW.

The amplification was performed in an Eppendorf thermal cycler with the following amplification conditions: initial denaturation at 95°C for 4 min. followed by 40 cycles of denaturation at 95°C for 50 sec., annealing at 60°C for 30 sec. and extension at 72°C for 1 min. followed by a final elongation at 72°C for 2 min. and stored at 4°C for further use (Fig. 1). After amplification, 5 µl PCR product was loaded in 2% agarose gel and 1X TAE buffer was used to run the gel for 40 min. in horizontal electrophoresis apparatus.

Restriction Analysis: After purification, the purified product was digested with *TaaI* (HpyCH4III, Thermo Scientific) that recognizes the nucleotide sequence (5'-AC ↓GT-3') site. The reaction mixture (15µl) consisting of 10 µl of PCR products, 1.5 µl of NFW, 1.5 µl of 10X Tango Buffer and 2 µl of *TaaI* enzyme was taken in a microtube. Then the microtube was incubated at 65°C in water bath for overnight. The whole digestion mixture was loaded in 2.5% agarose gel and run for 1 h to identify homozygous susceptible (SS) larvae (305 bp), heterozygous (rS) larvae (305, 257 and 48 bp and

homozygous resistance (rr) larvae (257 and 48 bp) as described by Tiwari *et al.* (2006).

Sequencing of PCR Product: To confirm the polymorphism in sequence of β-tubulin gene at codon 200 i.e. change of amino acid phenylalanine (TTC) by tyrosine (TAC) which is responsible for benzimidazole resistance in *H. contortus*, sequencing of one of the PCR product was performed in automatic sequencer to obtain the sequence of the β-tubulin isotype 1 gene.

Insilico Restriction Profile Method: The obtained sequence was also analysed by *Insilico* restriction digestion. For this, "NEB cutter" software was used. The sequence was put into software and was cut by *TaaI* restriction enzyme. The dry lab gel image was used to see the band size after restriction digestion of the product.

RESULTS AND DISCUSSION

The single nucleotide polymorphism (SNP) at codon 200 of β-tubulin gene is mainly responsible for the benzimidazole resistance in *H. contortus* (Elard and Humbert, 1999). The other SNPs may be present at codon 167 and 198 which could also play their role in benzimidazole resistance (Barrare *et al.*, 2012). Therefore, the present study was planned to detect the polymorphism in β-tubulin and its association with benzimidazole resistance.

The genomic DNA of L3 larvae of *H. contortus* was amplified with a product of 305 bp from the β-tubulin gene (Fig. 2). A total of 30 L3 larvae of *H. contortus* were genotyped for detecting polymorphism in β-tubulin gene. Upon restriction digestion by the enzyme *TaaI*, the PCR products of 29 larvae showed two bands of 305 bp and 257 bp, respectively (Fig. 3), thus confirming the presence of heterozygous individuals in the worm population at Central Sheep Breeding Farm, Hisar. The PCR product of one larvae showed single uncut band of 305 bp upon restriction digestion suggestive of homozygous susceptible larvae for benzimidazole resistance (Tiwari *et al.*, 2006). The sequence of amplicon was analysed for restriction endonuclease profiling with the help of *insilico* software (NEB Cutter) for further confirmation of results. Upon restriction digestion of amplicon by the enzyme *TaaI* the gel image showed two bands of size 257 bp and 48 bp (Fig. 4). However, 48 bp band was not visible in the gel which may be due to its smaller size

or may also be due to low concentration of the gel. The sequencing of one PCR product was further carried out in automatic sequencer by using forward and reverse primers. The sequences were aligned using BLAST software of NCBI. The obtained sequence showed the presence of point mutation or polymorphism in β -tubulin gene which was indicated by presence of tyrosine (TAC) at 200 codon (Fig. 5).

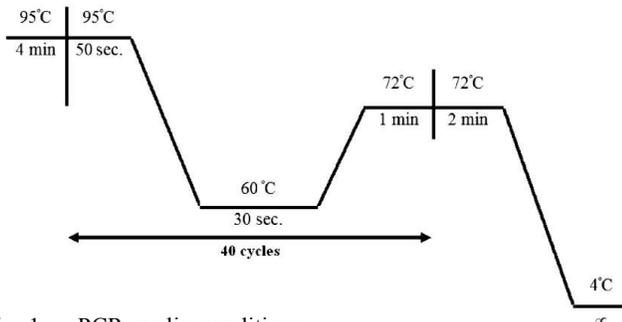


Fig. 1: PCR cyclic conditions

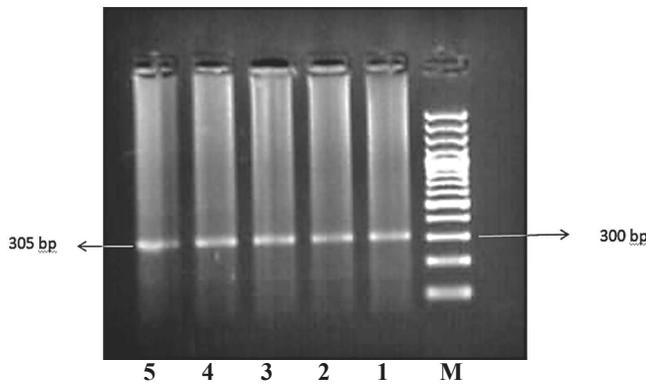


Fig. 2: PCR product of β -tubulin gene of *H. contortus*
Lane M = 100 bp DNA ladder
Lane 1, 2, 3, 4 and 5=amplified product of β -tubulin gene.

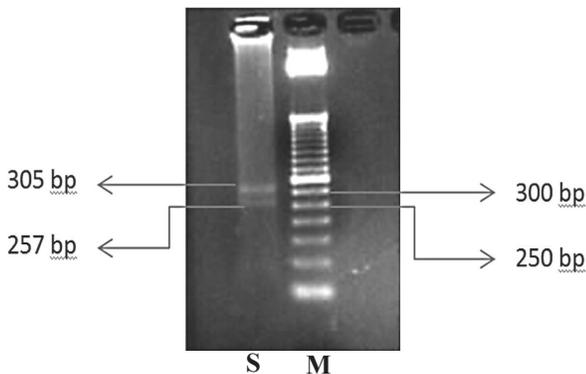


Fig. 3: RFLP-PCR profile of β -tubulin gene of L3 of *H. contortus* showing polymorphism. *In silico* restriction digestion.
Lane M = 50 bp ladder

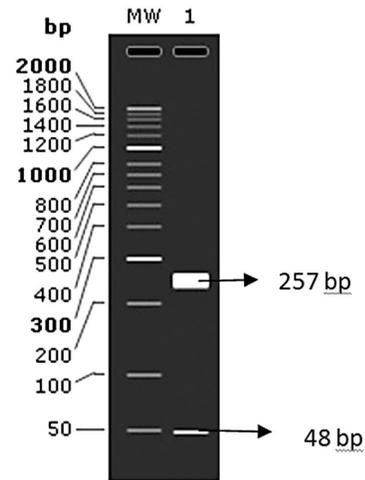


Fig. 4: *In silico* restriction profile of amplicon.
Lane MW = 50 bp ladder
Lane 1 = restriction profile of amplicon

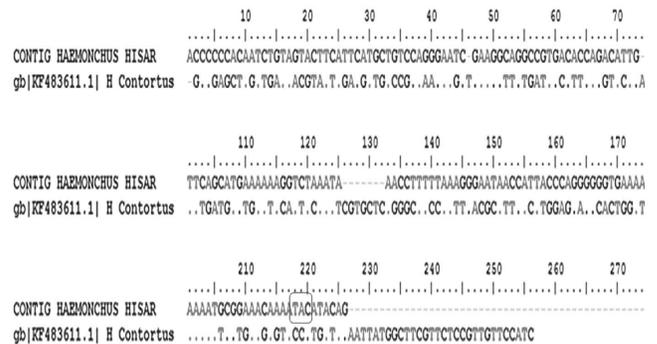


Fig. 5: The obtained sequence of β -tubulin gene of *H. contortus* shown by *Haemonchus (Hisar)*
The mutation is shown by encircling the TAC (tyrosine) at codon 200 in the sequence.

Severe resistance was detected for fenbendazole (FECR- 44.49%) in sheep at this farm by FECRT (Kumar and Singh, 2016). The post-treatment faecal cultures of animals treated with fenbendazole showed the survival of larvae of *H. contortus* only. This study clearly proved the presence of resistant *H. contortus* worm population in sheep at CSBF, Hisar. The PCR-RFLP based findings also confirmed the results of FECRT.

Many workers have supported PCR based molecular tools to detect the anthelmintic resistance as it is more accurate, quick and useful for the epidemiological studies of parasites (Elard *et al.*, 1999; Tiwari *et al.*, 2006). Moreover, these techniques are also helpful in species identification of various nematodes

(Silvestre and Humbert, 2000). Roeber *et al.* (2012) reported that PCR is useful as a rapid approach for detection of benzimidazole resistance in strongyles and, combined with FECRT, assessing the emergence of anthelmintic resistance. Therefore, these methods are helpful in making future strategies for use of anthelmintics as limited numbers of anthelmintic classes with different mode of action are currently available.

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