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RESEARCH ARTICLE
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Molecular diagnosis of benzimidazole resistance in *Haemonchus contortus* in sheep from different geographic regions of North India

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Abstract

Aim: To monitor the benzimidazole resistance in *Haemonchus contortus* in sheep from different agro climatic zones of India reared in extensive or intensive managemental conditions.

Materials and Methods: Faecal materials were collected from sheep from organized as well as private farms located in Rohilkhand region, Bundelkhand region, and Eastern Utter Pradesh. Larvae were obtained by coproculture and identified as L_3 of *Haemonchus contortus*. Exsheathment of larvae was done by incubating them in 180 µl of sodium hypochlorite for 5-20 minutes. Genomic DNA was isolated from single larvae and amplification of β-tubulin of *Haemonchus contortus* was done by primary and nested PCR. PCR-RFLP was done with *RsaI* enzyme for species identification. Genotyping was done by Allele specific PCR using Nested PCR product as template and resulting fragments were separated by gel electrophoresis in a 1.5% agarose gel in TAE buffer.

Results: Species was confirmed as *H. contortus* by PCR-RFLP which shows three fragments at 440 bp, 190 bp, and 150 bp. From Rohilkhand region out of 30 larvae, 19 (63%) were homozygous resistant (RR), 6 (20%) were homozygous susceptible (SS), and 5 (17%) were heterozygous (RS). From Bundelkhand 34 (85%) were RR, 4 (10%) were SS, and 2 (5%) were RS out of 40 larvae screened. From Eastern UP (Jaunpur and Varanasi) 30 larvae each from Jaunpur and Varanasi were genotyped;larvae from Jaunpur revealed 53% RR, 27% SS, and 20% RS whereas larvae from Varanasi genotyped showed 57% RR, 17% SS, and 26% RS.

Conclusion: Due to indiscriminate use of Benzimidazole group of drugs in organized and private farms, resistance to this group of drugs is increasing at the field level.

Keywords: β-tubulin, allele specific PCR, benzimidazole resistance, *Haemonchus contortus.*

Introduction

Benzimidazole group of anthelmintics are widely used for control of Haemonchosis in sheep due to their high therapeutic index and absence of toxic residues in milk and meat [1]. Benzimidazole (BZ) exhibit the anthelmintic activity by binding to β-tubulin which interferes with polymerization of the microtubule. Resistance to BZ is correlated with a conserved mutation at amino acid 200 in β-tubulin in isotype-1 with Phenylalanine being replaced by Tyrosine [2].

The diagnosis of BZ resistance in a worm population of *Trichostrongyle* parasites was based until now on the estimation *in vitro* of the lethal dose 50 (LD_{50}) on eggs (Egg hatch assay), on larvae (Larval development assay), or *in vivo* of the egg reduction in faecal sample after BZ treatment (FECRT) [3, 4, 5]. These methods have proved their efficacy but can only detect BZ resistant worm population when more than 25% of the parasites are BZ resistant [6].

Benzimidazole resistance in *Trichostrongylid* nematodes has been reported by many researchers. First report of Thiabendazole resistance in sheep in India was from Rishikesh (Uttarakhand) [7]. Other reports are from Himanchal Pradesh from goats [8], Uttar Pradesh, Uttarakhand [9-13], Rajasthan [14], and South India [15, 16].

In current study Allele-specific Polymerase Chain Reaction (AS-PCR) was employed to detect BZ resistance in larval population of *Haemonchus contortus* of sheep origin, and susceptible, resistant, and heterozygous population was genotyped.

Materials and Methods

Ethical approval: Adequate measures were taken to minimize pain or discomfort to animals while taking samples per-rectally. Experiments were carried out in accordance with the guidelines laid down by the Institutional ethics committee and in accordance with local laws and regulations.

Study area: Faecal samples were collected from organised and private farms per-rectally from sheep. These farms were located in Rohilkhand region (Bareilly), Bundelkhand (Jhansi), and Eastern UP (Jaunpur and Varanasi).

Harvesting of L_3 larvae of *H. Contortus*: L_3 of *Heamonchus*

were obtained from coproculture of sheep faeces from areas which had history of *Haemonchus* infection previously and larvae were identified [17]. The characters taken as criteria for correct identification of larvae of *Haemonchus* were length of larvae including sheath, anterior end of larvae, number and shape of intestinal cells and distance between tail of L_3 and sheath.

Genomic DNA extraction from larvae: Larvae were collected from culture and washed 3-4 times with distilled water. Approximately 200 larvae were ex-sheathed by incubation for 5-20 minutes in a petridish containing 4 ml of larval suspension and 180 µl of Sodium hypochlorite (aqueous solution, 4% active chlorine). Single larva was removed with 2 µl of suspension and killed by placing in a micro tube at –20ºC for 20 min. DNA was extracted by adding 5 µl extraction buffer (1 mM Tris-HCl, 0.1 mM EDTA and 5 mg/ml proteinase K) and incubating tubes at 50ºC for overnight. Proteinase K was inactivated by incubation at 95ºC for 20 minutes [18].

Oligonucleotide primers: Oligonucleotide primers (P_1 to P_7) were synthesised from published sequence [19]. P_1- 5'GGC AAG TAT GTC CCA CGT GC 3, P_2- 5'GAT CAG CAT TCA GCT GTC CA 3' P_3- 5'GGA ACA ATG GAC TCT GTT GT 3', P_4- 5'GGG AAT CGA AGG CAG GTC GT 3' P_5- 5'GGA ACG ATG GAC TCC TTT CG 3', P_6- 5' CTG GTA GAG AAC ACC GAT GAA ACA TA 3', P_7- 5' ATA CAG AGC TTC GTT GTC AAT ACA GA 3'

Amplification of β-tubulin

Primary polymerase chain reaction (PCR): PCR was carried out in thin wall PCR tubes in 25 µl reaction volume. Genomic DNA from third stage larvae was used as template for amplification of β-tubulin. The PCR mixture consisted of 5 µl of lysate as template, 20 pmol of each primer (P_1 and P_2), 1 mM $MgCl_2$, 100 µM of each dNTPs, and 2.5 µl of 10x Taq DNA polymerase buffer and 1 U of Taq DNA polymerase (Fermentas, Germany). The volume of the reaction was made up to 25 µl with autoclaved milli-Q water. Polymerase chain reaction was performed using PTC-200 with the following conditions.

Initial Denaturation at 94ºC for two minutes was followed by 20 cycles each at 94ºC for 55 s, 57ºC for 55 s and 72ºC for 55s. This was followed by 10 min final extension at 72ºC. The PCR amplicons were used as template for nested PCR.

Nested PCR: The β-tubulin PCR amplicons were used as template for nested PCR. The PCR mixture consisted of 3 µl of template (β-tubulin PCR product) 20 pmol of P_3 and P_4 primers, 100 µM of each dNTPs, 2 mM of $MgCl_2$, 2.5 µl of 10x Taq DNA polymerase buffer and 1 U of Taq DNA polymerase. The volume of reaction was made up to 25 µl with autoclaved milli-Q water. PCR was performed in PTC-200 with conditions as similar as β-tubulin amplification.

Restriction Fragment length polymorphism (RFLP) with *RsaI* enzyme: 10µl of the amplified nested product was digested with the restriction enzyme *RsaI* for species identification. The restriction enzyme digestion was performed in waterbath for 4 hours at 37ºC and, resulting fragments were separated by electro-phoresis on a 2.5% agarose gel and their lengths used for species identification [19].

AS-PCR: Two aliquots (1.5 µl) of the (P_3-P_4) nested product were used to determine the resistance of larvae against BZ group of drugs. The system was divided into two mixes, each containing two non-specific primers (P_4 and P_5) and one allele-specific primer. Each reaction generated one allele specific and one non - allele specific fragments. The PCR mixture volume is 25 µl. 1.5 µl of nested product used as template for amplification of allele specific PCR with 10pmol of P_4 & P_5, 20pmol of P_6 in one mix and P_7 in another mix, 1 mM $MgCl_2$, 80 µM of each dNTPs, 2.5 µl of Taq DNA polymerase buffer and 1 U of Taq DNA polymerase. The volume of the mixture was made up to 25µl with autoclaved milli-Q water.

PCR was performed with following conditions: Initial denaturation at 94ºC for 2 minutes was followed by 30 cycles each at 94ºC for 55 s, 55ºC for 55 s and 72ºC for 55 s. This was followed by 10 min final extension at 72ºC. The resulting fragments were separated by gel electrophoresis in a 1.5% agarose gel in TAE (1x) buffer for 1 hour at constant voltage (100V).

Statistical analysis: Statistical analysis was performed using the Chi test for analysis [20]. The values that were below $P < 0.05$ were considered as significant.

Results

Identification of *Haemonchus* larvae: The anterior end of larvae was bullet shaped intestinal cells were 16 in number and rectangular in shape. The tail of the sheath was showing kink in the tail just after the tail of the larva proper (Figure-1 and 2).

Amplification of β-tubulin gene of *H. Contortus*: The primary PCR size of genomic DNA isolated from pooled larvae of *H. contortus* was 840 bp (Figure-3). Nested PCR amplified approxi-mately 774 bp product (Figure-4). In PCR-RFLP the expected fragments were obtained from third stage larvae of *H. contortus.* One internal fragment of 440 bp, low intense 190 bp and 150 bp were confirmed the species as *H. contortus* (Figure-5). In Allele specific PCR the size of the specific bands were susceptible allele specific gene at 550 bp, resistant allele specific gene at 250 bp non allele specific gene at 750 bp (Figure-6).

The extent of resistance was found to increase at field level (Table-1). From Rohilkhand region 30 larvae were analyzed of which 19 (0.63) were homozygous resistant (rr), 6 (0.20) homozygous susceptible (SS) and 5 (0.17) heterozygous (rS). From Bundelkhand

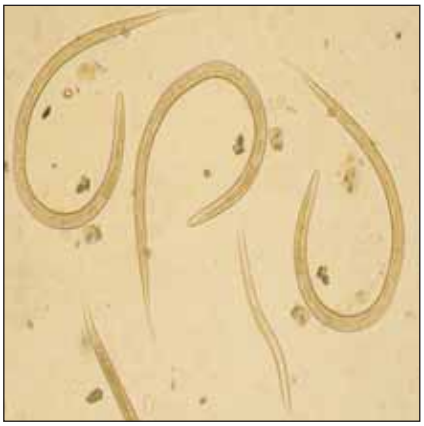

Figure-1: L_3 larvae of *H. contortus*

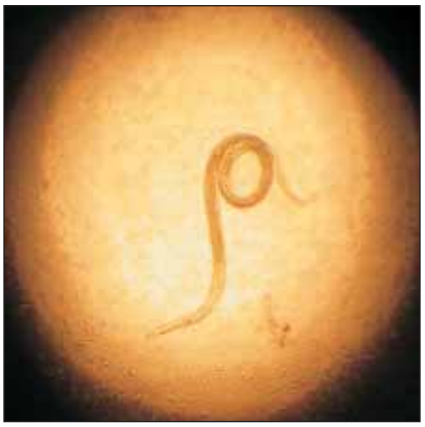

Figure-2: Exsheathed L_3 of *H. contortus*

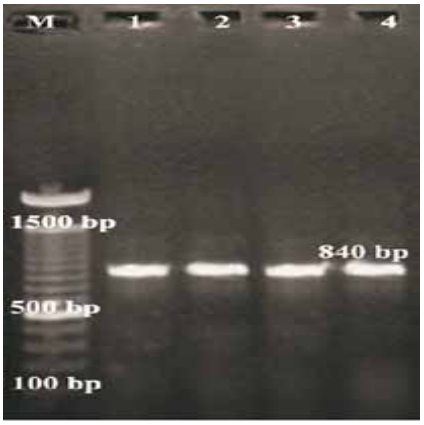

Figure-3: Standard PCR from pooled larvae of *H. contortus,* Lane M : 100 bp DNA ladder, Lane 1 : Rohilkhand strain, Lane 2 : Jhansi strain, Lane 3 : Jaunpur strain, Lane 4 : Varanasi strain

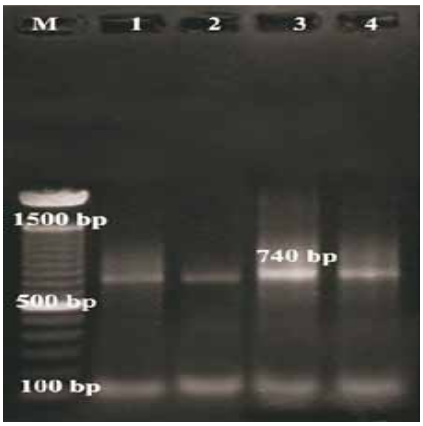

Figure-4: Nested PCR from L_3 of *H. contortus,* Lane M : 100 bp DNA ladder, Lane 1 : Rohilkhand strain, Lane 2 : Jhansi strain, Lane 3 : Jaunpur strain, Lane 4 : Varanasi strain

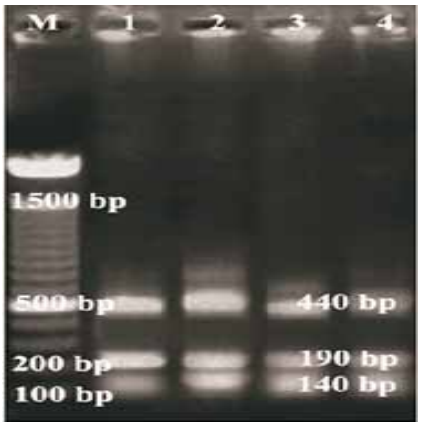

Figure-5: RFLP with RSa1 enzyme of L_3 *H. contortus,* Lane M: 100 bp DNA ladder, Lane 1 : Rohilkhand strain, Lane 2 : Jhansi strain, Lane 3 : Jaunpur strain, Lane 4 : Varanasi strain

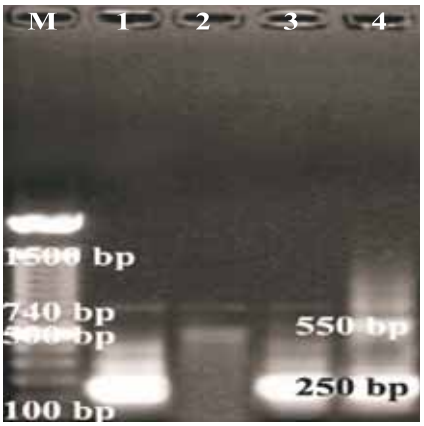

Figure-6: AS PCR of L_3 of *H. contortus,* Lane M : 100 bp DNA ladder, Lane 1 : Rohilkhand strain, Lane 2 : Jhansi strain, Lane 3 : Jaunpur strain, Lane 4 : Varanasi strain

Table-1: Genotyping of larvae of *H. contortus*

Location	Host	No. of L_3 larvae	Genotypic frequency			Allelic frequency	
			Homozygous Resistant (RR)	Homozygous susceptible (SS)	Heterozygous (RS)	Resistant (R)	Susceptible (S)
Rohilkhand (organized farm)	Sheep	30	19 (0.63)	6 (0.20)	5 (0.17)	0.73	0.27
Bundelkhand (organized farm)	Sheep	40	34 (0.85)	4 (0.10)	2 (0.05)	0.88	0.12
Jaunpur (Private farm)	Sheep	30	16 (0.53)	8 (0.27)	6 (0.20)	0.63	0.37
Varanasi (Private farm)	Sheep	30	17 (0.57)	5 (0.17)	8 (0.26)	0.70	0.30

region (Jhansi) 40 larvae from sheep were analyzed and result showed a high degree of resistance in organized farm of Jhansi of which more than 75 % larval population showed resistance (rr). From Eastern U.P. larvae were analyzed from Jaunpur and Varanasi. 30 larvae from sheep from Jaunpur were analyzed of which 16 (0.53), homozygous resistant (rr), 8 (0.27) homozygous susceptible (SS), 6 (0.20) heterozygous (rS). Larvae from sheep of Varanasi were showing more resistance.

Discussion

Benzimidazole resistance in India is a wide spread problem interfering with control of gastrointestinal nematodiasis in field as well as organised farm. Although, some workers have previously reported resistance employing conventional techniques. Very few reports are available on the application of molecular technique AS-PCR which can be very easily applied to L_3 larvae obtained from coproculture of faeces of infected animals not responding to this group of anthelmintic.

During the present study larval (L_3) population from different regions of Uttar Pradesh were genotyped. These regions were Rohilkhand, Bundelkhand, Jaunpur and Varanasi which are under different agro climatic zones of Uttar Pradesh. In nested PCR, the amplified

product was of 774 bp. In RFLP, for species identification DNA fragments were of 440 bp, 190 bp, and 150 bp when RSa1 enzyme was utilized. The fragments confirmed the larvae belonging to *H. contortus.* Our results of nested PCR are similar to earlier study [19] who also found DNA fragments of 441 bp, 190 bp and 155 bp with same enzyme in RFLP. In nested PCR, product size was 774 bp which is slightly different from earlier study [19]. In AS-PCR specific bands showing susceptibility to BZ was of 550 bp and resistance specific gene was of 250 bp and non specific gene at 750 bp. Since the genes are conserved in *H. contortus* it was found that the product size of AS-PCR were similar in larval samples collected from different regions. Our results are further confirmed by similar observation on larval population for detection of point mutation involved in BZ resistance applying AS-PCR [19]. The fragments generated by AS-PCR were similar to previous workers [11,12,13,16,19].

Genotypic frequency of larvae with reference to homozygous resistant (RR), homozygous susceptible (SS) and heterozygous (RS) differed in different regions. RR population was maximum (85%) in organised farm from Bundelkhand region from sheep. It was followed by organised farm in Rohilkhand region (63%). In private farms, RR population showed increasing trend which varied 53% (Jaunpur) to 57% (Varanasi). SS population varied from 10% (Bundelkhand) to 20% (Rohilkhand). In private farms, SS population varied from 17% (Varanasi) to 27% (Jaunpur). RS population in organised farms varied from 5% (Bundelkhand) to 17% (Rohilkhand). In private farms RS population varied from 20% (Jaunpur) to 30% (Varanasi).

The results indicated that in organized farms, due to frequent use of BZ group of drugs, RR population is increasing and susceptible population is decreasing. RS population also has decreased drastically and in due course of time complete resistant population may develop unless suitable measures to avoid anthelmintic resistance is taken up applying principle of grazing management and TST applying FAMACHA. Similar studies on BZ resistance has also been conducted utilizing RFLP-PCR and it was found 68% RR, 17% RS and 15% SS larvae out of 54 larvae [21]. The allelic frequency for gene for 'r' was 0.77 and gene for susceptibility 'S' 0.23. In the present study allelic frequency of 'r' gene varied from 0.63 (Jaunpur) to 0.88 (Bundelkhand) where as for 'S' gene it was 0.12 (Bundelkhand) to 0.37 (Jaunpur). Our results indicated that allelic frequency for resistant gene is increasing in organised farm in Bundelkhand region followed by Rohilkhand.

Conclusion

Molecular method (AS-PCR) is a quick and reliable method for diagnosis of Benzimidazole resistance at early stage of development of resistance in *H. contortus.* Prevalence of resistance in *H. contortus* has been detected against Benzimidazole group of drugs in Rohilkhand, Bundelkhand (Jhansi), and Eastern U.P. (Jaunpur, Varanasi). Benzimidazole resistance from Bundelkhand and Eastern U.P. has been recorded for the first time from both farm and field. Genotypic frequency of Benzimidazole resistance varies from 53% to 85%. Resistant population is present at organized as well as private farms; it is showing higher resistance in organized farms which may be due to continuous use of Benzimidazole group of drugs.

Author's contributions

AP and MS has designed and supervised the research. SC collected samples and carried out experiment. NY and SD participated in coproculture. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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