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Molecular Detection of Benzimidazole Resistance in *Haemonchus contortus* Larvae of Goats in Chhattisgarh, India

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ABSTRACT

Background: Benzimidazole resistance is one of the key problem in small ruminant production. A rapid, truthful and responsive system is required for detection benzimidazole resistance so that proper regulatory measure can be applied. Allele specific PCR is one of the tools to understand the mechanism and origin of benzimidazole resistance.

Methods: A total 198 larvae of *Haemonchus contortus* were isolated from goats of Chhattisgarh region, central India were genotyped by allele specific polymerase chain reaction (AS-PCR). Faecal samples of goats were collected from three Government farms and adjoining field goats and were subjected for faecal culture, separately. DNA of third stage larva was used for nested PCR for amplification of β- tubulin gene. Restriction fragment length polymorphism (RFLP) was applied on nested PCR product for species identification with RsaI enzyme. AS-PCR was applied on the nested-PCR product to know the genotypic and allelic frequency.

Result: The nested PCR amplified product showed approximately 820 bp in all cases and PCR-RFLP revealed 462 bp, 211 bp and 147 bp fragments, which confirmed the species as *H. Contortus*. Frequency of resistant allele ('r') was 49.7% and 50.3% for susceptible allele ('S'). Frequency of homozygous resistant (rr), heterozygous susceptible (rS) and homozygous susceptible (SS) genotype were 33.83 per cent, 31.81 per cent and 34.34 per cent, respectively. The frequency of homozygous resistant (rr) genotype was low (19.61%) in field compare to farm (48.96%) indicating refugia in field region.

Key words: AS-PCR, Benzimidazole resistance, Frequency, Goats, *Haemonchus contortus*.

INTRODUCTION

The problem of resistance to anthelmintics has attained clinical and economical importance particularly in trichostrongyloid nematodes of small ruminants and is a serious risk to profitable animal livestock production. There are cases where the prevalence of resistance and the cost of control failure are so high that livestock are in jeopardy. Anthelmintic resistance is outspread in many states of India (Singh and Swarnkar, 2008). Due to widespread emergence of anthelmintic resistance and ubiquitous nature of the organisms presents a difficult challenge in, management/ control of worms (Sanyal, 2014).

It is necessary to monitor the effectiveness of currently available anthelmintics, where there is no resistance and to keep away from further selection of resistance where it has already begun to become illusory (Shalaby, 2013). This is only achievable if there are qualified means for the diagnosis of anthelmintic resistance (AR) available (Pawar *et al.,* 2019). *In vivo* tests, like the controlled test, which involves necropsy of treated and untreated animals, or the faecal egg count reduction test (FECRT) are exorbitant and laborius. Alternatively, *in vitro* tests have been established, like the egg hatch assay (EHA) and the larval development tests (LDT) are there. These are quicker, less labor intensive and have been recognized, especially for AR prevalence studies but with lower degree of sensitivity (Singh *et al*., 2017).

In the present study Allele specific Polymerase Chain Reaction (AS-PCR) is applied to detect benzimidazole resistance in *Haemonchus contortus* of goats. AS-PCR offer the opportunity to detect the resistance at near the beginning level when only 1% of parasite populations have become resistant in comparison to conventional methods which are only reliable if more than 25% worms in a given population are resistant to benzimidazole (Roos *et al*. 1995). Molecular methods are of great value to understand the origin and mechanism of benzimidazole resistance.

Three single nucleotide polymorphisms (SNPs) in the isotype-1 b-tubulin gene at codon 167, 198 and 200 are accounts for benzimidazole resistance in various nematode species which correlate with benzimidazole resistance (Prichard, 2001; Ghisi *et al*., 2007; Silvestre and Cabaret, 2002; von Samson Himmelstjerna *et al*., 2007; Chaudhry *et al*., 2015; Ramünke *et al*., 2016).

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There were very few studies on anthelmintic resistance had been done in the above region (Sanyal *et al*., 2014 and Kumar *et al*., 2014) but, these studies uses conventional *in vitro* (Egg hatch test) and *in vivo* (Faecal egg count reduction test) methods for detection of anthelmintic resistance. Though, AS-PCR is expensive and required trained personal, but it can be applied in for early detection with sensitivity so that proper measures could be taken for sustainable control of gastrointestinal parasites.

MATERIALS AND METHODS

Place of study and sample collection

The proposed work has been taken in Chhattisgarh region, Central India. Chhattisgarh's geographical position is 17° 46 minutes north to 24° 5 minutes north and 80° 15 minutes east to 84° 20 minutes east with hot and humid climate. These prevailing conditions cause the animals vulnerable for parasitic infection. Faecal samples of goats were collected from three Government Farms Namely Pakaria Goat Farm (Bilaspur), Kawrdha Goat Farm and College Unit Goat Farm, Durg as well as from field goats of the adjoining area of the above three farms.

Benzimidazoles (albendazole/fenbendazole) and ivermectin were alternately used three to four times a year in farm animals, while the frequency of deworming in field goats was less than one or as per the clinical condition of the animal. It was known before the experiment that the chosen animals had not been de-wormed in the previous 3 months. About 10 to 20 grams of faecal samples were collected directly from the rectum of each goats in sterile polybags, labeled and brought to Department of Veterinary Parasitology, College of Veterinary Science and Animal Husbandry, Anjora, Durg (C.G) for further investigation.

In this study 198 infective larvae (L3) of *H. contortus* were genotyped to know the prevalence of genotypic frequencies of homozygous resistance (rr), heterozygous resistance (rS) and homozygous susceptible (SS) for both farm and field population of *H. contortus* larvae. The status of BZ resistance in populations of *H. contortus* larvae collected from goat flocks in semi-intensive and traditional farming in the Chhattisgarh region of Central India by AS-PCR was investigated. The above study was carried during the period of June 2018 to June 2019.

Genomic DNA extraction

DNA extraction from single larvae was done as per the method described earlier by Coles *et al*. (2006) and Chandra *et al*. (2015). The positive faecal samples were pooled as per their geographic location of collection and were mixed with activated charcoal powder for faecal culture by petri dish method (Soulsby, 1982). Development of the larvae in the culture was checked regularly, collected and washed 3-4 times with distilled water by centrifuging at 5000 rpm for 1 minute. For exsheathment of larvae, petri dish containing 4 ml larval suspension (less than 1000 larvae/ml) and 180 µl sodium hypochlorite (aqueous solution, about 3.5% active Chlorine) was added and the suspension was kept for 20-30 minutes and checked regularly under microscope for larval exsheathment. Single exsheathed larva was collected under stereo microscope with 2 µl of suspension by micropipette. The larva was killed by placing it in a PCR tube at -20°C for 20 min. 5 µl of DNA extraction buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0) and 5 mg/ml proteinase K) was added and incubated at 56°C for six hours. Inactivation of Proteinase K was done by incubating lysate at 95°C for 20 min. Further, the lysate was centrifuged at 4000 rpm for 2 min and 5 µl of supernatant was used for amplification.

Amplification of β-tubulin gene and detection of resistance by AS-PCR

Genomic DNA from third stage exsheathed larvae was used as template for amplification of β-tubulin isotype I gene as per Silvestre and Humbert (2000) and Coles *et al*. (2006) with minor modifications. The primary PCR product was used as nested PCR template using primers and reaction conditions as defined by Silvestre and Humbert (2000) and Coles *et al*. (2006). Consequently, the nested PCR amplicons were digested with the RsaI restriction enzyme (10 U/25 µl reaction) for correct species identification. Allele specific PCR was executed by adding *H. contortus* specific primers in two reactions per larval sample, one for resistant allele and the other for susceptible allele, using the nested PCR product as template for detecting BZ resistance (Coles *et al*., 2006).

The amplicons were isolated in the TAE (1x) buffer by 2.5% agarose gel electrophoresis and the resulting fragments were visualized under UV light as a compact fluorescent band of predicted size and recorded by the gel documentation system (Bio-Rad Gel Doc EZ Imager). Chi-square test was applied for statistically analyzing the genotyping of *H. contortus* resistant and susceptible larva (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

The Nested PCR amplified a product of approximately 820bp in all cases (Fig 1). The RsaI digested amplicons showed major fragments around 462 bp, 211 bp and 147 bp, which proved the species as *H. contortus* (Fig 2).

The size of the specific bands was around 603 bp for susceptible allele-specific gene while resistant allele-specific gene showed product size of 222 bp and another non-allele specific gene showed band size around 774 bp (Fig 3 and Fig 4). The results of larval genotyping using AS-PCR from various regions of Chhattisgarh were presented in Table 1. The AS-PCR results showed the overall prevalence of resistant allele 'r' 49.7% and susceptible allele 'S' 50.3%. The genotypic frequency of homozygous resistant (rr), heterozygous susceptible (rS) and homozygous susceptible (SS) were 33.83%, 31.81% and 34.34%, respectively. The samples from farm regions indicated that 12.5-66.67% of

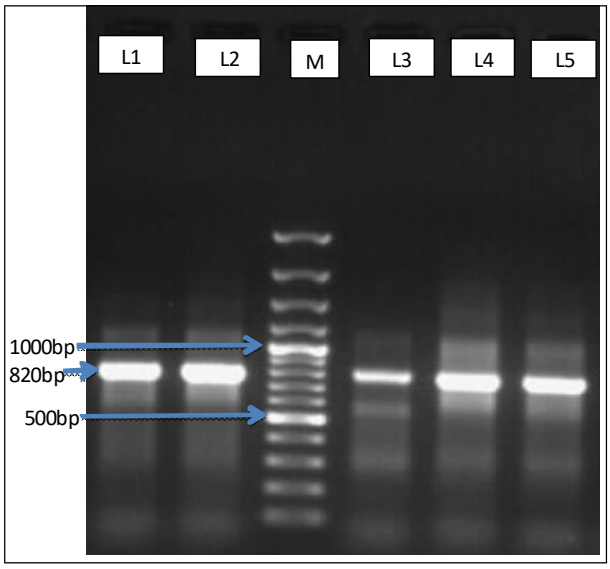

Fig 1: Nested PCR product at 820 bp showing amplification of β-tubulin gene Lane 1 100 bp plus DNA marker.

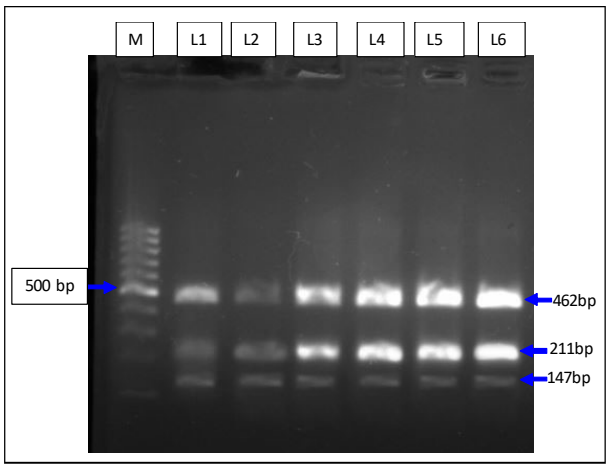

Fig 2: RFLP of larvae collected from different farm and field showing 3 bands at 462 bp, 211 bp and 147 bp confirming *Haemonchus contortus* Lane 1 100 bp plus DNA marker (M) Lane 2-7 (Larvae 1-6).

H. contortus larvae were homozygous resistant (rr, TTC), 16.67-41.67% homozygous susceptible (SS, TAC) and 16.67-45.83% heterozygous susceptible (rS, TTC/TAC).

In Durg, College Unit Goat farm, the genotypic frequency of larvae with regard to homozygous resistant (rr) was substantially greater (P<0.01). In the field region, the overall frequency of homozygous resistant (rr, TTC) was 6.67-25%, 33.33-41.67% heterozygous susceptible (rS, TTC/TAC) and 33.33-60% homozygous susceptible (SS, TAC). The genotypic frequency of homozygous susceptible (SS) *H. contortus* larvae was significantly higher (P<0.01) in Bilaspur field region.

In farms, where the treatment frequency was higher (3-4 times in a year) showed higher degree of resistance revealing 48.96% of homozygous resistance genotype (rr) and 60.96% of resistant allele (r) frequency. Whereas in field area the frequency of resistant individual (rr) was 19.6% which is significantly lower (P<0.01) than susceptible individual (SS). Among farm animals, *H. contortus* infecting goats of College Unit Farm, Durg region revealed highest (66.67%) genotypic frequency of homozygous resistant (rr) genotype and its corresponding field area, showing 25% genotypic frequency of homozygous resistant (rr) in the population of *H. contortus*. However, due to proper anthelmintic management and other mitigation strategies like pasture rotation *etc*. in Pakaria Goat Farm, Bilaspur there was lowest frequency (12.5%) of homozygous resistance (rr) genotype among all the farms. The corresponding field region showed lowest degree (6.67%) of resistance.

As the animals of field area were in the close proximity to the farm goats chances of using the same grazing land was higher thereby getting resistant nematodes. The results indicated that benzimidazole resistance is reached alarming level in farms and emerging in field condition. These findings confirmed that there was an impact of status of resistance of the farm area on field area and there is dissemination of the phenomenon of resistance from farm to field.

Many workers in India and abroad had used AS-PCR based genotyping for the detection of BZ resistance in strongyles of small ruminants, cattle and equines as this

Table 1: Genotyping of larvae of *H*aemonchus *contortus* in different farm and field region.

Location	No. of L3 larvae	χ^2 value	Genotypic frequency			Alleleic frequency	
			Homozygous resistant rr	Heterozygous susceptible rS	Homozygous susceptible SS	Resistant allele	Susceptible allele
Durg, College Unit Farm	48	33.38**	66.67% (32)	16.67% (8)	16.67% (8)	75.0%	25.0%
Bilaspur Farm	24	7.125^{NS}	12.50% (3)	45.83% (11)	41.67% (10)	35.0%	65.0%
Kawardha Farm	24	4.875^{NS}	50% (12)	20.83% (5)	29.17% (7)	61.0%	39.0%
Total (Farm Unit)	96	15.84**	48.96% (47)	25% (24)	26.04% (25)	61.46%	37.5%
Durg, Field Tegion	48	2.438^{NS}	25% (12)	39.58% (19)	35.42% (17)	45.0%	55.0%
Bilaspur Field Region	30	19.2**	6.67% (2)	33.33% (10)	60% (18)	23.0%	77.0%
Kawardha Field Region	24	1.5^{NS}	25% (6)	41.67% (10)	33.33% (8)	46.0%	54.0%
Total (Field Region)	102	13.32**	19.61% (20)	38.23% (39)	42.16% (43)	38.73%	61.27%
Total	198	0.85^{NS}	33.83% (67)	31.81% (63)	34.34% (68)	49.7%	50.3%

**Values indicate significance at P<0.01. NS; Non-significant.

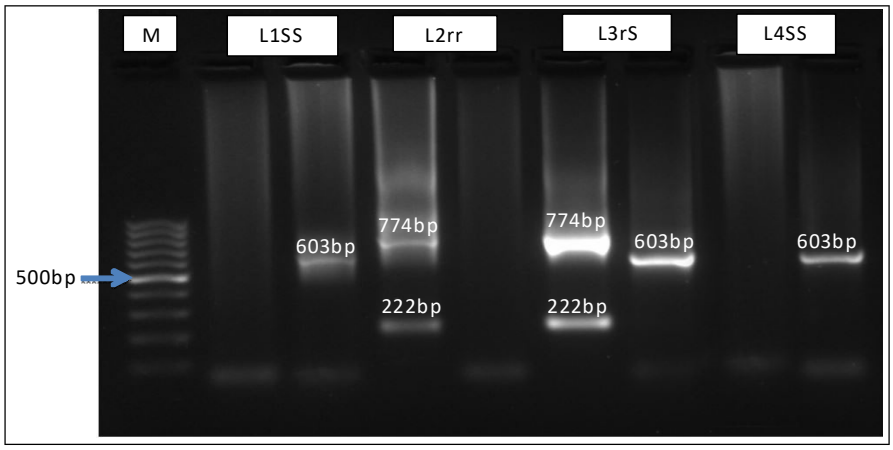

Fig 3: Genotyping of *Haemonchus contortus* for benzimidazole resistance of representative farm sample. Lane 1, 100 bp plus DNA marker (M) Lane 2-9: Larvae (4).

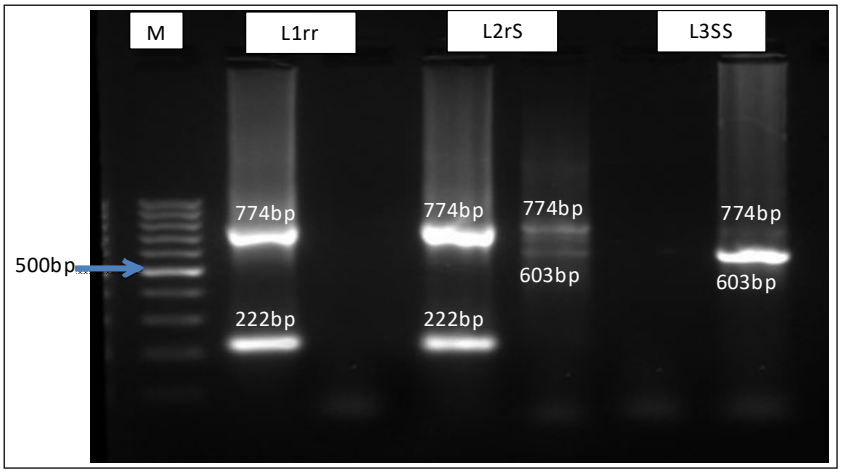

Fig 4: Genotyping of *Haemonchus contortus* for benzimidazole resistance of representative field sample. Lane 1, 100 bp plus DNA marker (M) Lane 2-7: Larvae (3).

test produced unerring results (Silvestre and Humbert, 2000; Winterrowd *et al*., 2003; von Samson-Himmelstjerna *et al*., 2007; Chandra *et al*., 2015; Dixit *et al*., 2017; Singh *et al*., 2019).

Unlike our results Singh *et al*. (2019) in the study of genotyping of *H. contortus* larvae collected from sheep from six districts of Punjab observed higher level of resistance showing overall genotypic frequency of 49.28% for homozygous resistant rr, 46.37% homozygous susceptible SS and heterozygous susceptible rS genotype showing low frequency of 3.62%. The allelic frequencies were 0.72 and 0.28 for resistant and susceptible allele respectively. Much the same results were discerned by Dixit *et al*. (2017) where they found 62% of *H. contortus* larvae were homozygous resistant (rr), 14% homozygous susceptible (SS) and 24% heterozygous susceptible (rS). The frequency of resistant allele was significantly (P<0.01) higher (74%) than susceptible allele (S) (26%). Chandra *et al*. (2015) in the study from different regions of Uttar Pradesh detected 55-85% of *H. contortus* as homozygous resistant rr, 10-21% homozygous susceptible SS and 5-24% heterozygous susceptible rS. The allelic frequencies were 67- 87.5% for resistant and 12.5-33% for susceptible.

Similar to our findings, significantly lower (P<0.005) overall frequency of resistant (rr) (17%) genotype than homozygous susceptible (SS) 61% *H. contortus* male was reported from two agro-climatic zones, *viz*. Tarai and Hill of Uttarakhand, India by Pandey and Vatsya (2013). Overall, prevalence of benzimidazole resistant allele (r) was significantly (P<0.005) lower (28%) than benzimidazole susceptible allele (S) (72%).

The present study only consider the F200Y mutation in the β-tubulin gene which could be identified by the AS-PCR technique, but it cannot recognize other mutations that could be responsible for the production of resistance to benzimidazole *viz*. E198A, F167Y *etc* (Prichard, 2001; Ghisi *et al*., 2007). If one of these polymorphisms is present in the *H. contortus* populations under study, then the degree of resistance could be much more in the areas under study.

CONCLUSION

In the present study, a moderate frequency of homozygous resistant 33.83%, (rr) and heterozygous genotypes 31.81% (rS) suggests that the resistance is establishing in this area at a quicker rate. Anthelmintic resistance is now accepted as inevitable phenomenon and the genes or alleles deliberating resistance to anthelmintics are conceived to be in perseverance in unselected worm population. Therefore, immediate regulatory measure must be given for anthelmintic administration so that spreading of resistant population is minimized.

Consequently, for all anthelmintics that have been made to date, it appears that the evolution of anthelmintic resistance is an inescapable consequence of their use but its development can be delayed. The felicitous measures like targeted selective treatment by employing FAMACHA need to be enforced urgently to disparage the dissemination of anthelmintic resistance.

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