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Original paper

Frequency of benzimidazole resistance in *Haemonchus contortus* populations isolated from sheep and goats in Bangladesh

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ABSTRACT. Anthelmintic resistance against gastrointestinal nematodes especially *H. contortus* of sheep and goat is a global issue. To address the gravity and extension of AR in Bangladesh, genotyping of 160 adult *H. contortus* parasites were performed to confirm benzimidazole resistance allele from different geographic zones of Bangladesh based on allele specific PCR (AS-PCR). The genotype frequencies were 9.4% for homozygous resistant (rr), 61.2% for heterozygous (rS) and 29.4% for homozygous susceptible (SS) among the selected areas. The allelic frequency of the mutation conferring resistance (r) ranged from 27.5% to 52.5% indicating substantial existence of benzimidazole resistance in *H. contortus* in small ruminant nematodes. Therefore, it can be concluded that genotyping the F200Y polymorphism can be used to monitor the resistance and thereby to enhance the control on the development of anthelmintic resistance against *H. contortus* in small ruminant nematodes.

Keywords: benzimidazole, resistance, β-tubulin gene, *Haemonchus contortus*

Introduction

Gastrointestinal nematode (GIN) parasites are recognized as one of the prime infectious obstacles to small ruminant health and productivity because of high prevalence (62.1%) in Bangladesh [1,2]. Globally sheep and goats farm profitability is substantially interrupted by these GINs infections [3]. Among GINs, hyper-endemicity of *H. contortus* in Bangladesh is reported by several authors [1,4,5]. This blood-sucking parasite can inflict significant impact on farm production and economy through host weight loss and mortality besides expense on anthelmintic treatment, drug residue, time and labor for disease management.

Today different anthelmintics are strategically used to control and treat GINs worldwide. Extensive and indiscriminate use of benzimidazoles (BZ) against GINs for the last five decades favored the development of resistance in parasites across the globe, including Bangladesh [6–9]. Different *in vivo* and *in vitro* tests are employed to detect the existence of BZ resistance. However, these detection methods are flawed due to requirement of more time and cost, lower sensitivity and presence of at least 25% of the resistant nematode population [10,11]. On the contrary, implication of molecular diagnostic methods can ease and improve the detection of the emergence of parasite resistance even at early stages. Molecular assays can identify mutation at the codon 200 of the β-tubulin isotype 1 gene which is known to be predominantly associated with BZ resistance in nematodes. The point mutation (TTC to TAC) brings alteration in the amino acid from phenylalanine to tyrosine [12]. Compared to the existing assays, AS-PCR is privileged with higher specificity and sensitivity, and prompt output with minimum input. Since,

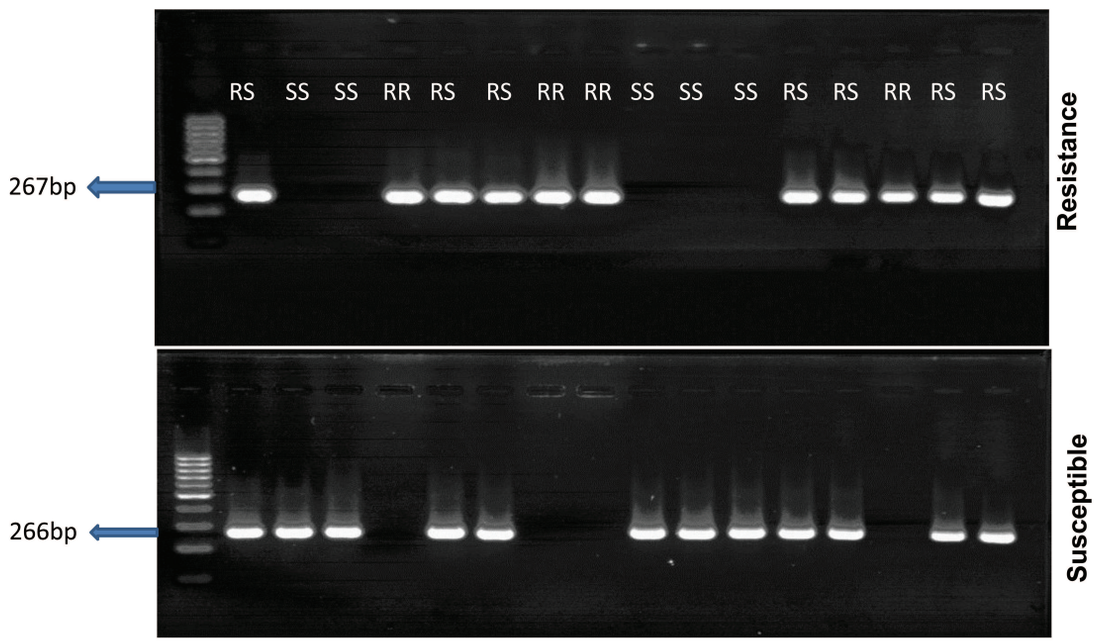

Figure 1. AS-PCR profile of *β-tubulin isotype 1* gene of *H. contortus*. Upper lane: resistant individuals, Lower lane: susceptible individuals, M: 100 bp ladder

precise and specific detection of AR is a prerequisite for developing sustainable and eco-friendly control management of parasites, the objective of this study was to determine the frequency of F200Y polymorphism in isotype 1 of the *β-tubulin gene* in field isolates of *H. contortus* from sheep and goats in different geographic areas of Bangladesh.

Materials and Methods

Collection of parasite materials

Adult *H. contortus* were collected from abomasa of slaughtered sheep and goats from eight different areas of Bangladesh (Mymensingh, Tangail, Sirajgonj, Dinajpur, Barishal, Khulna, Sylhet and Rangamati). Microscopic examination was conducted to identify and isolate a total of 160 male worms according to the procedures outlined by Soulsby [13]. Parasite samples were preserved in 70% ethanol and stored at −20°C, until DNA extraction was performed.

Isolation of genomic DNA

From 160 adult male worms, total genomic DNA was extracted individually using a QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. In short, single parasite tissues were disintegrated and lysed in Eppendorf tube. Proteinase K was applied to digest proteins followed by extensive washing. Elution buffer was used to elute trapped DNA. DNA quality and quantity were assessed in a Nanodrop spectrophotometer (ThermoFisher Scientifica, Germany). Extracted DNA samples were stored at −20°C until further use.

Detection of BZ resistance through AS-PCR assay

Benzimidazole resistance, which is linked to the mutation at the 200 codon of *β-tubulin isotype 1 gene*, was detected by allele specific PCR (AS-PCR). Two sets of primers were used for amplifications [14]; same reverse primer TGG 312 (5'-GGA ACC ATG TTC ACG GCT AAC-3') was used in two separate reactions with CAW 106 (5'-TAG AGA ACA CCG ATG AAA CAT T-3') as susceptible primer and TGG 331 (5'-G TAG AGA ACA CCG ATG AAA CAT A-3') as resistant primer. Primer CAW 106 annealed with complementary sequence with phenylalanine (TTC) codon, whereas primer TGG 331 annealed with complimentary sequence of tyrosine (TAC) at codon 200 of β-tubulin gene. Only the final base at the 3' end of each forward primer confers the specificity.

The PCR reaction was carried out in a total volume of 25 µl containing 12.5 µl of master mix (2× PCR Master Mix, Promega, USA), 2.5 µl (>30 ng/µl) of template DNA, 1 µl of each primer (10 pmol/µl) and 8 µl of water. The thermocycling conditions used were at 95°C for 10 min for the

Table 1. Genotypic and allelic frequencies of BZ resistance in *H. contortus* using AS-PCR

	No. of parasites genotyped	Genotype frequency			Allele frequency	
		Homozygus resistant (rr)	Heterozygus (rS)	Homozygus susceptible (SS)	Resistant (r)	Susceptible (S)
Mymensingh	20	0.00 (0)	0.80 (16)	0.20 (4)	0.4 (16)	0.6 (24)
Sylhet	20	0.20 (4)	0.65 (13)	0.15 (3)	0.525 (21)	0.475 (19)
Tangail	20	0.05 (1)	0.60 (12)	0.35 (7)	0.35 (14)	0.65 (26)
Sirajgonj	20	0.05 (1)	0.65 (13)	0.30 (6)	0.375 (15)	0.625 (25)
Dinajpur	20	0.15 (3)	0.55 (11)	0.30 (6)	0.425 (17)	0.575 (23)
Rangamati	20	0.00 (0)	0.55 (11)	0.45 (9)	0.275 (11)	0.725 (29)
Khulna	20	0.10 (2)	0.55 (11)	0.35 (7)	0.375 (15)	0.625 (25)
Barishal	20	0.20 (4)	0.55 (11)	0.25 (5)	0.475 (19)	0.525 (21)
Overall	160	0.094 (15)	0.613 (98)	0.294 (47)	0.4 (128)	0.6 (192)

initial denaturation followed by 50 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. Amplified products were analyzed on 2% agarose gel stained with ethidium bromide. The susceptible primer (CAW 106) and resistant primer (TGG 331) amplify a product of 266 bp and 267 bp, respectively. Worms that gave amplification only with BZ-susceptible primer were designated as homozygous susceptible (SS), the individuals that amplify only with BZ-resistant primer were designated as homozygous resistant (rr), the individuals gave amplification with both the primers were designated as heterozygous (rS) for BZ resistance (Fig. 1).

The Chi-square ($\chi 2$) test was used to statistically analyze the genotyping of *H. contortus* resistant and susceptible parasites [15].

Ethics statement

The research protocol was reviewed and approved by the Research Ethics Committee of The Faculty of Veterinary Science, Bangladesh Agricultural University. While carrying out this

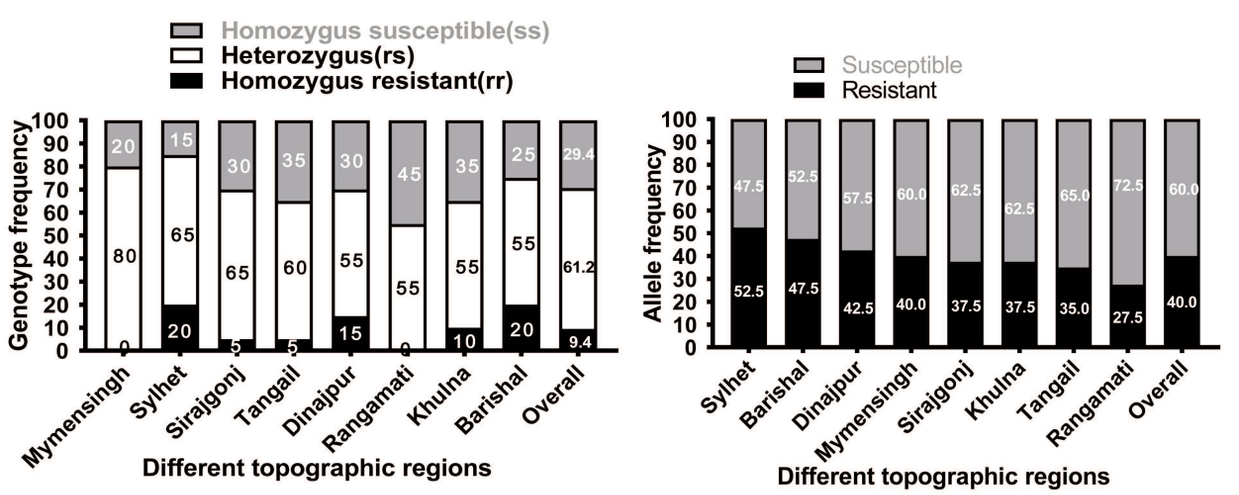

Figure 2. Genotypic and allelic frequency of BZ resistance in *H. contortus* in different geographic areas of Bangladesh

research, no animals were harmed or unethically injured/ killed.

Results

To ascertain BZ resistance against *H. contortus* in small ruminants of Bangladesh, AS-PCR was applied in this study for its high competency in diagnosis. For this, genomic DNA samples were isolated from a total of 160 adult male *H. contortu*s originating from eight topographic areas of Bangladesh. AS-PCR detected all three genotypes (rr, rS and SS) that suggested BZ susceptibility or resistance of *H. contortus* to BZ. The overall genotype frequencies of *H. contortus* adults were 9.4% homozygous resistant (rr, TAC), 61.2% heterozygous (rS, TAC/TTC) and 29.4% homozygous susceptible (SS, TTC) among different regions in Bangladesh (Tab. 1). The frequency of resistance genotype (rr) ranged from 0% to 20%. The allele frequencies were ranged from 27.5% to 52.5% for resistant, and 47.5% to 72.5% for susceptible among different regions (Fig. 2). The genotype frequencies (rr, rS and SS) and allelic frequencies (r and S) varied significantly (P<0.05) in different zones in Bangladesh. In all the regions, the frequency of heterozygous genotypes (rS) was higher (55% to 80%) as compared to that of homozygous resistant (rr) and homozygous susceptible (SS) genotypes.

Discussion

Benzimidazole, a broad-spectrum anthelmintic is widely used against parasitism in domestic animals [16]. Due to low cost and short withdrawal period, it has been commonly used for the control of GI nematodes [17]. Rigorous use of BZ to control GI nematodes led to the development of AR which is an alarming issue in many countries including Bangladesh [18,19]. Among the GIN, *H. contortus*, the most significant blood feeding nematode has the tendency to develop resistance against anthelmintics due to high gene flow between population and increased genetic diversity pattern within population [7,20]. This increased variability within-populations is especially true for β-tubulin genes, which account for resistance to BZ substance class of anthelmintic drugs [21].

Formulation of control strategies for GINs with regards to AR depends extensively on precise and early detection and regular monitoring of BZ resistance. Application of fast and authentic assays based on molecular tools is feasible to be applied for detection of BZ resistance both at laboratory and field practice. Molecular techniques, namely, AS-PCR, qPCR and pyrosequencing are now frequently exercised for diagnosis of BZ resistance [8,10,22]. In comparison to conventional faecal egg count reduction test (FECRT) and egg hatch assay (EHA), molecular diagnostic techniques are more sensitive and capable to mark resistance in isolates even when resistance conferring alleles are present in below 25% of the gene pool [23].

AS-PCR is an established technique that detects point mutation, small insertions and deletions, polymorphism and other variation in the sequence of parasite DNA. In *H. contortus*, three BZ resistance related single nucleotide polymorphism (SNPs) were recognized in *β-tubulin isotype 1* gene at codons 200 (TTC to TAC; F200Y) [12], 167 (TTC to TAC; F167Y) and 198 (GAA to GCA; E198A) [24]. BZ resistance linked with SNPs F200Y seems to be very common than F167Y and E198A [25].

Under this experiment, amplification of *β-tubulin isotype 1 gene* was performed by AS-PCR technique for the detection of BZ resistance of *H. contortus* male. The genotype frequency of adult parasites of *H. contortus* with reference to “rr”, “rS” and “SS” differed significantly from region to region in Bangladesh. Frequency of resistant allele (r) ranged from 27.5–52.5% whereas in our previous study, it was 25.0–47.0% [6]; thus indicates increasing development of AR against *H. contortus* in small ruminants in Bangladesh. Frequency of resistant allele (r, 28–87%) in *H. contortus* population were reported in India [14,26,27] by AS-PCR and in Brazil (r, 49–52%) by nested PCR [28,29]. The increasing frequency of resistant allele might be due to the continuation of frequent and generous use of BZ in the recent past. It is noteworthy that the pasture management system is not practiced in Bangladesh; thus also responsible for increasing development of AR. Farm animals including sheep, goats, cattle and buffaloes share common pasture, and subsequently increase the chance of transmission of same GIN and favor the spread of BZ resistant *H. contortus* among the animal species [30]. Hence, despite the fact that GI nematodosis is frequently encountered in animal farms, it remains widely disregarded. In addition to this, in most of treatment cases, animal body condition score are measured based on eye-

estimation or distant assumption. This might potentially result in inappropriate dosing (over or under) of treated animals and development of AR.

In conclusion, emergence of homozygous resistance (rr) population and rise of the number of heterozygous (rS) population of *H. contortus* was revealed in eight selected geographic zones of Bangladesh. The frequency of resistant allele was more than 40%. Therefore, target-specific and sustainable strategies should be high-priority to reduce further dissemination and growth of AR. Use of bio-rational anthelmintics, on-farm alternate management practices, preventing indiscriminate anthelmintic use and genetic improvement of hosts, with consideration to animal welfare and environment-friendly approaches, might facilitate AR control against GINs.

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