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Diagnosis of anthelmintic resistance against naturally infected gastrointestinal nematodes in cattle of Assam employing FECRT and molecular techniques

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Abstract
Faecal egg count reduction test (FECRT) and molecular techniques were employed to diagnose the anthelmintic resistance (AR) against naturally infected gastrointestinal nematodes in cattle of both organized and unorganized farm of in and around Guwahati, Assam. Twenty-four animals of each from organized and unorganized farm having more than 200 eggs per gram (EPG) of faeces were selected randomly and divided into four groups of six animals each to record the AR by FECRT. Animals of Group I, II, III were treated with levamisole (@7.5mg/kg body weight orally), fenbendazole (@5mg/kg body weight orally), albendazole (@7.5mg/kg body weight orally), respectively and group IV was kept as infected untreated control. Faecal samples were collected per-rectally on "0th" day (pre-treatment) and 10 and 14th day post-treatment from animals of all groups and individual faecal egg counts were determined by Modified McMaster technique. The study revealed FECR% of 100.00, 90.00 and 93.00% was in animals of Group I, II and III, respectively in organized farm while in unorganized farm it was 100.00, 92.00 and 92.00% in animals of Group I, II and III, respectively. It was observed that BZ group were found to develop resistance while levamisole treated group was 100% effective. The adult *Haemonchus* parasites was collected obtained from slaughtered male animals of the respective farm without any anthelmintic treatment and confirmed based on morphological features of the parasites and subjected to DNA extraction as per standard protocol. The partial β-tubulin isotype-1 gene encoding the codon 200, which was found to be responsible for BZ resistance, was amplified with the combination of P_1 and P_2 primers. An aliquot of first PCR amplicon was used for nested PCR with Pn3 and Pn4 primers combination and product size of 820 bp was visualised in 1.5% agarose gel electrophoresis. The Allele specific PCR was used for diagnosis of BZ resistance, which yielded 550 and 774 bp products in susceptible parasite and 250 and 774 bp in resistant parasite population.

Keywords: Anthelmintic resistance, Benzimidazole, FECRT, Allele specific PCR

1. Introduction
The gastrointestinal nematodes (GINs) parasitism occurs due to favourable weather condition as well as availability of larval stages on pasture throughout the year [1-3] causes enormous economic losses to livestock through morbidity, mortality and reduce productivity which is neglected because of the chronic nature of parasitism [4]. The control of GINs in ruminants is very difficult though used of highly efficacious BZ class of anthelmintic [5, 6]. When administered BZs, it inhibit the polymerisation of α and β-tubulin dimmers to microtubules [7] and restrain the glucose uptake leading to death of worms [8]. Therefore, the BZs are administered to exert their effect on the parasites by binding to the tubulin protein and preventing its polymerization into microtubules [9]. In field condition non-judiciously use of BZ drugs resulting in AR worldwide [10, 11] has been detected in parasitic nematodes of livestock since the early 1960s [12, 13] but in India it has been reported from many states in sheep, goat and cattle *viz.* Haryana [14, 15], Tamil Nadu [16] and Karnataka [17] and in cattle [18]. Thus spread of BZ resistance has led to major problems in livestock farming industries worldwide [10, 19-23].

BZ resistance in GINs are principally link to alteration in the gene that encodes for β-tubulin isotype 1 where mutation occurs. BZ resistance in *H. contortus* has been associated to different single nucleotide polymorphisms (SNPs) in the isotype-1 β-tubulin gene at codons 167 (TTC

to TAC; F167Y) [24], 198 (GAA to GCA; E198A) [25] and 200 (TTC to TAC; F200Y) [26] and F200Y single nucleotide polymorphism (SNP) in β-tubulin gene [26-29]. However, a F167Y SNP [30] and SNP, E198A has also been implicated in resistance in some isolates [25, 31] of *Haemonchus*. In addition P-glycoprotein may play a role in resistance to these drugs [32]. The identification and quantification of these mutations is the basis for the molecular diagnosis of BZs resistance is employed as molecular tests [33-37]. Among the various methods used to detect the AR in livestock [38, 39], the FECRT, egg hatch assay (EHA) and larval development assay (LDA) have proven to be suitable tests for detecting BZ resistance [40, 39] but they are time consuming, laborious and can detect resistance when at least 25% of the population has already become resistance [41]. Whereas by AS-PCR, it is possible to detect resistance very early when only 1% of the parasite populations has become resistance [42]. Therefore, this study was conducted to evaluate the AR against naturally infected GINs in cattle employing FECRT and molecular methods.

2. Material and Methods

A total of 250 cattle encompassing of 100 cattle from an organized farm (Instructional Livestock Farm, CVSc, Khanapara) and 150 cattle from the unorganized private farm in and around Guwahati city in Kamrup district of Assam were selected to collect faecal samples. The collected faecal samples were brought to the laboratory and proceed to record the prevalence of GINs by the floatation technique [43]. After ascertaining the prevalence, the therapeutic study was performed as per below mentioned.

2.1 Therapeutic study

Based on prevalence data 24 animals from each organized and unorganized farm with $\geq$ 200 EPG were selected randomly and divided into 4 groups of six animals each. Animals of Group I, II, III were treated with levamisole (@7.5mg/kg body weight (BW) orally), fenbendazole (@5mg/kg BW orally) and albendazole (@7.5mg/kg BW orally), respectively and group IV was kept as infected untreated control. Faecal samples were collected per-rectally on the 0^{th} day (pre-treatment) and 10 and 14^{th} day of post-treatment from animals of all groups and faecal egg counts of an individual sample were determined by Modified McMaster technique. The FECR% of the drugs and AR were performed by employing the FECRT as per the standard method [39, 44-46].

2.2 Identification of adult parasite

The collected adult parasites from the slaughtered male animals of the respective farm were examined at the laboratory for morphological studies and identified using the key of Zajac and Conboy (2012) [47].

2.3 Molecular study

Morphologically identified species was validated through the amplification of β-tubulin gene. The AR was diagnosed based on β-tubulin Isotype-1 gene, the targeted gene of *Haemonchus placei* obtained from cattle reared and managed in the respective study areas.

2.3.1 Isolation of genomic DNA from adult parasites

The high molecular weight DNA was extracted from the adult *H. placei* using DNeasy Blood and Tissue Kit (Quiagen®, Germany) according to manufacturer's protocol as well as conventional method.

2.3.2 Amplification of β-tubulin

The 25µl PCR mixture consisted of 4 µl of DNA template obtained from adult worm, 20pmol of each primer (Pn1: 5′ GGC AAA TAT GTC CCA CGT GC 3′ Pn2: 5′ GAA GCG CGA TAC GCT TGA GC 3), 1mM $MgCl_2$, 100µM of each dNTPs, and 2.5µl of 10x Taq DNA polymerase buffer and 1U of Taq DNA polymerase. The volume of the reaction was made up to 25µl with Nuclease free water. Initial denaturation at 94 ºC for two minutes was followed by 33 cycles each at 94 ºC for 55 sec, 57 ºC for 55 sec and 72 ºC for 55 sec. This was followed by 10 min final extension at 72 ºC and soak at 4°C in aTechnee-5000 thermal cycler (Bibby Scientific). The PCR amplicons were analyzed by agarose gel electrophoresis in 1.5% agarose gel in TAE (1×) buffer stained with ethidium bromide (0.0001%) for 1 hour at constant voltage (70V) and visualized under gel doc (DNR-Bio-Imaging System, MiniLumi).

2.3.3 Nested PCR

The obtained β-tubulin PCR amplicons in PCR were used as template for nested PCR. The PCR mixture consisted of 3µl of template (β-tubulin PCR product) 20 pmol of primers (5′ Pn3: GTG CTG TTC TTG TTG ATC TC 3′, PN4: 5′ GAT CAG CAT TCA GCT GTC CA 3′), 100µM of each dNTPs, 2mM of $MgCl_2$, 2.5µl of 10x Taq DNA polymerase buffer and 1U of Taq DNA polymerase. Finally, the volume of reaction was made up to 25µl with Nuclease free water. The reaction was performed with the initial denaturation at 94 ºC for two minutes followed by 33 cycles each at 94 ºC for 55 sec, 55 ºC for 55 sec and 72 ºC for 55sec. This was followed by 10 min final extension at 72 ºC and soak at 4ºC in aTechnee-5000 thermal cycler (Bibby Scientific). Further, the PCR amplicons were used as template for AS-PCR. Following DNA amplification, the resulting fragments were separated by gel electrophoresis as above.

2.3.4 Allele specific PCR

Two aliquots (1.5µl) of the (P_3-P_4) nested product were used to determine the resistance of the parasites against BZ group of drugs by the method of Humbert and Elard (1997) [48] and Coles *et al.* (2006) [45]. The system was divided into two mixes, each containing two non-specific primers (non-allele specific forward and reverse primer) and one allele-specific primer. Each reaction generated one allele specific and one non - allele specific fragments. The PCR mixture volume of 25µl was comprising of 1.5µl of nested product used as template for amplification of allele specific PCR with 7.5 pmol of non-allele specific forward and reverse primers, 20pmol of resistant allele primer in one mix and susceptible allele primer in another mix (Table 1), 1mM $MgCl_2$, 80µM of each dNTPs, 2.5µl of Taq DNA polymerase buffer and 1U of Taq DNA polymerase and finally made the volume of 25µl by adding the Nuclease free water.

The reaction was performed with the initial denaturation at 94 ºC for 2 minutes was followed by 30 cycles each at 94 ºC for 55 sec, 55 ºC for 55 sec and 72 ºC for 55 sec. This was followed by 10min final extension at 72 ºC. The resulting fragments were separated by gel electrophoresis as above.

Table 1: Primer used in the Allele specific PCR

Ph1 forward primer	5′ GGA ACG ATG GAC TCC TTT CG 3′
Ph2 reverse primer	5′ GGG AAT CGA AGG CAG GTC GT 3′
Ph3 resistant allele primer	5′ CTG GTA GAG AAC ACC GAT GAA ACA TA 3′
Ph4 susceptible allele primer	5′ ATA CAG AGC TTC GTT GTC AAT ACA GA 3

3. Results and Discussion

3.1 Therapeutic study and FECRT

The commonly used anthelmentics against GINs naturally infected in cattle were found to be significantly reduced the faecal egg counts for all the groups of treatment (Table 2). After administration of the different anthelmintic the EPG was found to be 0, 50.00 ±22.36 and 33.00±21.08 in group I, II and III, respectively in cattle of organized farm on 14th day of post-treatment, but in cattle of unorganized farm the EPG was found to be 17.00±16.66, 33.00±21.08 and 33.00±21.08 in group I, II and III, respectively. The EPG in untreated group was found to be higher as compare to 0 days (Table 2). The FECRT % was found to be in organized farm 0, 50.00±22.36, and 50.00±22.36 and in unorganized farm 33.00±21.08, 33.00±21.08, and 50.00±22.36in group A, B, and C, respectively on 10th day of post-treatment. Hafiz *et al.* [6] observed gradual reduction of EPG and complete elimination of eggs of strongyle on 14th day post-treatment with a single dose of albendazole orally unlike our findings. Almost similar findings were also reported by Bulbul *et al.* [11] in Kashmir and other workers from other parts of the country. The FECR % was found to be 100.00, 90.00 and 93.00% in organized farm and 100.00, 92.00 and 92.00 in unorganized farm for levamisole, fenbendazole and albendazole, respectively. This indicated development of AR against the BZ groups of drugs. This development of AR might be due to regular usage of the same group of anthelmintic, use of anthelmintics in sub-optimal doses, prophylactic mass treatment of domestic animals and frequent and continuous use of a single drug. Bansal [49] reported a reduction of EPG by 99.5% in fenbendazole treated animals @ 5 mg/ Kg body weight. In contrast, animals in control group showed an ascending EPG count were also successfully treated with levamisole @ 7.5 mg / Kg body weight orally after completion of the trial. de Soutello *et al.* [50] found 97.4% efficacy for levamisole after 24 hours of administration of the drugs as like our present study. As there are less number of reports available regarding the anthelmintic resistance developed against gastrointestinal nematodes in cattle and most of the results available in India and abroad were of small ruminants. Varshney and Singh [51] ascertained the phenothiazine and thiabendazole resistance against *H. contortus* in sheep in Central Wool and Research Station. Similarly, Uppal *et al.* [52] and Yadav *et al.* [14] had reported benzimidazole, levamisole, morantel and thiophanate resistance in *H. contortus* in goat in Haryana. Fenbendazole and Morantel were found resistance against *H. contortus* in sheep, respectively. Jayanthilakan *et al.* [53], Arunachalam *et al.* [16] and Easwaran *et al.* [54] reported the AR in nematode of sheep flock in Tamil Nadu based on FECRT and they found AR for fenbendazole, albendazole, levamisole and morantel in *H. contortus*, *Oesophagostomum columbianum*, *Bunostomum* spp. and *Trichostrongylus* spp. They also recorded FECR % for fenbendazole, albendazole and levamisole ranged from 65-75%, 69-80% and 70-82%, respectively. From the present study, it can be easily concluded that all three drugs were effective against naturally acquired GI nematodosis in ruminants is an effective anthelmentics against nematodosis in ruminants but resistance arisen might be due to faulty uses. For prevention and control of helminth parasites, appropriate anthelmintic drugs should be administered in right way. Hence, FECRT might be used for diagnosis of AR in livestock in field condition as our recommendation which is supportive statements of other workers [39, 55, 56].

Table 2: FECR per cent of anthelmentics against naturally acquired gastrointestinal nematodosis in cattle

Cattle of	Group I					Group II					Group III					Group IV		
	EPG ± SE			FECR%		EPG± SE			FECR %		EPG± SE			FECR %		EPG± SE		
	Pre-treatment	Post-treatment				Pre-treatment	Post-treatment				Pre-treatment	Post-treatment				Untreated		
	0 day	10th day	14th day	10th day	14th day	0 day	10th day	14th day	10th day	14th day	0 day	10th day	14th day	10th day	14th day	0 day	10th day	14th day
Organized farm	300± 44.71	0	0	100 ±0	100 ±0^{aA}	267± 33.33	50± 22.36	50± 22.36	88± 5.59	90± 4.74^{bA}	250± 34.15	50± 22.36	33± 21.08	88± 5.27	93± 4.55^{b}	350± 34.15	433± 21.08	483± 40.13^{cA}
Unorganized farm	367± 42.15	33± 21.08	17± 16.66	89± 6.95	100± 0^{aA}	317± 30.73	33± 21.08	33± 21.08	92± 5.27	92± 5.27^{bA}	317± 30.73	50± 22.36	33± 21.08	85± 6.89	92± 5.27^{b}	300± 25.81	333± 21.52	450± 42.81^{cA}

Means bearing in a row and column with common superscript do not differ significantly

3.2 Detection of anthelmintic resistance by molecular techniques

The partial β-tubulin isotype-1 gene encoding the codon 200, which was found to be responsible for BZ resistance, was amplified with the combination of P_1 and P_2 primers. An aliquot of first PCR amplicon was used for nested PCR with Pn3 and Pn4 primers combination and product size of 820bp was visualised in 1.5% agarose gel electrophoresis (Fig1).

3.2.1 Allele specific PCR for genotyping of *Haemonchus placei*

Allele specific PCR was used for diagnosis of BZ, which yielded 550 and 774 bp products in susceptible parasite and 250 and 774 bp in resistant parasite population (Fig 2). Hence this polymorphism at codon 200 of the β-tubulin gene marker can be used to detect the BZ resistance against *H. placei* in cattle. Coles *et al.*, (2006) [45] tested the BZ resistance against seven strongyles of sheep, goats, horses and cattle with the allele specific PCR and found all resulted in the amplification of specific product as our findings. Similar findings were also reported by Humbert *et al.* [41] who reported first time development of an allele specific PCR, the mutation involved at residue 200 of the isotype 1 beta- tubulin gene. Thus, BZ resistance can be described quantitively ranging from 0 to 100%. The AR might be due to the fact of the mutation of Tyr in position 200 of isotype 1 β-tubulin gene is the major genetic mechanism that confers BZ-resistance in

Trichostrongylid nematodes, in field conditions. In *H. contortus* a single nucleotide polymorphism at codon 200 of the β-tubulin gene (TTC to TAC), causing a phenylalanine to tyrosine amino acid substitution, has been found to be involved in many cases of resistance [35]. Data from a recent survey in India and Pakistan confirmed the TAC SNP in codon F200Y as the most prevalent one, but in contrast to the results from previous studies in America and Europe, no mutations were found at codon F167Y (TAC) and only a small number of populations displayed the SNP in codon E198A (GCA) in India [57].

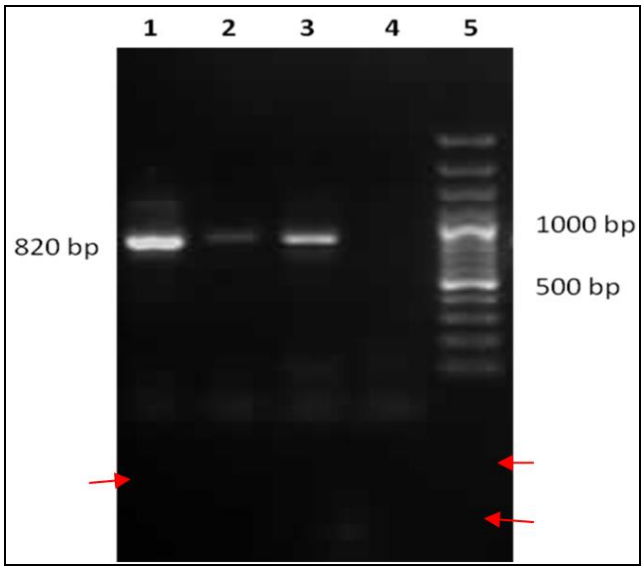

Fig 1: Nested PCR amplification for partial β-tublin gene of *H. Placei*, where Lane 1 (DNA of *H. Placei* collected from cattle of organized farm), Lane2 (DNA of *H. Placei* collected from cattle of unorganized farm) Lane 3 (DNA of *H. Placei* collected from slaughtered cattle) showing PCR amplicons of 820 bp; Lane-4 negative control and Lane-5 1kb ladder

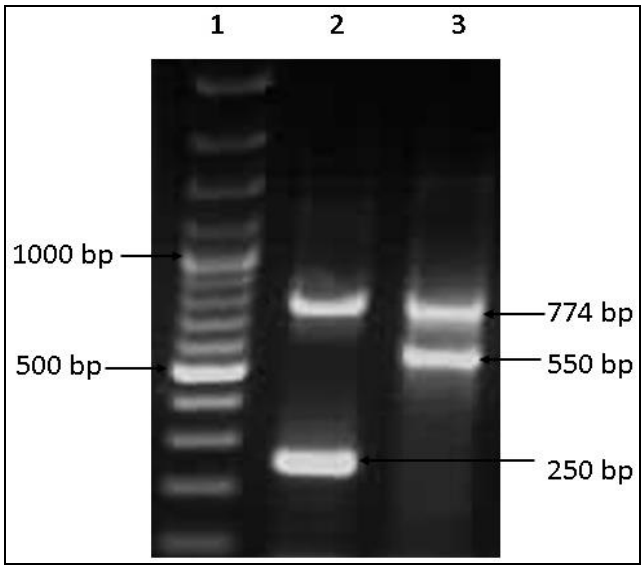

Fig 2: AS-PCR amplification for BZ resistance in diagnosis of *Haemonchus placei*, where Lane1-Molecular marker of 100 bp ladder, Lane 2- resistant worm (250 and 774 bp) and Lane 3- susceptible parasite (550 and 774 bp)

4. Conclusion

BZ resistance arisen against GINs in Assam which can be detected by FECRT. So before treating with anthelmintic the FECRT must be done by the veterinarian. The detection polymorphism at codon 200 of the β-tubulin gene in *H. placei* by AS-PCR collected from both organized and unorganized farm, which is usually the case in field situations, is an efficient marker for large-scale surveys for BZ resistance. This method can also be utilized to develop equally useful assays for GIN infections.

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