Diagnosis of benzimidazole resistance in *Haemonchus contortus* using allele-specific PCR technique

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**ABSTRACT**

Molecular tools can be of great use in understanding the origin and nature of underlying mechanisms of benzimidazole resistance. In the present study an allele-specific PCR was standardized to diagnose the mutation (Phe to Try) at residue 200 of the isotype 1 beta tubulin gene responsible for benzimidazole resistance in *Haemonchus contortus* adult and infective larvae. Once standardized, AS-PCR proved to be a powerful tool to detect point mutations at codon 200 of isotype 1 B-tubulin gene of *H. contortus* adult worms and infective larvae. The technique can be effectively used for genotyping of benzimidazole susceptible and resistant alleles of *H. contortus* from sheep.

**Key words**: Allele specific PCR, benzimidazole resistance, *Haemonchus contortus*

Anthelmintic resistance is a serious problem confronting the successful control of haemonchosis in ruminants, especially in small ones, throughout the world, including India (Singh *et al.* 2002, Yadav and Garg 2005). The currently used methods for its diagnosis India include controlled anthelmintic efficacy test, faecal egg count reduction test (*in vivo*), egg hatch assay and larval development assay (*in vitro*). Of these, controlled efficacy test is the most reliable method for confirming resistance and is considered to be a gold standard (Coles *et al.* 2006). But the expense involved in conducting this test usually excludes its use. Moreover, classical methods are only reliable if more than 25% worms in a given population are resistant (Roos *et al.* 1995). To overcome these limitations, molecular techniques which are highly sensitive and specific are being explored for diagnosis of anthelmintic resistance (Elard *et al.* 1999, Silvestre and Humbert 2000, Alvarez-Sanchez *et al.* 2005, Tiwari *et al.* 2006). These methods have several advantages over classical methods, viz., they provide immediate estimation of resistance and frequency of resistant alleles can be estimated (Roos *et al.* 1995). The most common molecular mechanisms that confer benzimidazole resistance in trichostrongylid nematodes in small ruminants involves a phenylalanine (TTC) to tyrosine (TAC) mutation at residue 200 of the isotype 1 and 2 beta tubulin genes (Elard *et al.* 1996, 1999).

Keeping the above background information in mind and considering the increasing number of reports of anthelmintic resistance in gastrointestinal nematodes of small ruminants in India, the present investigation was planned to standardize a molecular technique for the diagnosis of benzimidazole susceptible and resistant strains of *Haemonchus contortus*.

**MATERIALS AND METHODS**

Benzimidazole resistant and susceptible strains of *Haemonchus contortus*: Benzimidazole resistant strain of *H. contortus* was obtained from the sheep flock of Department of LPM, C.V.A.Sc., Pantnagar, where from repeated failure of fenbendazole medication was reported. Benzimidazole resistance was confirmed by egg hatch assay (Coles *et al.* 1992) and faecal egg count reduction test (Coles *et al.* 1992).

Benzimidazole susceptible strain of *H. contortus* was collected from the abomasum of a sheep slaughtered at Rudrapur slaughterhouse after confirming its origin. Live female *H. contortus* were allowed to lay eggs *in-vitro* in RPMI–1640 medium for 6 h. The eggs were separated by centrifugation after removal of the worms and washed in water twice. The collected eggs were cultured in sterilized buffalo faeces, which was free from any parasitic eggs. The larvae thus obtained were used to infect GI nematode free experimental sheep. Susceptibility to fenbendazole was confirmed by faecal egg count reduction test (Coles *et al.* 1992) and egg hatch assay (Coles *et al.* 1992).

Faecal cultures were made to harvest benzimidazole resistant and susceptible infective larvae from sheep for molecular diagnosis. Resistant and susceptible adult worms were also collected from the abomasum of sheep for molecular diagnosis.
Genomic DNA extraction from adult worms: Genomic DNA was isolated from individual benzimidazole susceptible and resistant adult worm separately as per the method described previously (Anonymous 1999) with modifications. After thorough washing of worms in distilled water, individual worm were suspended in 500μl of worm lysis solution (100 mM Tris-Cl, 100 mM NaCl, 50 mM EDTA, 1% SDS, 1% β-mercaptoethanol, 100μg/ml Proteinase K and 20μg/ml RNaseA) and frozen at –60°C for 1 h. The tubes were then thawed and incubated at 65°C for 1 h with occasional agitation. After incubation, phenol: chloroform: isoamyl-alcohol (25:24:1) extraction was performed twice as per the standard protocol. The DNA was precipitated with chilled absolute ethanol and 5 M sodium acetate and the tubes were centrifuged at 12,000 r.p.m. for 15 min. The resulting DNA pellet was washed with 70% ethanol. The DNA pellet was then air dried in open tubes at room temperature for 30 min. and suspended in 30 μl of TE buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8.0). The concentration and purity of the isolated DNAs were determined by UV/VIS spectrophotometry. The quantity of the DNA was measured by using standard reading of 1 OD at A260 for 50μg DNA per ml. The resolution and purity of DNA samples were further analysed by 0.8% agarose gel electrophoresis in a submarine horizontal electrophoresis unit.

Genomic DNA extraction from infective larvae: DNA from individual third stage larva was isolated using the method described by Silvestre and Humbert (2000) with minor modifications. The larvae were exsheathed by incubating them for 5 min in a petri-dish containing 4 ml of larval suspension in distilled water (about 1000 larvae/ml) and 180μl of 4% aqueous solution of sodium hypochlorite. Once the larvae had exsheathed, they were washed twice with distilled water and resuspended in triple distilled water. Individual larva was picked with 2 μl of water using a micropipette under microscopic surveillance and placed in a Proteinase K. The tubes containing the digested larvae were then transferred at 95°C for 20 min to inactivate the tubes were placed at 50°C for 8 hours. The tubes were then thawed and incubated at 65°C for 1 h with occasional agitation. After incubation, phenol: chloroform: isoamyl-alcohol (25:24:1) extraction was performed twice as per the standard protocol. The DNA was precipitated with chilled absolute ethanol and 5 M sodium acetate and the tubes were centrifuged at 12,000 r.p.m. for 15 min. The resulting DNA pellet was washed with 70% ethanol. The DNA pellet was then air dried in open tubes at room temperature for 30 min. and suspended in 30 μl of TE buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8.0). The concentration and purity of the isolated DNAs were determined by UV/VIS spectrophotometry. The quantity of the DNA was measured by using standard reading of 1 OD at A260 for 50μg DNA per ml. The resolution and purity of DNA samples were further analysed by 0.8% agarose gel electrophoresis in a submarine horizontal electrophoresis unit.

For amplification of isotype 1 β-tubulin gene from infective larvae the PCR mixture consisted of 7μl of larval suspension as template DNA, 1.25μl of 10X PCR buffer (minus Mg), 90μM of each dNTP, 7.5 pmol of each primer (P1 and P2), 2.0 mM MgCl2 and 1.0 U of Taq DNA polymerase. The volume of the reaction mixture was made up to 12.5μl with autoclaved milli-Q water. The cycling conditions included initial denaturation at 94°C for 3 minutes, followed by 25 cycles each of denaturation, annealing and extension as described above. This was followed by final extension for 10 min at 72°C. The PCR tubes containing β-tubulin PCR amplions were properly labeled and stored at–20°C till further use.

Nested PCR: The products of 1st PCR reaction (amplified β-tubulin gene) were used for nested PCR using the primer sequences (P3: GGA ACA ATG GAC TCT GTT CG and P4: GGG AAT CGA AGG CAG GTC GT) found in isotype 1 β-tubulin gene. The PCR reaction mixture consisted of 4μl of β-tubulin PCR product from adult worms, 5μl of 10X PCR buffer (minus Mg), 0.25 mM of each dNTP, 15 pmol of each primer (P3 and P4), 3 mM MgCl2 and 2.5U of Taq DNA polymerase. The volume of the reaction mixture was made up to 50μl with autoclaved milli-Q water. PCR was performed with initial denaturation at 94°C for 2 min, followed by 30 cycles each of denaturation at 94°C for 1 min, annealing at 56.5°C for 1 min and extension at 72°C for 1 min. This was followed by final extension for 10 min at 72°C. The β-tubulin PCR amplons were analysed by agarose gel electrophoresis in 1.5% agarose gel stained with ethidium bromide. The PCR tubes containing β-tubulin PCR amplions were properly labeled and stored at–20°C till further use.

For nested PCR on β-tubulin PCR product of each larva, 3μl of β-tubulin PCR product was placed in 22μl reaction mixture containing 2.5μl of 10X PCR buffer (minus Mg), 0.2 mM of each dNTP, 10pmol of each primer (P3 and P4) as described under section 3.3.1.5), 1.5 mM MgCl2 and 2.0U of Taq DNA polymerase. Polymerase chain reaction was performed with initial denaturation at 94°C for 2 min was followed by 33 cycles each of denaturation, annealing and extension as described above. This was followed by final extension for 5 min at 72°C. The PCR amplons were analysed by agarose gel electrophoresis in 1.5% agarose gel stained with ethidium bromide. The PCR tubes containing the nested products from adult worms were properly labeled and stored at–20°C till further use.

For nested PCR on β-tubulin PCR product of each larva, 3μl of β-tubulin PCR product was placed in 22μl reaction mixture containing 2.5μl of 10X PCR buffer (minus Mg), 0.2 mM of each dNTP, 10pmol of each primer (P3 and P4) as described under section 3.3.1.5), 1.5 mM MgCl2 and 2.0U of Taq DNA polymerase. Polymerase chain reaction was performed with initial denaturation at 94°C for 2 min was followed by 33 cycles each of denaturation, annealing and extension as described above. This was followed by final extension for 5 min at 72°C. The PCR amplons were analysed by agarose gel electrophoresis in 1.5% agarose gel stained with ethidium bromide.

Identification of species of individual larva: For molecular identification of species of larva, PCR-RFLP was done. 10μl of nested product was digested with 20 units of restriction enzyme EP-Gradient5 thermocycler with initial denaturation at 94°C for 5 min, followed by 30 cycles each of denaturation at 94°C for 1 min, annealing at 56.5°C for 1 min and extension at 72°C for 1 min. This was followed by final extension for 10 min at 72°C. The β-tubulin PCR amplons were analysed by agarose gel electrophoresis in 1.5% agarose gel stained with ethidium bromide. The PCR tubes were properly labeled and stored at–20°C for further use.
enzyme Rsal and 2.0 μl of enzyme buffer for 4 h at 37°C. The resulting fragments were separated by electrophoresis in 2.0% agarose gel. The sizes of resulting fragments were used as a key for identification of species of larva. Only the nested products showing specific profile for *H. contortus* were used further.

**Allele specific PCR:** The nested PCR products from both adults and infective larvae were further used for allele specific PCR for diagnosis of benzimidazole susceptibility and resistance (Silvestre and Humbert 2000). The allele non-specific primers (P5: GGA ACG ATG GAC TCC TTT CG and P2: GAT CAG CAT TCA GCT GTC CA), specific resistant-allele primer (P6: CTG GTA GAG AAC ACC GAT GAA ACA TA) and specific susceptible-allele primer (P7: ATA CAG AGC TTC GTT GTC AAT ACA GA) were used for AS-PCR amplification. The reaction was divided into two mixtures of 25 μl each. Each reaction mixture contained two non-specific primers (P5 and P2) and either of specific resistant or susceptible allele primer (P6 or P7). Two aliquots of 1.5 μl of nested product from adult and infective larvae were used as templates for amplification of allele specific products in a set of two PCR tubes. Besides this, each PCR reaction mixture consisted of 2.5 μl of 10X PCR buffer (minus Mg), 0.085 mM of each dNTP, 10 pmol of each non-specific primer (P5 and P2), 25 pmol of specific resistant-allele primer (P6) in one mixture and 25 pmol of specific susceptible-allele primer (P7) in another mixture, 1.7 mM MgCl2 and 1.5 U of Taq DNA polymerase. The volume of each reaction mixture was made up to 25 μl with autoclaved milli-Q water. Polymerase chain reaction was performed with initial denaturation at 94°C for 2 min, followed by 30 cycles each of denaturation at 94°C for 55 sec, annealing at 55°C for 55 sec. and extension at 72°C for 55 sec. This was followed by final extension for 10 min. at 72°C. The PCR amplions were finally analysed for presence or absence of benzimidazole resistance alleles by agarose gel electrophoresis in 1.5% agarose gel stained with ethidium bromide.

**RESULTS AND DISCUSSION**

**Benzimidazole resistant and susceptible strains of Haemonchus contortus:** The sheep, that were harbouring resistant strain of *H. contortus*, showed 1.7% FECR on 10th day post-treatment (DPT) after treatment with fenbendazole. EHA revealed an ED50 value of 0.8987 μg/ml. The sizes of resistant worms were used as a key for identification of species of larva. Only the nested products showing specific profile for *H. contortus* were used further.

**Molecular diagnosis of benzimidazole resistance in H. contortus:** The average A260: A280 ratio of the extracted DNA from individual susceptible and resistant adult worms was 1.824 indicating high purity of DNA without RNA and protein contamination. The concentration of DNA ranged from 2.839 to 5.642 μg/ml. The quality of DNA was further evaluated by agarose gel electrophoresis using 0.8% agarose gel. The isolated DNA were intact and without shearing. The genomic DNA from larvae could not be visualized on electrophoresis due to very low concentration in the larval suspension.

The isotype 1 β-tubulin gene of each DNA sample (homozygous susceptible, homozygous resistant and heterozygous) was amplified by PCR using specific forward (P1) and reverse (P2) primers in 50 μl reaction mixture. Amplification yielded partial β-tubulin gene PCR product of approximately 840 bp in all the cases. However, the β-tubulin gene was at very low concentrations in the amplified product from larval suspension, it could not be demonstrated on agarose gel electrophoresis.

Nest PCR was performed to generate enough quantity of β-tubulin gene product for further analysis. Nested PCR amplified a product of approximately 770 bp in all cases as evidenced by 1.5% agarose gel electrophoresis analysis.

Nest product from larvae were digested with 20 units of restriction enzyme Rsal for identification of species of the larvae used. On electrophoresis, 3 bands of approx. 430 bp, 190 bp and 145 bp were obtained, which confirmed the identity of larvae to be of *H. contortus*.

Analysis of allele specific PCR products revealed a susceptible specific band of approximately 550 bp from susceptible worms, whereas resistant worms revealed a resistant specific band of approximately 250 bp. Besides this a non-specific band of about 650 bp was also observed in some cases. In heterozygous, all the 3 bands of approximately 650 bp, 550 bp and 250 bp were visible. Similar strategy was used previously to detect mutation at residue 200 of isotype 1 β-tubulin gene in *H. contortus* adult worms (Kwa et al. 1994) and larvae (Silvestre and Humbert 2000). This method can also be used to detect first benzimidazole resistant individuals in a population (Elard et al. 1998).

AS-PCR is actually a type of multiplex or competitive PCR. For *H. contortus*, two sets of PCR mixtures were used, each comprising 3 primers (2 non-specific primers and either of susceptible or resistant allele specific primers). This results in generation of one non-allele specific fragment of about 650 bp size as an internal standard and one allele specific fragment. The susceptible specific fragment is of 550 bp and resistant specific fragment is of 250 bp in size.

The most critical part of standardization of AS-PCR is the adjustment of primer concentrations in the PCR mixture to ensure efficient competition between the primers (Coles et al. 2006). Silvestre and Humbert (2000) reported that false positive results might be obtained when primer concentrations are unbalanced. Besides primer concentration of Mg ions, dNTP's and Taq DNA polymerase must also be carefully optimized to get accurate, specific and good results. Also, annealing temperature is very critical. Once standardization of AS-PCR was done, it proved to be a
powerful tool to detect point mutations at codon 200 of isotype 1 β-tubulin gene of *H. contortus*.

Another advantage of this procedure is that the species of the parasite can also be determined simultaneously by simply applying PCR-RFLP technique with *RsaI* restriction enzyme on the nested product of β-tubulin gene obtained from infective larvae (Silvestre and Humbert 2000), thus excluding the morphological identification of larvae that requires a great expertise. In the present study, *H. contortus* infective larvae yielded 3 fragments of 430, 190 and 145bp after digestion of nested products with *RsaI*, which are specific for *H. contortus*. PCR-RFLP with *RsaI* using nested product for the diagnosis of *H. contortus* has also been used in India by Sankar *et al.* (2006) with good results.

However, AS-PCR strategy for diagnosis of benzimidazole resistance has it’s own limitations too. It cannot detect mutations other than those at position 200 in β-tubulin gene with the same set of primers, which may be responsible for development of resistance (Prichard 2001), viz. mutation at codon 167 (Prichard 2001). Also, the number of worms to be processed in order to find at least one resistant individual (P=0.002) should be 100, 50, 35 and 20 for 4, 8, 10 and 12% resistant worms in a given population, respectively (Elard *et al.* 1999).

Before the test can be used routinely in the field, it will have to be developed as a real-time PCR or pyrosequencing assay or should be developed within the framework of DNA chip technology which can detect point mutations responsible for resistance.

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