Genotyping of benzimidazole resistant and susceptible isolates of *Haemonchus contortus* from sheep by allele specific PCR

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Abstract Extensive and indiscriminate use of the benzimidazole class of drugs has led to the onset of anthelmintic resistance. In tropical countries like India, *Haemonchus contortus* is the most pathogenic parasite infecting sheep and goats. The widespread presence of resistant helminths (especially *H. contortus*) threatens the livestock farming. The use of various drugs has led to single nucleotide polymorphism that causes specific amino acid substitutions in β -tubulin protein of *H. contortus* to confer resistance. This emphasizes the need for a survey on the present status of resistance in India. In this study, allele specific PCR was employed to screen the presence of a SNP, a thymine-to-adenine transversion which leads to substitution of amino acid in codon 200 of β -tubulin gene that is correlated specifically with BZ resistance. Third stage larvae (L3) from pooled faecal cultures of four organized sheep farms served as a source of genomic DNA for identification of *H. contortus* and further genotype analysis. A total of 1000 larvae was screened, out of which 673 larvae were identified as *H. contortus*. Among 673 *H. contortus* larvae, 539 larvae (80 %) were genotyped as homozygous resistant (rr) and remaining 134 (20 %) were heterozygous susceptible (Sr) by allele specific PCR. The concluded resistance status reasons out the failure of anthelmintic drug in treating ruminants. Immediate steps are needed to avoid further aggravation of the problem. Target selective treatment by reviewing the resistance status of individual drugs, appropriate use of anthelmintic drugs and other control strategies

will provide a pragmatic option for delaying the further spread of anthelmintic resistance.

Keywords Sheep · Nematode · *Haemonchus contortus* · Anthelmintic resistance · Benzimidazole · Allele specific-PCR

Introduction

Gastrointestinal parasitism is a foremost socioeconomic problem and global concern in farm animals due to the production losses incurred by various helminthic parasites. Hematophagous gastrointestinal nematodes cause emaciation, anemia and even death of infected animals, ultimately leading to substantial economic losses to the livestock production (Ghisi et al. 2007). Among various helminthic infections of small ruminants, major threat and impediment to ruminant livestock farming occurs in the form of gastroenteritis caused by *Haemonchus contortus* (Santos et al. 2012). McLeod (2004) approximated the annual treatment cost due to *H. contortus* to be around \$103 m in India. The ubiquitous prevalence of *H. contortus* makes it a predominant helminth in tropical as well as temperate conditions. *H. contortus* adult parasites ingest about 0.05 ml of blood/helminth/day (Rowe et al. 1988) causing significant blood loss and marked reduction in PCV, which is a common finding in haemonchosis (Williamson et al. 2003). Till date, the control of this blood feeding nematode infection relies only on chemotherapy i.e., use of anthelmintic drugs. But empirical dosing of animals without estimating weight, frequent and arbitrary use, lack of proper veterinary assistance and knowledge are the predominant factors responsible for the emergence of anthelmintic resistance. The poorly elucidated, yet the complex genetic mechanism

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of helminthic worms, helps in evading drug binding by crucial mutations and thus, inducing resistance (Kaplan 2004). Benzimidazole (BZ) is the most widely used broad spectrum class of drugs for treating helminthic infections (Prichard 1994). This broad spectrum anthelmintic inhibits the cell division of parasites by binding to tubulin monomers. This binding prevents the self-polymerization of monomers leading to microtubule instability (Lacey 1988; Lubega and Prichard 1990). Initially, BZ group of drugs were highly efficacious in controlling helminth parasites affecting domestic animals. Due to its extensive use, the emergence of anthelmintic resistance in veterinary helminths was reported from all over the world (Wolstenholme et al. 2004; von Samson-Himmelstjerna and Blackhall 2005).

It has been well authenticated by various experiments that nucleotide polymorphism in β -tubulin coding sequence are correlated with BZ resistance (Kwa et al. 1993a, b, 1994; Wolstenholme et al. 2004). Point mutations in tubulin coding gene lead to the following amino acid substitutions—F200Y (TTC to TAC) (Elard et al. 1996), F167Y (TTC to TAC) (Silvestre and Cabaret 2002) and E198A (GAA to GCA) (Rufener et al. 2009). With drug treatment, the frequency of these substitutions increases, leading to treatment failure (Beech et al. 2011). Of late development of anthelmintic resistance has become a common event in *H. contortus* (Varady et al. 2007). The emergence of drug resistant *H. contortus* has become a major concern in developing countries due to the heavy economic burden it inflicts on the farmers. With the unavailability of new classes of anthelmintic drugs, a survey on the status of current resistance scenario in different geographical locations becomes a prerequisite to formulate strategies to delay further spread of anthelmintic resistance. Moreover practices like co-grazing of ruminants, poor knowledge on anthelmintic drug usage, incorrect use by farmers and high prevalence of *H. contortus* in tropical countries like India demand for the need to evaluate the resistance status of *H. contortus* populations.

Perusal of literature shows that benzimidazole resistance is a well recognized problem and various conventional in vivo (faecal egg count reduction test) and in vitro (egg hatch assay, micro-agar larval development test) methods are available to identify resistant trichostrongylids (Martin et al. 1989; Elard et al. 1999; Silvestre and Humbert 2000). But these methods are time consuming; laborious and specific for certain group of drugs. Hence, the current study was undertaken by exercising a PCR based detection method to identify and genotype resistance–susceptibility status of *H. contortus* isolates from four organized sheep farm in Tamil Nadu, India. This study using Allele specific PCR (AS-PCR) as described by Silvestre and Humbert (2000) would be highly useful to know the first hand

knowledge of BZ resistance in Tamil Nadu, India to better appraise the existing drug treatment strategies and herd management protocols.

Materials and methods

Collection of parasitic larvae

A total of 1000 fresh faecal samples were randomly collected per rectum from 250 sheep maintained in each of the four organised sheep farms namely Government sheep farm, Sattur, Mecheri sheep research station, Pottaneri, Sheep breeding research station, Ooty and District livestock farm, Hosur. The samples were transported to the laboratory in airtight zip lock covers under refrigerated conditions. Copro-cultures were set up individually for each farm using 250 g of pooled faecal samples. The faecal cultures were maintained in a BOD incubator (Forma Scientific Inc., USA) set at 37 °C with a relative humidity of 65–70 %. Larvae were recovered from these cultures after about 3–4 days (MAFF 1986). Ex-sheathment of larvae was done by incubating larval suspension for 5 min in a petri dish containing 3.5 % sodium hypochlorite (Silvestre and Humbert 2000) at 37 °C. Individual larvae were picked under microscopic surveillance and placed in 2 μ l of sterile water in a 0.2 ml micro centrifuge tube. Presence of single larva in each micro centrifuge tube was ensured invariably by microscopy.

Extraction of genomic DNA from larvae

DNA was extracted by adding 5 μ l DNA extraction buffer (1 mM TrisHCl, 0.1 mM EDTA and 5 mg/ml of Proteinase K) to the microcentrifuge tubes containing single larva. The tubes were incubated overnight at 41 °C in a water bath followed by incubation at 95 °C for 20 min to inactivate proteins K (Silvestre and Humbert 2000). This larval digests containing DNA was stored at –20 °C until used as a template for both species identification and amplification of beta-tubulin gene.

Design of oligonucleotide primers based on internal transcribed spacer (ITS)-1 sequences of *H. contortus*

New set of oligonucleotides was designed based on an ITS-1 region of rDNA to specifically identify *H. contortus*. The forward and reverse primers were chosen manually from homologous sequences available in NCBI, GenBank. The primers designed are listed as below:

HC ITS F-5' GTATCACCCCGTTTAAAGCTC 3'
HC ITS R-5' GATCCATCGCTGAAGCTAATC 3'

Screening of *H. contortus* by PCR

The species identification was carried out using 2 µl of larval digest as template in a total reaction volume of 25 µl containing 10× Taq Buffer, 1.5 mM MgCl₂ (New England Biolabs, MA), 0.2 mM of dNTP mix, 0.2 U Taq DNA polymerase (New England Biolabs, MA), 10 pmol of each forward and reverse primer (Eurofins MWG Operon) as mentioned in 2.3. Initially the samples were subjected to gradient PCR (55–65 °C) to optimize annealing temperature. The cycling conditions were 1 cycle of initial denaturation at 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 55–65 °C for 30 s and 72 °C for 30 s; this was followed by a final extension at 72 °C for 10 min. Negative controls without template DNA were also included. The specificity of the primers was checked using DNA extracted from other parasites infecting sheep prevalent in this geographical location. The PCR products were electrophoresed through 2 % agarose gel.

Purification, cloning and sequencing of PCR product

The PCR Product was purified using the QIAQuick PCR Purification Kit (Qiagen, Germany) and ligated into the plasmid pTZ57R/T (InsTAclone PCR Cloning Kit, Fermentas). Plasmid DNA from single *E. coli* DH5α colonies was purified using FastPlasmid Mini kit (Eppendorf, Germany). The plasmids were sequenced on both strands using the Big Dye Terminator v. 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) on a PRISM 3100 Genetic Analyzer (Applied Biosystems).

Detection of BZ resistance status of individual larvae

Amplification of β-tubulin gene by nested PCR

The larval DNA found positive for *H. contortus* by species specific PCR were then subjected to nested PCR to generate a significant amount of β-tubulin gene for genotyping analysis. The cyclic reaction as described by Coles et al. (2006) was performed with the 5 µl of the larval digest as the template in a 10.25 µl reaction mixture containing 10× Taq Buffer (without MgCl₂), 80 µM dNTP mix (New England Biolabs), 1.5 mM MgCl₂ (New England Biolabs), 0.5U Taq DNA polymerase (New England Biolabs), 6.5 pmol of each forward Pn1 and reverse primer Pn2. An aliquot of 1 µl of the [Pn1–Pn2] PCR product was used as a template in a reaction mixture of 25 µl containing 10× Taq Buffer (without MgCl₂), 80 µM dNTP mix (NEB), 1.5 mM MgCl₂ (NEB), 1.0 U Taq DNA polymerase (NEB), 12.5 pmol of each forward Pn3 and reverse primer Pn4.

Allele specific PCR

Allele specific PCR was employed to determine the resistant–susceptible status of individual larva. The PCR Program was followed as described by Coles et al. (2006). The reaction was carried out in two sets of reaction i.e. one set using Ph1, Ph2 and Ph3res primers (resistant allele) and Ph1, Ph2 and Ph4sus (susceptible allele) primers for the second set. An aliquot of 1.5 µl of [Pn3–Pn4] PCR product was used as a template in a reaction mixture of 25 µl containing 10× Taq Buffer (without MgCl₂), 80 µM dNTP mix (New England Biolabs), 1.5 mM MgCl₂ (New England Biolabs), 1.0 U Taq DNA polymerase (New England Biolabs), 8.5 pmol of each forward Ph1F, reverse primer Ph2R and Ph3res for one set and Ph4sus for another set.

Gel electrophoresis

The PCR products were electrophoresed on 2.5 % agarose gel stained with ethidium bromide in Bio Rad Gel electrophoresis unit and photographed using BIO RAD Gel Doc XR+.

Results

Standardization of species specific PCR

PCR was standardized using a known positive control (adult *H. contortus* parasites) and a negative control (DNA from *Mecistocirrus digitatus* and *Trichostrongylus* spp.). Specific amplification (345 bp) was observed with the positive control at 63 °C and 1.5 mM MgCl₂, while the negative control did not show any amplification. The PCR could detect as low as 2.79×10^{-2} ng/µl (Biophotometer plus, Eppendorf) of DNA. Hence, the newly designed primers were found to be both species specific and sensitive. A BLAST search was carried out to confirm the identity of the sequence and it revealed 98 % identity with the existing *H. contortus* sequences in GenBank, NCBI, thus confirming the identity of the strains. The sequence is

Table 1 Screening of *H. contortus* population in sheep farms

Name of the farm	Number of larvae screened	Number of larvae identified as <i>H. contortus</i>
Government sheep farm, Sattur	250	145
Mecheri sheep research station, Pottaneri	250	167
Sheep breeding research station, Ooty	250	172
District livestock farm, Hosur	250	189

Fig. 1 Agarose gel electrophoresis of species-specific PCR. Lane 2–29 and 32–48 DNA from Individual larvae. Lane 1, 30, 31 and 50 DNA ladder (100–1000 bp), Fermentas. Lane 49: NTC

Table 2 Resistance status of *H. contortus* in sheep farms

Name of the farm	Percentage and number of homozygous resistant <i>H. contortus</i>	Percentage and number of heterozygous susceptible <i>H. contortus</i>
Government sheep farm, Sattur	116 (80 %)	29 (20 %)
Mecheri sheep research station, Pottaneri	129 (77 %)	38 (23 %)
Sheep breeding research station, Ooty	136 (79 %)	36 (21 %)
District livestock farm, Hosur	158 (84 %)	31 (16 %)

available in the GenBank database under the accession number GenBank ID: JX289536.

Screening of *H. contortus* larvae by species specific PCR

Out of the 1000 larvae (L3) screened using the optimized PCR conditions, 673 larvae were positive for *H. contortus*. The percentage of the *H. contortus* population in the individual farms are given in the Table 1. A gel picture depicting the amplicon of 345 bp confirming the presence of *H. contortus* species is shown in Fig. 1.

Determination of BZ resistance status

Out of the 673 *H. contortus* larvae that were screened, 539 larvae was found to be homozygous resistant (rr) and the

remaining 134 were heterozygous susceptible (Sr). The resistance status of the individual farms are described in Table 2. Figure 2 shows the gel picture depicting the allelic status of the screened larvae. Few of the individual larvae which were not positive for *H. contortus* were also subjected to nested PCR. The negative samples did not give any of the characteristic 250 or 550 bp bands when subjected to allele specific PCR. This step was done to rule out any false positive results.

Discussion

Way back in 1961, thiabendazole was discovered for the treatment of worm infections. But the rapid development of drug resistance in *H. contortus* population was observed within 3 years of drug use (Drudge et al. 1964). The trend continued with the introduction of a new generation of drugs in the same class as well as other classes like imidathiazole and macrocyclic lactones. This has led to the development of multiple drug resistant (MDR) strains of *H. contortus* in many parts of the world viz., South America (Nari et al. 1996), South Africa (van Wyk et al. 1999), Malaysia (Chandrawathani et al. 2003), Southeast USA (Mortensen et al. 2003), Australia (Besier and Love 2003), New Zealand (Leathwick et al. 2001) and India (Gill 1996; Swarnkar et al. 1999; Easwaran et al. 2009). In Tiwari et al. (2006) reported the prevalence rate of BZ resistance in *H. contortus* of Indian isolates by RFLP-PCR technique. As *H. contortus* is an economically important parasite affecting sheep and goats in India, it is highly essential to review the benzimidazole resistance status, so that an effective

Fig. 2 Agarose gel electrophoresis of AS-PCR. Lane 8–17, 20–35 resistance status of individual larvae. Lane 2–7, 24–25: NTC. Lane 1, 18, 19 and 36: DNA ladder (200–2000 bp), Bio Basic

control strategy can be planned. Hence, an attempt was made for the first time in southern India to investigate the resistance status of *H. contortus*.

ITS is one of the most variable nuclear loci and hence has been a popular choice in developing highly sensitive techniques for detection of helminths (Mochizuki et al. 2006). Hence, oligonucleotides were designed to amplify precisely the ITS-1 region of the *H. contortus* rDNA. L3 larvae were preferred over adult worms to avoid the gDNA contamination from eggs in adult female worms as described by Beech et al. (1994). The species specific PCR revealed 67 % of larval population as *H. contortus*. Similar kind of result was observed by Raman et al. (1996) which inferred 60–70 % of larvae as *H. contortus* by morphological identification. The need for identification of species is of prime importance because of the mixed infections found in sheep of Tamil Nadu (Easwaran et al. 2009). This PCR was found to be very specific in identifying *H. contortus* from larval pool of different nematodes. Thus, the ITS based PCR assay can be more specific and sensitive in detecting *H. contortus* than just identifying the larvae based on its taxonomy with variable results.

Silvestre and Humbert (2000) described the principle of genotyping larvae by allele-specific PCR (AS-PCR). The same was followed in the present study. The three primer genotyping strategy to detect the Phe/Tyr substitution at position 200 was used to ascertain the resistance status as it yielded more reliable results for *H. contortus*. The study revealed that about 80 % of larvae were homozygous resistant (TAC, TAC, rr), 20 % were heterozygous susceptible (TTC, TAC, Sr) and no homozygous susceptible (TTC, TTC, SS) larvae were found. The reason for absence of homozygous susceptible worms in our screen may be attributed to the fact that any susceptible worm that might be present in the animals would have been killed by the routine anthelmintic treatment. The resistance status of *H.*

contortus in the four farms were almost similar. Tiwari et al. (2006) reported similar genotypic frequency using RFLP-PCR in Rajasthan, India. In addition to the SNPs of β -tubulin, genetic changes in P-glycoprotein and cytochrome P-450 also contribute to the development of anthelmintic resistance (Kerboeuf et al. 2003). So, the true percentage of BZ resistant larvae might be more than the actual number detected in this study.

The present study undoubtedly signifies that anthelmintic resistance is present in *H. contortus*, a predominant nematode infecting sheep. This is a serious concern for farmers especially in the developing countries like India. The perception of the majority of farmers that the benzimidazole anthelmintics are performing unsatisfactorily holds good and if the control practices are not changed, then the resistance is anticipated to escalate rapidly in the very near future. The presence of high percentage of resistant *H. contortus* larvae in an organized sheep farm raises serious questions about the status of resistance in unorganized farms. Due to the poor farming practices, nomadic culture and unavailability of proper veterinary service, the resistance status is expected to be even worse. Studies of a similar kind will be useful in selecting anthelmintic drugs on the alternative basis to control the spread of anthelmintic resistance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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