An allele specific PCR assay for screening for drug resistance among *Wuchereria bancrofti* populations in India

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**Background & objectives:** Albendazole, a commonly used anthelmintic drug that targets the polymerization of α- and β-tubulin dimer is currently co-administered with the antifilarial drug, diethylcarbamazine citrate (DEC) in the ongoing Global Programme for Elimination of Lymphatic Filariasis (GPELF). The experience in veterinary field has shown that there can be a rapid development of resistance to this drug, which therefore, needs to be monitored regularly in GPELF. Hence, we investigated the nucleotide polymorphism in the albendazole-binding domain of the isotype 1 β-tubulin gene from several populations of *Wuchereria bancrofti* and developed an AS-PCR assay useful in screening for sensitive/resistance alleles among parasite populations and also evaluated its utility.

**Methods:** For studying the polymorphism of isotype 1 β-tubulin gene, a 475 bp fragment spanning exon 5 and 6 of the gene was amplified and sequenced from the genomic DNA of *W. bancrofti* collected from six geographic regions of India. An allele specific (AS) PCR for screening albendazole sensitivity/resistance was developed and a total of 55 mf samples from blood smears on slides collected from Thiruvannamalai, Thanjavur and Puducherry were screened. Selective therapy with DEC was in place in three areas, mass drug administration (MDA) with DEC alone was implemented in four areas, while DEC plus albendazole was administered in one district.

**Results:** The analysis of the nucleotide sequence of the fragment from 20 *W. bancrofti* populations showed the domain to be highly conserved. An allele-specific PCR assay developed was used to detect sensitive/resistance alleles among 55 isolates of *W. bancrofti* and no albendazole resistance alleles were detected among the populations tested.

**Interpretation & conclusion:** The drug-binding domain of isotype 1 β-tubulin gene of *W. bancrofti* from different geographical locations was highly conserved. The AS-PCR developed showed potential application as a tool for monitoring albendazole sensitivity/resistance alleles among *W. bancrofti* populations, in areas where combination therapy of DEC-albendazole is being mass administered in the LF elimination programme.

**Key words** Albendazole - anthelmintic resistance - benzimidazole - β-tubulin - *Wuchereria bancrofti*

Lymphatic filariasis (LF) remains a considerable cause of clinical morbidity and disability in many developing countries. More than 600 million subjects in the world are at the risk of LF infection. *Wuchereria bancrofti* is responsible for approximately 90 per cent (128 million estimated cases) of the disease in the
world, while the remaining burden is due to *Brugia malayi* and *B. timori*. A global programme for the elimination of LF has been launched employing mass drug administration (MDA) of annual single dose of a combination of diethylcarbamazine citrate (DEC)/ivermectin and albendazole.

Albendazole, a safe, broad-spectrum benzimidazole anthelmintic has long been used to combat many nematodes, trematodes and cestode parasites occurring in human and animals. Benzimidazoles (BZs) are also preferred drugs in human mass chemotherapy programmes due to their low cost, broad spectrum of activity, relative high efficacy, and ease of administration. Resistance to all commonly used anthelmintic class of compounds has been observed worldwide and its prevalence appears to be increasing. BZs bind to β-tubulin, thereby ultimately preventing polymerization of tubulin into microtubules. β-tubulin, which is considered to be the major target of these broad-spectrum anthelmintics, has been described for several species, including the most important gastrointestinal nematodes of ruminants. Genetic and biochemical evidence suggests a critical role for β-tubulin in the development of resistance to BZs by nematodes. Further, mutations or deletions in genes encoding β-tubulin have been found to be associated with a change in phenotype from susceptibility to resistance in *Haemonchus contortus*. Subsequent studies have shown that a single nucleotide change at amino acid 200 [TTC (phenylalanine) to TAC (tyrosine)] of β-tubulin isotype-1 results in resistance to BZ. This discovery led to the introduction of molecular techniques to monitor BZ resistance using PCR assays.

Since albendazole is currently a component of MDA in the global LF elimination programme, it is necessary to monitor the development of drug resistance in *W. bancrofti* populations to this drug. Simple and inexpensive tools are necessary for monitoring the development of drug resistance on a large scale. Earlier, we reported a method for detecting albendazole sensitive alleles at amino acid position (200) among *W. bancrofti* populations. This method was based on sequencing the drug-binding domain comprising exon 5, intron 5 and exon 6 of isotype 1 β-tubulin gene. Real time PCR method for detecting resistance for this drug among *W. bancrofti* populations is also reported. Both are expensive and require sophisticated equipment. The LF endemic countries, generally with poor resources require less expensive method for monitoring drug resistance. In view of this we looked at the nucleotide sequence variations, if any, of the drug-binding domain of isotype1 β-tubulin gene of the *W. bancrofti* populations from different geographical location of India and also developed a simple allele-specific (AS) PCR for detecting albendazole resistance/sensitivity among these.

**Material & Methods**

All chemicals used in this study were procured from Sigma-Aldrich (USA), PCR enzymes and reagents were from Finzyme (Finland) and gel purification kits were from Qiagen (USA). Primers were custom synthesized by Metabion International AG (Germany).

**Study sites:** Six different localities viz., Varanasi (Uttar Pradesh State), Jagdalpur (Chhattisgarh State), Rajahmundry (Andhra Pradesh State), Kozhikode (Kerala State) Athipakkam (Tamil Nadu State) and Puducherry endemic for bancroftian filariasis in India were selected for studying the polymorphism in the drug binding domain of isotype 1 β-tubulin gene. For screening for albendazole drug resistance alleles among *W. bancrofti* populations, two localities of Tamil Nadu viz., Thanjavur and Thiruvannamalai and also Puducherry were chosen. MDA with annual single dose of DEC plus albendazole was implemented in Thanjavur, while DEC alone was administered in Thiruvannamalai and Puducherry. In the remaining locations, selective treatment of DEC was in place for the past half of century.

**Blood sample collection and mf purification:** For studying gene polymorphism of drug-binding domin of isotype 1 β-tubulin gene, blood samples were collected from microfilaria (mf) carriers residing in different geographical locations viz., Varanasi, Jagdalpur, Rajahmundry, Kozhikode, Athipakkam (during 2001) and from Puducherry (during 2001-2007) between 0900 and 1200 h. Written consent was obtained from the carriers before collecting the blood samples and the study protocol was approved by the institutional ethical committee (IEC). The mf were purified from blood by membrane filtration, followed by Percoll gradient centrifugation. For screening albendazole resistant alleles by AS-PCR, mf were obtained from blood slides collected during 2003 in Thiruvannamalai and Thanjavur and from Puducherry during the year 2002-2007 using the method reported by Bisht.

**Isolation of genomic DNA:** The DNA from 5-100 mf was extracted by digestion in a buffer containing...
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50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 20 mM EDTA, Proteinase K (1 mg/ml) and SDS (1%), at 56°C overnight, followed by standard phenol-chloroform procedure and the concentration was measured using a spectrophotometer (Gene Quant, USA). DNA was also extracted from 55 W. bancrofti populations purified from blood slides collected from mf carriers residing in Thanjavur, Thiruvannamalai and Puducherry (Table) for detecting albendazole sensitivity/resistance alleles by AS-PCR assay.

**Amplification of drug-binding domain:** The drug-binding domain containing exons 5 and 6 of isotype 1 β-tubulin gene (Fig. 1a) was amplified from 20 mf samples collected from different geographic areas of India using wbbt2F (5’ TATCAGATGTTGTGGTTGG 3’) and wbbt2R (5’ CTGTTGAGTAAAGTTGAGCTC-3’) oligonucleotide primers specific for isotype 1 β-tubulin gene fragment of W. bancrofti. The reaction was performed in thermal cycler (Mastercycler gradient, Eppendorf, USA) in a reaction mixture (30 µl) containing 3 µl 10x buffer, 0.2 mM of each dNTPs, 50 pmol each of forward and reverse primers, 2 units of DyNAzyme (Finnzymes, Finland) and 1 µl of 50 mM MgCl₂. The thermal profile used for amplification was: initial denaturation of 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. The final extension was at 72°C for 8 min. After amplification, 10 µl of PCR products was visualized with ethidium bromide staining after electrophoresis on 1 per cent agarose/tris acetate EDTA (TAE) gel.

**Sequencing of the amplicons and sequence analysis:** The PCR amplicons obtained were purified using gel purification kit (Qiagen, Germany). Sequencing of the double stranded DNA was done by dideoxy chain termination method in an automated cycle sequencer (Applied Biosystems, USA). DNA and amino acid sequences were analyzed using BLAST programme and aligned using clustalW software (www.ebi.ac.uk/clustalw).

**Allele specific PCR:** The AS-PCR (Fig. 1) was performed using W. bancrofti genomic DNA to detect the albendazole resistance/sensitivity alleles located in the drug-binding domain (spanning exons 5 and 6) of isotype 1 β-tubulin gene, in two separate reactions. Two sets of primers were used; one specific for sensitive and other for resistant alleles. While the reverse primer (Wbbt2R-5’-ACGACTTGAATGAGTTGTC-3’) was same for both the reactions, the forward primer for sensitive allele has TT at the 3’ end (BTUB/A 5’:

<table>
<thead>
<tr>
<th>Sample locations</th>
<th>Year of sampling</th>
<th>Drug administered (No. of rounds of MDA)</th>
<th>No. of parasite populations tested</th>
<th>Screening method and sample size</th>
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<tr>
<td>Athipakkam</td>
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<td>Selective therapy with DEC</td>
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<td>Gene polymorphism</td>
</tr>
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<td>MDA with DEC (4)</td>
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<td>2001</td>
<td>Selective therapy with DEC</td>
<td>2</td>
<td></td>
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<tr>
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<td>Screening drug resistance by AS-PCR</td>
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<tr>
<td>Thiruvannamalai</td>
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<td>MDA with DEC (5)</td>
<td>15</td>
<td>AS-PCR</td>
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<tr>
<td>Thanjavur</td>
<td>2003</td>
<td>MDA with DEC+Alb (5)</td>
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<td>N=55</td>
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</table>

MDA, Mass drug administration; DEC, diethlcarbamazine citrate; Alb, albendazole

**Table.** Details of parasite samples used for gene polymorphism studies and AS-PCR for screening drug sensitivity/resistance; locations of blood smear collection, year of collection, number of populations and chemotherapy

**Fig. 1.** (a) Structure of isotype 1 β-tubulin gene showing albendazole drug binding region. (b) The principle of allele specific (AS) PCR method to detect drug sensitive/resistance alleles among W. bancrofti populations. This AS-PCR utilizes 3 primers in which reverse primer is common for both the alleles, whereas A-primer for detecting sensitive allele and B-primer for detecting resistant allele. The 3’ end of the sensitive primer is specific for TTC (which code for phenylalanine) and resistant primer specific for TAC (which code for tyrosine) at 200th amino acid position.
GTTGAAAAACCTGACGAAACTTT-3’) and that for resistance allele had TA at the 3’ end (BTUB/T 5’GTTGAAAAACCTGACGAAACTTA-3’). The reaction volume and concentration of PCR ingredients were same as indicated above for the amplification of exons 5, intron 5 and exon 6 of isotype 1 β-tubulin gene. Amplification was carried out for 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and final extension of 72 °C for 8 min using thermal cycler (Mastercycler gradient, Eppendorf, USA).

Results & Discussion

Albendazole is a potent antihelminthic drug widely used for the control of human as well as animal helminthes. It can be given orally, lacks major side effects, and has broad efficacy against most nematodes. It acts by blocking the synthesis of microtubules by inhibiting β-tubulin polymerization. However, its wide spread use has led to the development of resistance among veterinary helminthes. Resistance has also been reported for many other anthelmintics drugs. Molecular techniques have proved more sensitive tools and are used widely for screening for drug resistance among different parasites.

Albendazole is currently used in combination with DEC for LF elimination to enhance the community acceptance of the latter drug. Development of albendazole resistance among W. bancrofti parasites is quite likely to occur in the near future due to its long-term use. Hence, detection and monitoring of albendazole resistance among W. bancrofti populations is very important.

Polymorphism in drug-binding domain of isotype1 β-tubulin gene of W. bancrofti: An amplicon of 475bp encoding drug-binding domain of isotype 1 β-tubulin gene (consists of exon 5, intron 5 and exon 6 regions) was obtained by PCR using genomic DNA extracted from 20 W. bancrofti populations collected from different and distant locations in India viz., Puducherry (P), Varanasi (V), Jagadalpur (J), Rajahmundry (R) Athipakkam (T) and Kozhikode (C). Nucleotide sequences of these populations matched with the β-tubulin sequences reported earlier. Clear nucleotide sequence of the drug-binding domain obtained from sequencing the fragment amplified from different populations varied in length between 393-441bp (Ac. No. EF190199-190209, EF492870-492878). The alignment of these sequences (Fig. 2) showed very high conservation (average identity ~99%) of the drug-binding domain of these parasite populations. However, among 20 W. bancrofti parasite populations from different parts of the country only 3 populations showed 1-2 nucleotide variations in the gene fragment (Fig. 2). The sequence variation in Jagadalpur isolates were non-synonymous and at nucleotide positions 2839 (Leu215Trp; TTG/ TGG) in population J5, 3091 (Val 258Gly; GTA/ GGA) and 3175 (V286; GGT/GGT) in J9 population (Fig. 3). One of the isolate (C8) from Kozhikode (Kerala State) showed a variation in the intron 5 at nucleotide position 2975 (T=C). None of the sequences of the other populations exhibited any variation. Thus, the drug-binding domain of the isotype 1 β-tubulin of the W. bancrofti populations from different geographical areas of India appeared to be highly conserved.

Several nucleotide variations in the β-tubulin gene of nematodes as well as fungi belonging to different genera, leading to amino acid changes have been reported. These changes were at amino acid positions 6, 165, 198, 200 and 241. But amino acid changes only at positions 167, 198, 200 and 368 are of importance as far as drug resistance in nematodes is concerned. In the present study, nucleotide variations were found in exonic regions of two isolates from Jagadalpur (J5 and J9) and presently it is difficult to infer what kind of phenotypic changes these might confer, at least in relation to drug sensitivity. At least one amino acid change (215) in the Jagadalpur isolate was close to the amino acid residue which is a major determinant of the sensitivity/resistance to albendazole (200). Bennet et al have also reported a change at 189 residue in the β-tubulin gene of Trichuris trichura. It will be interesting, with further investigations on phenotypic changes to see what influence it may have on the binding efficacy of the drug.

Allele specific (AS) - PCR assay: An allele-specific PCR was designed and was employed for screening albendazole resistant alleles among 55 W. bancrofti populations collected from two towns in Tamil Nadu viz., Thiruvannamalai and Thanjavur, and also Puducherry town, where MDA was launched during 2000. In Thanjavur, a combination of DEC and albendazole was administered, while in Thiruvannamalai and Puducherry only DEC was administered, as MDA annually. Out of 55 W. bancrofti samples, 20 each were from Puducherry and Thanjavur towns and 15 from Thiruvannamalai town. W. bancrofti mf were isolated from 55 archived blood smears collected from these towns as reported earlier. DNA was extracted from mf and the drug-binding domain, containing the locus
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<th>Position</th>
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Fig. 2. Alignment of nucleotide sequences of the drug-binding domain of isotype 1 \( \beta \)-tubulin gene fragment (exon 5, intron 5 and 6) of *W. bancrofti* isolates collected from different locations. Sequences were aligned using ClustalW. Only the isolates having sequence variation (J5, J9 and C8) are shown by comparing nucleotide sequence of Puducherry isolate (P2) as reference. Variations in *W. bancrofti* sequences are marked in box. P, Puducherry; J, Jagadalpur; C, Kozhikode; BP, Brugia pahangi.
Fig. 3. Comparison of amino acid sequences of the drug-binding domain of isotype 1 β-tubulin of W. bancrofti isolates from different locations. The amino acid sequence of Jagadalpur isolates (J9, J5) and Kozhikode isolate (C8) were aligned with Puducherry isolate (P2) and B. pahangi. The amino acid variations are shown in box.

was amplified. All samples yielded an amplicon of ~425bp (Fig. 4) with the sensitive primer and none of the samples gave amplification with resistance specific primers, indicating the absence of albendazole resistance alleles among the parasite populations tested. Thus, the AS-PCR assay results demonstrated that there was no albendazole resistance, associated with a Phe200Tyr change, among these W. bancrofti populations. The same results were obtained through sequencing the drug-binding domain of isotype 1 β-tubulin gene fragment earlier.

The AS-PCR developed in the present study may not be as sensitive as RT-PCR assay developed by Schwab et al. However, it is expected that the frequency of drug resistance alleles will be significantly high among W. bancrofti populations even with the administration of a few rounds of albendazole. Schwab et al. have reported that the resistance associated with a change at amino acid position 200 increased significantly under drug pressure. They have also reported that the percentage of resistance alleles was 26.2 per cent in an untreated population, which increased to 60.2 per cent after treatment once with albendazole plus ivermectin and further to 86.2 per cent after two rounds of treatment. But it was quite low in Ghana because the parasites collected from patients had not been treated with albendazole or ivermectin. This indicates that even though there is a less chance of detecting the low frequency of drug resistance alleles by AS-PCR assay, it may be useful for screening drug resistance after several rounds of MDA when the frequency of resistance alleles is expected to be significantly high.

Thus, the present study showed highly conserved nature of albendazole-binding domain in the isotype 1 β-tubulin gene of W. bancrofti populations from different geographical regions of India. The AS-PCR assay developed appears to have potential application in monitoring albendazole resistance in areas where co-administration of albendazole with DEC/ivermectin is implemented under global LF elimination programme for several rounds. However, the weakness of the assay was lack of a positive control. This problem can be circumvented by sequencing the amplicons spanning exon 5 through exon 6 from the samples that test negative for albendazole sensitive AS-PCR assay. Further, more samples need to studied for the complete picture of the genetic variation and drug resistant alleles in the benzimidazole drug-binding domain of β-tubulin gene from W. bancrofti.

Fig. 4. Sample gel showing detection of albendazole sensitive alleles among Wuchereria bancrofti populations from Puducherry, using AS-PCR assay. M-Marker, lane 1-7 –amplicons with W. bancrofti isotype 1 β-tubulin with sensitive primers and NC-negative control.
Acknowledgment

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References


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