DETECTION OF BENZIMIDAZOLE RESISTANCE-ASSOCIATED MUTATIONS IN THE FILARIAL NEMATODE WUCHERERIA BANCROFTI AND EVIDENCE FOR SELECTION BY ALBENDAZOLE AND IVERMECTIN COMBINATION TREATMENT

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Abstract. The Global Program to Eliminate Lymphatic Filariasis has been implemented to reduce human microfilariae to levels low enough to break the transmission of the disease by using single annual doses of albendazole in combination with diethylcarbamazine or ivermectin. Many veterinary helminth parasites have developed resistance against both albendazole and ivermectin. Resistance to albendazole in veterinary nematodes is known to be caused by either of two single amino acid substitutions from phenylalanine to tyrosine in parasite β-tubulin at position 167 or 200. We have developed assays capable of detecting these single nucleotide polymorphisms (SNPs) in Wuchereria bancrofti, and have applied them to microfilaria obtained from patients in Ghana and Burkina Faso. One of the SNPs was found in worms from untreated populations in both locations. Worms from treated patients had significantly higher frequencies of these mutations. These findings indicate that a β-tubulin allele associated with benzimidazole resistance is being selected in these populations.

INTRODUCTION

The Global Alliance to Eliminate Lymphatic Filariasis relies on chemotherapy with the anthelmintic albendazole in combination with ivermectin or diethylcarbamazine to block transmission of Wuchereria bancrofti, the main causative agent of lymphatic filariasis.1 Avermectins and benzimidazoles have been used extensively in veterinary medicine, and this has led to high levels of drug resistance.2 Although resistance against anthelmintics has not yet impeded the treatment of human helminth infections,3 the development of drug resistance has had an adverse impact on the control of other parasites, such as Plasmodium falciparum.4 The development of drug resistance in lymphatic filariae could severely compromise the international control program, which will involve an estimated 350 million people.

The mode of action of benzimidazoles was found to be their interference with tubulin polymerization into microtubules by binding to β-tubulin.5-7 Resistance against benzimidazoles in a number of nematode species has been shown to be caused by a phenylalanine (TTT/TTC) to tyrosine (TAT/TAC) substitution at either position 167 or 200 of nematode β-tubulin.8-10 The position 200 tyrosine mutation appears to be more common in parasitic nematodes and was found to be recessive.11,12 Resistance to benzimidazoles was also found to be associated with 200Tyr or 167Tyr substitutions in normally benzimidazole-susceptible fungi,17-20 and the 200Tyr mutation in β-tubulin was also found in benzimidazole-resistant strains of protozoa.21 However, the position 200 mutation was not found in Necator americanus hookworms from Pemba Island in which benzimidazole resistance was suspected.22 Eukaryotes that normally code for tyrosine at codon 200 appear to be uniformly tolerant for benzimidazole. Phenylalanine at codons 167 and 200 appears to be critical for the high-affinity binding of benzimidazole anthelmintics to nematode tubulin and for sensitivity to these anthelmintics.12

In a study carried out in India,23 pooled W. bancrofti microfilariae from 14 patients from five geographic regions were sequenced to detect the codon 200 resistance mutation. The consensus sequences of the 14 pooled samples failed to show the codon 200 resistance mutation. However, sequencing the pooled samples would not have shown the mutation unless it was abundant, since sequencing pooled DNA samples is not a sensitive tool for the detection of rare single nucleotide polymorphisms (SNPs). The sequencing result of a pooled sample will show the most abundant sequence.

The objectives of this study were to assess whether mutations associated with benzimidazole resistance in other nematodes are present in W. bancrofti, to examine their frequency in untreated populations from Ghana and Burkina Faso, and to determine their frequency in microfilaria obtained from patients treated with albendazole and ivermectin.

MATERIALS AND METHODS

The study and microfilarial collections were reviewed and approved by the McGill University, Faculty of Medicine Institutional Review Board, the Noguchi Memorial Institute for Medical Research Institutional Review Board, and the Ministry of Health of Burkina Faso. Informed consent was obtained from all participants.

Amplification of Brugia malayi and W. bancrofti β-tubulin. Primers were designed based on the B. pahangi β-tubulin sequence (GenBank accession no. M36380) to amplify the full length β-tubulin cDNA from B. malayi. Total RNA was extracted from bulk adult worms (obtained from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) using TRIzol reagent (Invitrogen, Carlsbad, CA) and methods described by the manufacturer. Total RNA was reverse transcribed with the adaptor primer (5’-3’) GGC CAC GCG TCG ACT AGT AC(T) and 200 units of murine moloney leukemia virus reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. cDNA was used to amplify a fragment of β-tubulin using a reaction containing 2.5 μL of 10 × polymerase chain reaction (PCR) buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 1 μL (50 μM) of MgCl2, 0.5 μL each of primers sense (5’-3’) GGT ACC ATG GAT TCT ATT CG (AY705382

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position 211-230) and antisense (5'-3') GAT CGG CGT TCA ACT GTG ACA AAG CC (AY705383 position 672-694) at a concentration of 20 μM, 1 unit of Taq polymerase, 1 μL (10 μM) of dNTPs, and sterile water to give a final volume of 25 μL. The amplification conditions were 94°C for 2 minutes, followed by 30 cycles at 94°C for 55 seconds, 55°C for 55 seconds, and 72°C for 55 seconds, and a final extension at 72°C for 10 minutes.

The full-length sequence was obtained using a 3' rapid amplification of cDNA ends (RACE) procedure. Briefly, 5 μL of cDNA was amplified in a nested reaction containing 2.5 μL of 10× PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1.5 μL (50 μM) of MgCl₂, 0.5 μL each of the sense primers (5'-3') GGC AAA TAT GTG CCA CGA GC (position 169-188) and (5'-3') GGT ACC ATG GAT TCT ATT CG (position 211-230) at a concentration of 20 μM, 1 unit of Taq polymerase, 1 μL (10 μM) of dNTPs, and sterile water to give a final volume of 25 μL. The amplification conditions for both steps were 94°C for 2 minutes, followed by 40 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 75 seconds, and a final extension at 72°C for 10 minutes. The 5' end of the gene was amplified using the same conditions as the 3' RACE, but with different primers: sense primer, which corresponded to the nematode splice leader sequence SL1 (5'-3') GGT TTA ATT ACC AAG TTT GAG, and antisense primers (5'-3') AGG GCT CGA TAA GCA GCA GC (position 832-851) and (5'-3') GAT CGG CGT TCA ACT GTG CA (position 729-748). The resulting fragments were cloned into a PCR 2.1 vector using a TA cloning kit (Invitrogen) as per the manufacturer's instructions and sequenced with a Beckman (Palo Alto, CA) CEQ DNA sequencer.

Primers to amplify genomic DNA from W. bancrofti were designed based on the full-length B. malayi β-tubulin cDNA to amplify by PCR a region surrounding the mutations to be diagnosed from W. bancrofti microfilaria in a reaction containing 2.5 μL of 10× PCR buffer, 1 μL (50 μM) of MgCl₂, 0.5 μL each of the primers sense (5'-3') GGC AAA TAT GTG CCA CGA GC (AY705382 position 169-188) and (5'-3') AGG GCT CGA TAA GCA GCA GC (AY705382 position 832-851) at a concentration of 12.5 μM, 1 unit of Taq polymerase, 1 μL (10 μM) of dNTPs, and sterile water to give a final volume of 25 μL. The amplification conditions were 94°C for 2 minutes, followed by 30 cycles at 94°C for 55 seconds, 55°C for 55 seconds, and 72°C for 55 seconds, and a final extension at 72°C for 10 minutes. All reactions were carried out in a PTC-200 Thermal Cycler (MJ Research, Inc., Waltham, MA). Resulting fragments were cloned and sequenced as described earlier in this report.

**Diagnosis of resistance-associated mutations in W. bancrofti.** Blood samples (10 mL) containing microfilaria were transferred to centrifuge tubes and centrifuged at 1,100 rpm (300 x g) for 10 minutes at 4°C. The pellet was resuspended in 25 mL of red blood cell lysis solution, incubated on ice for 10 minutes, and centrifuged again as above. The final pellet was resuspended in 1.5 mL of 70% isopropanol alcohol. To separate individual worms, a small amount of the sample was transferred to a small Petri dish. Single microfilariae were washed in 70% ethanol, separated into individual PCR-microcentrifuge tubes using a micropipette under an inverted microscope, and identified visually as W. bancrofti. Extraction of DNA was carried out directly in the PCR tube using the Qiagen (Valencia, CA) DNeasy tissue kit at one-fourth the recommended volume and following the manufacturer's instructions.

To detect mutations using a pyrosequencer, a smaller fragment surrounding the SNPs was amplified using microfilarial DNA or, when the DNA content was small, the product of the PCR amplification performed earlier was used as a template. The reaction contained 5 μL of 10 × PCR buffer, 2 μL (50 μM) of MgCl₂, 0.5 μL each of primers sense (5'-3') (AY705383 position 308-328) GGG AAC ATT GTG CTC GAA and antisense (5'-3') GGA AGC AGA TGT CAT ACA AAG CC (AY705383 position 672-694) at a concentration of 20 μM, 1 unit of Taq polymerase, 2 μL (10 μM) of dNTPs, and sterile water to give a final volume of 50 μL. The second primer had biotin attached to its 3' end to generate a single-stranded template using streptavidin beads. The amplification conditions were 94°C for 2 minutes, followed by 40 cycles at 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. The sequencing primers used for SNP analysis in a Pyrosequencer® (Pyrosequencing, Inc., Westborough, MA) were (5'-3') CAT ACA AAG CC (position 907-926) at a concentration of 12.5 μM, 0.2 μL each of the probes 5'-CCG TGA GGA GTA TCC GGA TCG AAT TAT G (fluorescein)-3' (position 332-359) and 5'-LC Red 640)CTC TTT TTC GGT GTG GCC GTC G (phosphate)-3' (position 362-382) for position 167 and 5'-ACT TCT TGC ATT GAT AAC ACG GC (fluorescein)-3' (position 652-674) and 5'-LC Red 705) TGT ATG ACA TCT GGT TCC GAA CGT (phosphate)-3' (position 677-700) for position 200 at a concentration of 20 μM. The reaction conditions were 20 sec at 95°C, followed by 40 cycles at 95°C for 10 seconds, 55°C for 10 seconds, and 72°C for 40 seconds, a melting step from 50°C to 80°C, and a final cooling step to 40°C. All temperature changes were with a ramp speed of 20°C/second. Melting temperatures for the position 200 probes are 67°C for the wild-type gene and 63°C for the mutant. Melting temperatures for the position 167 probes are 63°C for the wild-type gene and 58.5°C for the mutant.

Both diagnostic tests were optimized with a plasmid containing the wild-type gene and plasmids containing the mutation(s), generated by site-directed mutagenesis, using the QuickChange kit (Stratagene, La Jolla, CA) and the following mutagenesis primers: sense (5'-3') ATG AGC TCT ATT TCG GTT GTG CCG TCG (AY705383 position 357-373) and antisense (5'-3') ACA ACC GAA TTA GAG CTC ATA ATT CG (position 351-377) for the mutation at codon 167, and sense (5'-3') CGA AAC TTA CTG CAT TGA TAA CG (position 648-670) and antisense (5'-3') CTG TAT CAA TGC AGT AAG TTG CC (position 648-670) for the mutation at codon 200, following the manufacturer's instructions.

**Experimental design and statistical analyses.** Microfilariae from patients infected with W. bancrofti were collected in Burkina Faso and Ghana. Treatment programs for lymphatic
filariasis have been instituted in communities in Ghana and Burkina Faso and records and patient questionnaires were available to establish the treatment histories in the individuals sampled. We analyzed microfilaria from patients before treatment, after one round of treatment, and after two rounds of treatment with 400 mg of albendazole and 200 μg/kg of ivermectin. In the samples obtained from Burkina Faso, 5–15 microfilariae per patient were genotyped. Microfilariae from Ghana were obtained as pooled samples from several patients. Two pooled samples were obtained that had been collected at different times in 2001. These two samples were treated separately because they were sampled from different populations. A chi-square test was used to detect significant differences in genotype frequencies and in allele frequencies between the three treatment groups within each country. It was determined whether genotype frequencies of the whole population and within each patient were in Hardy-Weinberg equilibrium using a chi-square analysis to compare observed and expected values.

RESULTS

We used fresh B. malayi to obtain high-quality RNA and synthesize full-length β-tubulin cDNA (Genbank accession no. AY705382). The high sequence homology between B. malayi and W. bancrofti allowed us to design primers, based on the B. malayi sequence, to clone and sequence a partial genomic sequence of W. bancrofti β-tubulin containing the regions coding for both amino acid 167 and 200 (Genbank accession no. AY705383). Only one isotype of β-tubulin was detected. Based on these sequences, two diagnostic tests for mutations at positions 167 and 200 of β-tubulin of W. bancrofti were developed. The first assay was developed with a pyrosequencer. This technique is able to sequence short fragments of DNA very rapidly; the pyrosequencer can process 96 samples in less than one hour and the results are reliable and easy to interpret. A second test made use of fluorescence resonance energy transfer (FRET) probes and melting point analysis for mutation detection on a Light Cycler (Roche). This test is able to identify both mutations in a single Light Cycler reaction. Both assays are capable of genotyping single microfilaria. We evaluated these tests with the help of several plasmids. These plasmids contained either the wild-type β-tubulin gene, or the β-tubulin gene containing the position 167 or the position 200 mutations introduced by site-directed mutagenesis. The absence or presence of the mutations was verified by sequencing. Although the FRET assay is not as quick and easy to perform as the pyrosequencer assay, it is more accessible for large-scale use since real-time PCR technology is now available in many research facilities.

We obtained several hundred microfilaria from Ghana and Burkina Faso. Ghanaian samples consisted of microfilaria from patients with lymphatic filariasis who had not been treated with albendazole and ivermectin as part of the Global Alliance to Eliminate Lymphatic Filariasis. Blood samples containing W. bancrofti collected in Burkina Faso were from either untreated patients or patients treated once or twice with albendazole and ivermectin. Samples were taken at least seven days after the last anthelmintic treatment. Microfilaria was low in the treated patients, although the patients were still microfilaria positive despite recent treatment. The first two batches of microfilaria from Ghana were analyzed using the FRET assay. We were able to identify several microfilariae that had the position 200 tyrosine genotype. The frequency of the mutant allele in the first group of 130 microfilariae was 2.7%. The frequency of the mutant allele in the second group of 300 microfilariae was 0.33% (Table 1). Because of a lack of information regarding the size of the human population from which the microfilariae were pooled, we cannot make a reliable estimate of the frequency of these resistance-associated mutations in the entire parasite population. However, these findings indicate that the allele, which potentially causes albendazole resistance, is present in the population prior to treatment with albendazole. This suggests that with the advent of the mass treatment program in Ghana, selection for this allele in the population could occur.

We have also analyzed 400 microfilariae from 48 patients residing in the villages of Gora, Perigban, Bandongo and Tangonko in Burkina Faso. Thirty of these patients had not received any treatment for lymphatic filariasis, and 14 had received one round of treatment with 400 mg of albendazole in combination with 200 μg/kg of ivermectin. Four patients had received two yearly doses of the same treatment combination. Microfilarial counts obtained from these villages are shown in Table 2. The microfilarial counts from the patients treated twice were taken seven days after treatment.

Using the pyrosequencer, we found the resistance-associated mutation at position 200 at an allele frequency of 26.2% in the untreated population. In the microfilaria from patients treated once, the allele frequency of this mutation was 60.2%. In worms from patients treated twice, the allele frequency was 86.2%. These allele frequencies were significantly different ($\chi^2 = 139.8$). The genotype frequencies for all three groups from Burkina Faso are shown in Figure 1. Worms from untreated patients were 63% homozygous wild type (200phe/200phe), 21.5% heterozygous (200phe/200tyr), and 15.5% homozygous for the resistance mutation (200tyr/200tyr). Microfilaria from patients treated once were 26.3% homozygous wild type, 27.1% heterozygous, and 46.6% homozygous for the resistance mutation. Microfilaria from patients treated twice were 0% homozygous wild type, 13.8% heterozygous, and 86.2% homozygous for the resistance mutation. All genotype frequencies were significantly different using chi-square analysis (Figure 1). A resistance mutation was not detected at codon 167.

DISCUSSION

These results of this study indicate that the resistance-associated mutation at codon 200 was present in the populations of W. bancrofti sampled, especially those from Burkina Faso, at a reasonably high frequency even before the mass treatment program had been initiated. It has been found that

<p>| Table 1 |
| Frequencies of codon 200 genotypes in β-tubulin in two populations of Wuchereria bancrofti from Ghana* |</p>
<table>
<thead>
<tr>
<th>FF (%)</th>
<th>FY (%)</th>
<th>YY (%)</th>
<th>Total Y (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>96.15</td>
<td>1.54</td>
<td>2.31</td>
</tr>
<tr>
<td>Group 2</td>
<td>99.67</td>
<td>0.33</td>
<td>0.33</td>
</tr>
</tbody>
</table>

*F = phenylalanine, Y = tyrosine. Homozygous FF and heterozygous FY have been associated with a benzimidazole-susceptibility phenotype and homozygous YY has been associated with a benzimidazole-resistance phenotype. Total Y is the allele frequency of the resistance-associated allele in the population. Group 1, n = 130; Group 2, n = 300.
alleles associated with benzimidazole resistance in *Haemonchus contortus* were found in an unselected population at an allele frequency of 46%.\(^{24}\) indicating that such resistance alleles may commonly be in the population prior to drug treatment at relatively high frequencies. However, it must be taken into consideration that benzimidazole resistance is recessive and that albendazole or mebendazole are common treatments for intestinal helminths, and yearly deworming of school children is common in many parts of west Africa.\(^{25}\)

Thus, parasites may have been previously exposed to benzimidazoles, and some selection for the 200 tyrosine mutation may have occurred prior to the onset of the mass treatment program.

We have shown that there was a significantly higher allele frequency of the 200 tyrosine genotype in worms collected from patients who had been treated with albendazole in combination with ivermectin. This indicates that this mutation may be selected for with drug treatment. The total allele frequency was 31.6% higher in the treated versus the non-treated worm population. In worms collected from patients who had been exposed to two rounds of albendazole/ivermectin treatment, the allele frequency was an additional 25.9% higher. Such selection is a strong indication of the development of drug resistance. Because pretreatment microfilarial counts were not taken, we cannot make conclusions about the success of treatment. However, it was only those microfilaria that were still present in the treated patients after treatment that were genotyped. In the case of the patients treated twice, microfilaria were harvested seven days after their last treatment and could therefore represent a population of microfilarial survivors of treatment or progeny of adult worms that were able to maintain microfilarial production despite treatment.

In some parasitic nematodes, a second β-tubulin gene appears to be present,\(^{26,27}\) which may contribute to benzimidazole sensitivity and resistance.\(^{24}\) We did not detect a second β-tubulin isotype in *W. bancrofti*. However, if present, it could contribute to maintaining susceptibility to albendazole.

It must also be noted that this was not a prospective study because parasites from different treatment groups were collected from different patients residing in separate villages. Therefore, we cannot rule out natural genetic variation, which may occur between populations, as being responsible for the differences in polymorphism between the treated and untreated populations.

We tested whether alleles of this gene were in Hardy-Weinberg equilibrium. When considering all worms in a treatment group (including untreated patients) as part of one population, the frequencies were not in balance, but showed an excess of homozygotes. When considering all worms sampled from an individual patient as being one population, the sum of the resulting allele frequencies from all patients was in Hardy-Weinberg equilibrium. The resistant-associated allele appeared to be aggregated within certain patients, rather than evenly distributed throughout the population. This result may be due to the fact that random mating does not occur between worms in different hosts. An excess of homozygotes may strongly accelerate selection for resistance, since it is believed to be a completely recessive trait,\(^{10}\) thus a larger proportion of the 200tyr as homozygotes could lead to a greater proportion of treatment failures.

Combination treatment with ivermectin and albendazole could be expected to reduce the rate of selection for resistance to either of these anthelmintics provided that the resistance mechanisms involve different genes. However, it has recently been found that ivermectin selects on β-tubulin in the filarial nematode *Onchocerca volvulus* (Eng KJL and Pri-chard RK, unpublished data). Although ivermectin selection is not associated with the phe200tyr SNP examined here, selection by the two anthelmintics can involve the same gene. Thus, there could be linkage between the region implicated in ivermectin selection and possible albendazole selection for the phe200tyr SNP, which could modify the rate of selection for albendazole resistance. The implications of combination treatment on genetic selection need to be examined in future studies. Based on these findings, it is imperative to continue monitoring for the presence of this mutation, in other treatment zones, to detect early evidence of possible resistance selection and to correlate this with responses to treatment.

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**Table 2**

<table>
<thead>
<tr>
<th>Village</th>
<th>Treatment history</th>
<th>No.</th>
<th>Geometric mean MF/ml</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tangonko</td>
<td>Untreated</td>
<td>16</td>
<td>15,091</td>
<td>27,050</td>
</tr>
<tr>
<td>Bandongo</td>
<td>Untreated</td>
<td>14</td>
<td>7,674</td>
<td>39,250</td>
</tr>
<tr>
<td>Gora</td>
<td>Treated once</td>
<td>1</td>
<td>9,300</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Treated twice</td>
<td>4</td>
<td>964</td>
<td>350</td>
</tr>
<tr>
<td>Perigban</td>
<td>Treated once</td>
<td>13</td>
<td>1,608</td>
<td>262</td>
</tr>
</tbody>
</table>

* N = sample size; MF = microfilaria. Range is the difference between the highest and lowest counts.

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**Figure 1.** Genotype frequencies of β-tubulin codon 200 of *Wuchereria bancrofti* microfilaria from Burkina Faso before and after treatment with albendazole/ivermectin. Y = tyrosine. F = phenylalanine. Genotype frequencies (YY = homozygous 200tyr; YF = heterozygous 200tyr/200phe; FF = homozygous 200phe) for different treatment groups were significantly different from each other (χ² = 106.5, P = 0.001). YY is associated with a resistance phenotype, and YF and FF code for the susceptibility phenotype. Untreated, n = 246; treated once, n = 118; treated twice, n = 36.

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