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Short communication

Molecular analysis of the β-tubulin gene of human hookworms as a basis for possible benzimidazole resistance on Pemba Island☆

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Infections caused by intestinal nematodes are responsible for significant morbidity both in human and in domestic animal populations [1,2]. Treatment of these infections relies mainly on a limited number of chemotherapeutic agents but in domestic animals, large-scale use, frequent treatment of confined populations, and improper dosing have led to the development of resistance against benzimidazoles (BZ), levamisole and ivermectin [3,4]. The benzimidazoles in particular are widely used in school-based deworming programmes for human gut nematodes [5]. Such biannual or triannual mass, single-dose treatments administered by schoolteachers reduce intensity of infection but have low cure rates for *Trichuris trichiura* and hookworms [6]. The increasingly widespread use of such programmes, including the addition of albendazole to the anti-filarial drugs ivermectin or diethylcarbamazin in the mass treatment Programme for the Elimination of Lymphatic Filariasis [7], raises the possibility of the selection of drug resistant strains of human gut nematodes. In Pemba Island, Zanzibar, a region in which regular school-based targeted BZ chemotherapy has been practised since 1994, we have recently observed reduced sensitivity of hookworms to mebendazole compared with pre-treatment campaign values or with efficacy data from the neighbouring island of Mafia [8,9]. The present study was to develop methods to look for molecular evidence of BZ drug resistance in this population.

BZ disrupt microtubule organisation and resistance is associated with reduced binding to tubulin [10]. Resistance in the sheep nematode *Haemonchus contortus* correlates with selection of strains possessing β-tubulin isotype 1 gene polymorphism [11–14] and resistance is believed to be caused by amino acid changes near the GTP binding domain, modulating BZ-β tubulin interaction [12]. Several amino acid differences have been reported [12], but Kwa et al. [15] identified a conserved mutation at amino acid 200, Phe (BZ-Sensitive)/Tyr (BZ-Resistant), which was correlated with resistance. Mutation at the same position has also been reported in BZ resistant strains of fungi [16,17] and other nematodes *Teladorsagia circumcincta* [18], *Caenorhabditis elegans* [19], *Trichostrongylus colubriformis* and *Ostertagia ostertagia* [20]. In selection experiments using *H. contortus* the resistant allele was found to be already present at a low frequency in the susceptible population but following the first round of selection the susceptible allele (Phe) was totally lost [20]. Subsequent rounds of selection for higher resistance resulted in loss of isotype 1 alleles and then loss of the isotype 2 locus. The early selection of Tyr at aa 200 implies that this mutation is a prerequisite for even low levels of resistance. In the present study we set out to determine if the aa 200 Phe → Tyr mutation could be demonstrated in the population of human hookworms recovered from standard 5 Pemban schoolchildren who had received 13 rounds of mebendazole treatment since starting school and so who we considered most likely to harbour resistant worms if these had arisen in the population. The methods described will allow monitoring for appearance of the aa 200 BZ resistance mutation during implementation of subsequent mass BZ treatment campaigns.

DNA sequence around the homologous aa 200 region of the human hookworm β-tubulin gene was obtained by

☆ *Note:* Nucleotide Sequence data reported in this paper have been deposited in the GenBank™ database with the accession number AF453524 (*Necator*) and AF453525 (*Ancylostoma*).

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RT–PCR (Fig. 1). The 606 bp sequence obtained, which was confirmed using a separate RT–PCR product, showed a high degree of homology to known β-tubulin 1 gene sequences. Compared with the *H. contortus* sequence there were 102 nucleotide differences and these resulted in two amino acid changes: aa 165 (Ser/Ala) and aa 284 (Leu/Ser) (numbering as in *H. contortus* sequence, GenBank accession no. M76493) [21]. At amino acid 200 the nucleotide sequence was TTC (Phe) corresponding to the BZ-sensitive allele of *H. contortus* and other nematodes [20].

To look for allelic variants, individual hookworms recovered from Std 5 children were preserved in 70% ethanol, speciated morphologically and DNA sequence around the aa 200 position was obtained as described in Fig. 1. In total 72 worms were processed and of these 71 were *Necator americanus* and only one *Ancylostoma duodenale*. Of the

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            N   N   W   A   K   G   H   Y   T   E   G   A   E   L   V   D   N   V   L   D   118
HW              AAT AAC TGG GCG AAG GGG CAC TAC ACT GAA GGA GCT GAG CTA GTC GAT AAT GTT CTT GAT
Hc      GGT AAC AAT TGG GCG AAG GGC CAC TAT ACT GAG GGA GCC GAG CTA GTT GAT AAC GTA TTA GAC   374

            V   V   R   K   E   A   E   G   C   D   C   L   Q   G   F   Q   L   T   H   S   L   139
HW      GTA GTT CGC AAA GAA GCT GAA GGA TGT GAC TGT CTC CAG GGC TTC CAA TTG ACC CAC TCT CTT
Hc      GTT GTC CGC AAA GAA GCT GAA GGT TGT GAT TGC CTT CAG GGC TTC CAA TTG ACG CAT TCA CTT   437

            G   G   G   T   G   S   G   M   G   T   L   L   I   S   K   I   R   E   E   Y   P   160
HW      GGA GGA GGT ACC GGA TCA GGA ATG GGA ACA CTT CTT ATT TCT AAA ATT CGG GAA GAA TAC CCT
Hc      GGA GGA GGC ACT GGA TCT GGA ATG GGC ACT TTG TTA ATT TCA AAA ATT CGT GAA GAG TAC CCT   500

            D   R   I   M   S   S   F   S   V   V   P   S   P   K   V   S   D   T   V   V   E   181
HW      GAT AGA ATC ATG TCC TCG TTT TCC GTT GTA CCC TCG CCA AAG GTT TCC GAC ACT GTG GTT GAG
Hc      GAT AGA ATT ATG GCT TCG TTC TCC GTT GTT CCA TCA CCC AAG GTA TCC GAC ACT GTC GTA GAA   563
                        A

            P   Y   N   A   T   L   S   V   H   Q   L   V   E   N   T   D   E   T   F   C   I   202
HW      CCA TAC AAT GCT ACA CTC TCT GTT CAC CAG TTA GTT GAG AAT ACA GAT GAG ACC TTC TGT ATT
Hc      CCC TAC AAT GCT ACC CTT TCC GTC CAT CAA CTG GTA GAG AAC ACC GAT GAA ACA TTC TGT ATT   626

            D   N   E   A   L   Y   D   I   C   F   R   T   L   K   L   T   N   P   T   Y   G   223
HW      GAT AAT GAA GCT TTG TAT GAT ATC TGC TTC CGA ACA TTG AAG CTT ACG AAC CCA ACA TAT GGA
Hc      GAC AAC GAA GCT CTG TAT GAT ATC TGC TTC CGC ACT TTG AAA CTC ACA AAT CCA ACC TAT GGA   689

            D   L   N   H   L   V   S   V   T   M   S   G   V   T   T   C   L   R   F   P   G   244
HW      GAT CTG AAT CAT CTT GTG TCT GTA ACA ATG TCT GGT GTT ACT ACC TGT CTT CGC TTC CCC GGT
Hc      GAT CTC AAC CAC CTT GTG TCT GTC ACA ATG TCT GGT GTC ACG ACC TGC CTT CGA TTC CCT GGA   752

            Q   L   N   A   D   L   R   K   L   A   V   N   M   V   P   F   P   R   L   H   F   265
HW      CAG TTG AAT GCT GAT CTT CGT AAG TTG GCT GTC AAC ATG GTT CCA TTC CCA CGT CTT CAC TTC
Hc      CAG CTG AAT GCT GAT CTT CGC AAG TTA GCC GTG AAC ATG GTT CCA TTC CCT CGT CTT CAC TTC   815

            F   M   P   G   F   A   P   L   S   A   K   G   A   Q   A   Y   R   A   L   T   V   286
HW      TTC ATG CCC GGT TTT GCT CCA CTC TCA GCT AAG GGT GCT CAA GCC TAC CGT GCT CTT ACT GTA
Hc      TTC ATG CCC GGT TTT GCT CCA CTG TCT GCA AAG GGT GCT CAA GCA TAT CGC GCT TCG ACA GTT   878
                                                                                S

            A   E   L   T   Q   Q   M   F   D   A   K   N   M   M                           300
HW      GCT GAA CTG ACT CAG CAG ATG TTT GAC GCA AAA AAC ATG ATG
Hc      GCT GAG CTT ACA CAG CAA ATG TTC GAT GCA AAG AAC ATG ATG                               920
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Fig. 1. Alignment of portion of the cDNA and deduced amino acid sequence of human hookworm (HW) β-tubulin 1 (AF453524) with portion of the cDNA sequence of *H. contortus* β-tubulin (Hc) (GenBank accession number M76493) [21]. Nucleotide differences are underlined and amino acid differences at positions 165 and 284 are indicated. The 606 bp of hookworm β-tubulin 1 cDNA sequence was obtained by RT–PCR. Hookworm eggs were purified by salt flotation of liquidized faeces from Standard 5 children, stored in RNA*Later* (Ambion) and total RNA prepared using Tri-reagent (Sigma). Degenerate primers were based on conserved regions of the β-tubulins from *H. contortus*, *T. colubriformis*, *T. circumcincta*, *Onchocerca volvulus*, *Brugia pahangi*, and *Ancylostoma caninum*. The 5′ primer (β-Tub 5′) was: 5′-AAC/T AAC/T TGG GCI AAA/G GGI CAC/T TA-3′ and the 3′ primer (β-Tub 3′) was: 5′-CAT CAT A/GTT C/TTT IGC G/ATC G/AAA-3′ (Amersham Pharmacia Biotech, UK) (corresponding respectively to nucleotides 315–338 and 900–920 of the *H. contortus* β-tubulin sequence). RT–PCR was carried out using the Superscript™ Preamplification System (GIBCO BRL) and the high fidelity, proof-reading enzyme HF2 (Clontech). The 603 bp cDNA obtained was subcloned into pGEM-T (Promega) and individual recombinants sequenced using automatic dye terminator sequencing [ABI PRISM Big Dye Terminator kit (PE Applied Biosystems, UK)]. Based on this sequence and on the position of introns in the *H. contortus* genomic sequence [14] 5′ and 3′ primers were designed (shown in bold) to allow PCR sequencing of a region flanking aa 200 (shaded) from individual hookworm gDNA. Individual hookworms were preserved in 70% ethanol and individually digested overnight at 42 °C in a 36 μl solution made by 25 μl 100 mM Tris, 50 mM EDTA buffer (pH 8.0), 1 μl Tween-20 and 10 μl 10 mg ml^{-1} Proteinase K. The digestion was terminated by heating at 95 °C for 1 h. PCR reactions (50 μl) contained 0.5 μl of worm digest, 0.4 μM primers, 0.3 μl Bio-X-Act (Bioline), Bio-X-Act buffer and 0.2 mM nucleotides. PCR conditions were 2 min 95 °C, 45 s 58 °C, 90 s 68 °C; then 34 cycles of 45 s 95 °C, 45 s 58 °C, and 90 s at 68 °C. PCR products were purified (Qiagen) and sequenced as above using the PCR primers.

Necator worms, 40 were sequenced in both directions and overlapping sequence between aa 190 and 210 was reliably obtained. The remainder were sequenced in the forward direction only. Within the region 190–210 the nucleotide sequence of all 71 of these worms was identical to the cDNA sequence. PCR product was also obtained from the single *A. duodenale* worm by the same process and several different PCR products were sequenced. Within the region, six nucleotide differences compared with the cDNA sequence in Fig. 1 were observed, 5 third base pair changes (C at 623, 629 and 680, G at 632 and A at 671) and one first base pair change (C at 636); none of which gave amino acid changes. Although based only on a single worm, this relatively large number of differences, together with the identity of the sequences of all the *Necator* worms sequenced with the cDNA sequence, demonstrates that the cDNA sequence corresponds to that of *N. americanus* β-tubulin.

Of particular note is the finding of TTC (Phe) in all of the worms at aa 200. The failure to find the codon TAC (Tyr) typical of BZ-resistant nematodes, indicates that, if present in the population, this allele is at a low frequency. To try to select for BZ resistant worms in vitro purified eggs were exposed to concentrations of thiabendazole that would kill 80% (ED_{80}) following incubation for 48 h (0.2 μg ml^{-1} [22]). This was done using a modified Baermann technique and the L1 larvae recovered were preserved in 70% ethanol. DNA extraction, PCR and sequencing was as described in Fig. 1 except that digestion was carried out in 3 μl and all of this used in the PCR. Of 16 larvae sequenced, all showed TTC (Phe) at aa 200.

In a further attempt to look for the TTC → TAC (Phe → Tyr) mutation at aa 200 we applied the PCR methods which had been developed for distinguishing Sensitive (TTC) from Resistant (TAC) *H. contortus* β-tubulin alleles, based on use of primers terminating at either the T, or the A of the second base of codon 200 [15]. Similar primers (Sens 5′ and Res 5′) were designed for *N. americanus* (Sens 5′ underlined in Fig. 1) and these used in PCR with the 3′ primer (bold in Fig. 1) using the same conditions as described in Fig. 1. The expected 102 bp band was obtained using the Sens 5′ primer but no product was visible using the Res 5′ primer. The resistance-specific PCR for *H. contortus* was able to detect the resistant allele at a frequency of down to 1% [20] and so the homologous mutation could be present in Pemban hookworms but probably at a frequency of less than 1%.

In conclusion, we have characterised the sequence surrounding aa 200 in *N. americanus* and *A. duodenale* and described methods for the PCR and sequencing of the region containing aa 200 from individual hookworms or larvae. The Phe–Tyr (TTC–TAC) mutation typical of resistant strains of other nematodes [20] was not found by sequencing individual worms derived from schoolchildren who had been repeatedly treated with mebendazole. Since single dose mebendazole only has around 30% cure rates even in populations not previously exposed to BZ [9], such repeatedly exposed children would be likely to harbour drug resistant worms if such drug selection had arisen in the population. The mutation was also not found in larvae selected in vitro using ED_{80} of thiabendazole nor by application of a PCR protocol similar to that developed for demonstrating the aa 200 Sens and Res alleles in other nematodes [20]. Therefore, the lower mebendazole efficacy observed in recent drug efficacy tests in Pemba [8] compared with the efficacy prior to the introduction of the school-based treatment programmes or those recently achieved on the adjacent island of Mafia [9] appear not to be due to selection of the typical mutation at aa 200. However, this should be investigated further using sensitive high-throughput methods for detection of single nucleotide polymorphisms such as use of sequence-specific oligonucleotide probes (SSOP) [23]. In addition, BZ resistance might arise in other ways: resistance has also been linked to other point mutations in, or deletions of, β-tubulin 1 [12,14,24–27]; other β-tubulin isotypes—yet to be characterised—may display differential sensitivities towards BZ [28,29]; microtubule associated proteins (MAPs) may be involved [30,31]. To have forewarning of which of these various mechanisms may develop/be developing in human hookworms it would be most valuable if BZ-resistant populations could be selected for molecular analysis from the laboratory maintained *N. americanus*/hamster cycle [32].

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