Available online at www.sciencedirect.com
ScienceDirect
Molecular & Biochemical Parasitology 156 (2007) 167–174
MOLECULAR & BIOCHEMICAL PARASITOLOGY

Characterization of beta-tubulin genes in hookworms and investigation of resistance-associated mutations using real-time PCR

Jan M. Schwenkenbecher[a,*], Marco Albonico[b], Quentin Bickle[c], Ray M. Kaplan[a]

[a] Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, 501 D.W. Brooks Drive, Athens, GA 30602, USA
[b] Ivo de Carneri Foundation, Milan, Italy
[c] Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, United Kingdom

Received 4 June 2007; received in revised form 24 July 2007; accepted 27 July 2007
Available online 3 August 2007

Abstract

Human hookworms (*Ancylostoma duodenale*, *Necator americanus*) are a major cause of malnutrition and anemia, particularly in children, and high worm burdens can lead to stunted growth and mental retardation. Mass drug administration (MDA) with benzimidazole (BZ) anthelmintics has the potential to greatly reduce morbidity and infection prevalence. However, such treatment strategies may apply significant selection pressure on resistance alleles. In several Strongylid parasites of livestock, resistance to BZ drugs is associated with single nucleotide polymorphisms (SNPs) in the beta-tubulin isotype-1 gene at codons 167 and 200. As an initial investigation into the possible development of BZ resistance in hookworms, we have cloned and sequenced the beta-tubulin isotype-1 genes of the canine hookworm *Ancylostoma caninum* and the two human hookworm species *A. duodenale* and *N. americanus*. The genomic sequences are highly conserved as evidenced by a similar structure of exons and introns; the 10 exons are of the same length in all three species and code for the same amino acids. The genomic sequences were then used to develop a real-time PCR assay for detecting polymorphisms in codons 167 and 200 in all three species. Hookworm specimens previously obtained from Pemba Island school children who had demonstrated a reduced response to treatment with mebendazole were then examined using the real-time PCR assay. None of the samples revealed significant levels of polymorphisms at these loci. If BZ resistance is present in the hookworm populations examined, the results do not support the hypothesis that changes in codons 167 and 200 of beta-tubulin isotype-1 are responsible for any resistance.
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Keywords: Hookworms; Beta-tubulin isotype-1; Genomic organization; Anthelmintic resistance; Real-time PCR

1. Introduction

Intestinal helminth infections cause significant morbidity both in humans and domestic animals. Hookworms are the second most prevalent intestinal helminth parasite of humans, infecting an estimated 740 million people in mostly underdeveloped countries throughout tropical and subtropical regions of the world [1]. Hookworms reside in the small intestine causing blood loss which may trigger iron deficiency anemia, especially in iron-deprived populations. In children, this can lead to nutritional deficiency, growth stunting, and cognitive deficits. Women of childbearing age are at risk of severe anemia, and in pregnant women, hookworm infection can cause severe damage to the mother and the unborn [2,3]. Hookworm infections in humans are caused by the species *Necator americanus* and *Ancylostoma duodenale*. Hookworms are also extremely important pathogens in dogs, with *Ancylostoma caninum* being the most prevalent species. Zoonotic infections with *A. caninum* in humans may produce a severe dermatologic condition known as cutaneous larva migrans, and less commonly, infection has been associated with an eosinophilic enteritis [4,5].

Human clinical studies have shown that treatment of at risk communities with anthelmintic drugs resulted in important health and nutritional benefits [6]. However, there are only a very limited number of different anthelmintic chemical classes. Hookworm disease is usually treated with anthelmintic drugs of the BZ class, which are safe, effective, cheap, and easy to administer in pill form [7]. BZ drugs are also used in the Global Program for the Elimination of Lymphatic Filariasis (GPELF), frequently in combination with ivermectin or diethyl-carbamizine (DEC). Mass treatment of communities for lymphatic filariasis (LF) has been shown to produce collateral

* Corresponding author. Tel.: +1 706 542 8447; fax: +1 706 542 5771.
E-mail address: jschwenk@vet.uga.edu (J.M. Schwenkenbecher).

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doi:10.1016/j.molbiopara.2007.07.019

benefit by dramatically reducing the incidence and intensity of infections with intestinal helminthes [8].

Treatment strategies that minimize refugia (proportion of parasite population unexposed to the drug treatment) are known to select very strongly for resistance [9]. Evidence of this is seen in gastrointestinal nematode parasites of livestock, which have developed multiple-resistance to all classes of anthelmintics [10]. Although the situation in humans is different due to other treatment schedules and higher refugia of the nematode populations, it can be assumed that drugs administered in community-wide approaches will apply selection pressure on resistance alleles in populations of human parasites. Classically, drug resistance is monitored by fecal egg counts, and recently progress has been made in the development of hookworm-specific egg hatch assays and larval development assays applicable under field conditions [11,12]. However, these classical methods can only detect drug resistance once resistance allele frequencies are at high levels and resistance becomes phenotypically expressed as reduced drug efficacy. Therefore, to prevent the development and spread of anthelmintic resistance in populations of human hookworms, thus ensuring that treatment programs will be sustainable, it is important to investigate the genetic basis for resistance.

BZ drugs act on nematodes by binding to beta-tubulin, causing interference with tubulin polymerization into microtubules [13–15]. It is generally accepted that mutations in the beta-tubulin isotype-1 are a major determinant of BZ resistance in many nematode species. A single non-synonymous mutation in codon 200 producing a phenylalanine (TTT/TTC) to tyrosine (TAT/TAC) substitution is strongly associated with BZ resistance in many veterinary parasites [10] and, recently, this same mutation has been demonstrated in the filarial parasite *Wuchereria bancrofti* after application of albendazole and ivermectin in the GPELF [16]. However, no substitution in codon position 200 was detected in hookworms from Pemba Island [17], which demonstrated a reduced response to treatment after receiving 13 rounds of mebendazole administration [18]. It is therefore possible that resistance in hookworms is caused by a mutation in codon position 167 [19], 198 [20] or in other positions that are not yet identified [21].

It is very difficult to obtain fresh hookworm specimens with intact RNA. Therefore, one must rely on DNA for use in molecular diagnostics. Consequently, a thorough knowledge of genomic sequences is needed in order to develop PCR-based assays to detect and monitor the presence of resistance-related polymorphisms. Here, we characterize the genomic sequences of beta-tubulin isotype-1 of three hookworm species and demonstrate the development and application of real-time PCR for monitoring sequence changes in codons known to be associated with BZ-resistance in other parasitic nematodes.

2. Materials and methods

2.1. Acquisition of parasite material

Adult *A. caninum* were collected at necropsy from random source dogs in Georgia, USA as previously described [22]. *N. americanus* adults, larvae and DNA prepared in a previous study [17] were received from the Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, UK. Adult *A. duodenale* were received from the University of Pennsylvania, USA.

2.2. Isolation of parasite DNA and RNA

DNA from adult and larval hookworms was isolated as previously described [22]. RNA from adult *A. caninum* was isolated using Trizol Reagent (Invitrogen, USA). Following a 10 min incubation at room temperature, the samples were extracted with chloroform, and RNA was precipitated with isopropanol. RNA pellets were then washed with 75% ethanol, resuspended in sterile water and stored at −80 °C.

2.3. PCR and sequencing

To obtain cDNA of the *A. caninum* beta-tubulin gene, RNA was reverse transcribed using the Omniscript RT Kit (QIAGEN, USA). Gene-specific primers derived from partially published sequences which appeared to be very conserved among nematodes [17] were used for amplification of the cDNA ends downstream or upstream from the specific primer annealing sites. To obtain the 3′-end of the cDNA rapid amplification of cDNA ends (RACE) was performed by annealing an oligonucleotide consisting of the M13F annealing site and a polyT-stretch (5′-GTAAAACGACGGCCAGTTTTTTTTTTTTTTTT-3′) to the polyA-site of the cDNA, and then using HW1 (5′-AATAACTGGGCGAAGGGGCACTA-3′) and the M13F-oligonucleotide as the reverse primer. For amplification of the 5′-end of the cDNA upstream from the known sequences, we used the forward primer SL1 (5′-GGTTTAATTACCCAAGTTTG-3′), which is specific for the splice leader sequence that is added to the mRNAs of many nematode species by trans-splicing [23], and HW2 (5′-CATCATGTTTTTGCGTCAAA-3′) as the reverse primer.

For PCR amplification of the genomic sequence of *A. caninum* beta-tubulin, primers were designed using the previously obtained cDNA-sequence. For large intronic sequences that could not be completely sequenced in a single reaction, additional sequencing reactions were performed using primers designed on the basis of the retrieved sequence. Based on the extremely high level of identity between the cDNA sequences of *A. duodenale* and *A. caninum*, primers designed for *A. caninum* beta-tubulin could be readily used for PCR amplification of the *A. duodenale* genomic sequence. For amplification of the *N. americanus* gene, primers designed for *A. caninum* and based on the published partial cDNA sequence of *N. americanus* [17] were used. Because the splice leader sequence is not part of the genomic sequence, sequences downstream of the first 20 bp of *A. caninum* DNA sequence were used to derive primers for the human hookworms.

All PCRs were performed using the HotStarTaq Mastermix Kit (QIAGEN, USA) as described previously [22]. PCR products were purified using Qiaquick columns (QIAGEN,

USA). Sequencing was performed at the Northwestern University Biotechnology Laboratory (Chicago, Illinois, USA).

2.4. Real-time PCR

Real-time PCR reactions were performed using the QuantiTect SYBR Green PCR Kit (QIAGEN, USA). Kinetic PCR reactions were run on a Stratagene Mx3000P real-time PCR system.

To determine whether polymorphisms associated with drug resistance can be detected by real-time PCR, control clones for the resistance alleles were designed using a site-directed positional mutagenesis approach. To produce a "resistance" SNP in codon position 167, the oligonucleotide 5′-GATAGAATCATGTCCTCGTATTCCGTTGTACCCTCGCC-′3 was used in PCR with reverse primers 5′-GCTGCAGTGAAGAAACCTCT-3′, 5′-TTACCAGTACTTCCTCAGGG-3′ and 5′-GATGGGCCTGCGCTGTGCCG-3′ for *N. americanus*, *A. duodenale* and *A. caninum*, respectively. For codon position 200 the oligonucleotide 5′-GAGAATACAGATGAGACCTACTGTATTGAT-3′ was used in PCR with reverse primer 5′-CATCATCTGGTCATCGAC-3′ for all three species.

To measure the allele specificity, PCR specific for SNPs of codons 167 and 200 were performed with primers specific for either the sensitive or resistant allele, differing in the last nucleotide, with a common reverse primer (Table 1). To test if the detection of resistance alleles works in assays with DNA prepared from diagnostic specimens, the "resistance" clones were used in dilutions of 1:100 and 1:1000 in reactions with genomic DNA.

Estimation of allele frequencies were done as described by [24]. Briefly, the difference in threshold cycles (Ct) between the two PCR reactions (ΔCt) is a measure of the allele frequency. A difference of one cycle means that the ration of the amount of one allele to the other is 1:2, or in general 1:$2^{\Delta Ct}$ [25]. Converting a ratio to a frequency by adding the numerator to the denominator results in

$$\text{Frequency of the allele} = \frac{100}{2^{\Delta Ct}+1} \quad \text{where} \quad \Delta Ct = (\text{Ct allele 1} - \text{Ct allele 2}).$$

The ΔCt of the different cycles were estimated as triplicates and averaged.

2.5. Bioinformatics

Sequences were analyzed using the basic local alignment of sequences tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST). The Alignment of the sequences and estimation of GC-contents were done in Bioedit (http://www.mbio.ncsu.edu/BioEdit). The exon-intron structure was retrieved by aligning the retrieved full length genomic sequence with the cDNA using SPIDEY (http://www.ncbi.nlm.nih.gov/spidey/). The translation of the retrieved coding DNA sequences into protein sequences was done using Transeq (http://www.ebi.ac.uk/emboss/transeq/). Searches for protein coding or functional sequences in intronic sequences were performed with Genescan (http://genes.mit.edu) and Grailexp (http://compbio.ornl.gov/grailexp).

3. Results

3.1. Cloning and sequencing of beta-tubulin cDNA of A. caninum

A. caninum was used for production of cDNA because this hookworm was the only species in which we had freshly preserved material containing good quality RNA. The 3′-RACE resulted in the amplification of an amplicon of 1150 bp. Sequencing and subsequent BLAST of the *A. caninum* beta-tubulin cDNA demonstrated very high identitiy to beta-tubulin isotype-1 sequences of other nematodes. Subsequently, the 5′-portion of the cDNA was amplified. The overall length of the cDNA was 1449 bp (including SL1, but not polyA). The translated region was 1344 bp, with 40 and 65 bp for 5′- and 3′-UTR, respectively (Fig. S1).

3.2. Generation of genomic sequences for three hookworm species

Primer sets designed from the cDNA-sequence were used to PCR amplify the genomic DNA sequences. Since the SL1 sequence is trans-spliced to the mRNA as part of post-

Table 1
Primers used for allele-specific real-time PCR in different hookworm species

Codon	Species	Primer name	Oligonucleotide sequence	T_{ann} (°C)	T_{diss} (°C)
167	*Necator americanus*	Forward: NA167res/sense Reverse: NARtr1	GATAGAATCATGTCCTCGTT/A GGGTGGTTCCAGGCTGATGC	58	80.4
	Ancylostoma duodenale	Forward: AD167res/sense Reverse: ADRtr1	GATAGGATTATGTCCTCGTT/A ACCAGTGTCTCGGCCTTTTC	58	81.4
	Ancylostoma caninum	Forward: AC167res/sense Reverse: ACRtr1	GATAGGATCATGTCCTCGTT/A GCTGGCGCCTTCGCCTTTTC	58	78.6
200	*N. americanus*	Forward: HW3/200res	GAGAATACAGATGAGACCTT		76.2
	A. duodenale	Reverse: HW4	AAGATGATTCAGATCTCCATA	55	75.5
	A. caninum				74.4

T_{ann}, primer annealing temperature; T_{diss}, dissociation point of PCR product.

trancriptional editing and not present in the genomic sequence, primers were derived from the 20 bp downstream of the SL1 site. Therefore, because primers were derived from the *A. caninum* cDNA, the first 40 bp may not show the real species-specific sequence for *A. duodenale* and *N. americanus*.

The longest genomic sequence was retrieved from *A. duodenale* (3889 bp), while the genes of *A. caninum* and *N. americanus* were 3613 and 3106 bp in length, respectively. Analysis of genomic organization revealed that in each species the beta-tubulin genes comprise 10 exons of exactly the same size for all three species ranging from 96 to 227 bp. All introns started with a GT at the 3′-acceptor and ended with an AG at the 5′-donor. The exon-intron boundaries were the same in all three species (Table 2). Most of the introns were small, with the shortest being 53 bp in *A. duodenale*. A major feature of all species is a large intron 4, which is 1536 bp in *A. duodenale*, 1215 bp in *A. caninum* and 765 bp in *N. americanus*, respectively (Fig. 1A). No functional domains could be found in the big intron. Of the nine introns, six were class 0 (where the splice occurs between the codons) and three were class 1 (where the intron interrupts the codon between the first and the second base) (Table 2).

In all three species, the GC-content was clearly higher in the exons varying from 49.37% (*N. americanus*) to 51.54% (*A. duodenale*) compared to the introns which varied from 40.64% (*N. americanus*) to 41.46% (*A. caninum*). The GC-content was particularly high in exon 10 in all three species. Overall, the beta-tubulin gene of *N. americanus* is more AT-rich compared to the other two species (Fig. 1B).

The genomic sequences of *A. duodenale* and *A. caninum* had a 92.1% identity, and the coding sequences were 97.2% identical. There was less identity between the two genera of hookworms. The coding sequence of *N. americanus* had 88.2% identity with *A. duodenale* and 86.8% identity with *A. caninum*, while the intron-containing sequences had only 62% and 62.6% identity, respectively. At the amino acid level, the sequences of all three species were 100% identical (the GenBank acknowledged *A. duodenale* sequence DQ055415 differed in two positions therefore we favored our own sequence for comparison).

3.3. Differences in codon usage bias among the species

The usage of degenerated codons for the identical amino acid sequence differed between the three species. A great identity was apparent between the two *Ancylostoma* species, where numbers of the same codon used differed maximally by one, except in TCT for serine with a difference of two. In contrast, eight amino acids differed strikingly between the two *Ancylostoma* species and *N. americanus*. In these, the codon usage corresponded to the GC-content that was estimated for the genes. Codons with higher GC-usage were present in *Ancylostoma* spp. for asparagine, glutamate, phenylalanine, isoleucine, threonine, leucine and serine. For lysine, the preferred codon in *N. americanus* had a higher GC-usage than in the other two species. Two codons were not used in any of the species, three codons were used in *N. americanus* but not in the *Ancylostoma* species, and one codon was used in the *Ancylostoma* species and not in *N. americanus* (Table S1).

3.4. Real-time PCR

To determine whether real-time PCR was able to detect mutations in codon positions 167 and 200 of beta-tubulin isotype-1, control clones for the resistance alleles were designed using a site-directed mutagenesis approach. Sequencing of these clones confirmed that we have produced the desired nucleotide changes (data not shown). These “resistant” clones were then tested in PCR which contained genomic DNA of each hookworm species. PCR reactions containing resistance controls and using primers specific for the resistant SNP amplified products with an earlier threshold as compared to PCR reactions using the same DNA templates but containing primers specific for the sensitive allele. PCR reactions containing genomic DNA only amplified at the same magnitude as the PCR containing resistant controls but sensitive allele primers (Fig. 2).

The dissociation curve of each SNP-specific PCR product showed species-specific melting points. The melting temperatures proved to be a function of the GC-content; for codon 167, *A. duodenale* had the highest and *N. americanus* the lowest temperatures. The same species-specific differences were also apparent for PCR of codon 200, but to a lesser degree. No differences could be observed between peaks of products of sensitive or resistance allele-specific PCR (Fig. 3).

This real-time PCR assay was then used to screen *N. americanus* samples from Pemba Island. Samples tested included 14 DNA samples from a previous study [17], DNA from 16 individual adult worms retrieved from the stools of five children after mebendazole treatment and DNA extracted from a pool of 400 larvae (8 pools of 50 each) derived from eggs recovered from

Table 2
Characterization of the 5′ and 3′ splice sites for the different hookworm species

Intron number	Phase	*N. americanus*	*A. duodenale*	*A. caninum*
1	0	AG/GT...AG/TT	AG/GT...AG/TT	AG/GT...AG/TT
2	1	CG/GT...AG/GA	TG/GT...AG/GA	TG/GT...AG/GA
3	0	AG/GT...AG/GG	AG/GT...AG/GG	AG/GT...AG/GG
4	0	AG/GT...AG/GT	AG/GT...AG/GT	AG/GT...AG/GT
5	1	TG/GT...AG/TG	CG/GT...AG/TG	CG/GT...AG/TG
6	0	AG/GT...AG/AT	AG/GT...AG/AT	AG/GT...AG/AT
7	0	GG/GT...AG/GA	GG/GT...AG/GA	GG/GT...AG/GA
8	1	TG/GT...AG/CC	CG/GT...AG/CT	AG/GT...AG/CT
9	0	AG/GT...AG/GA	AG/GT...AG/GA	AG/GT...AG/GA

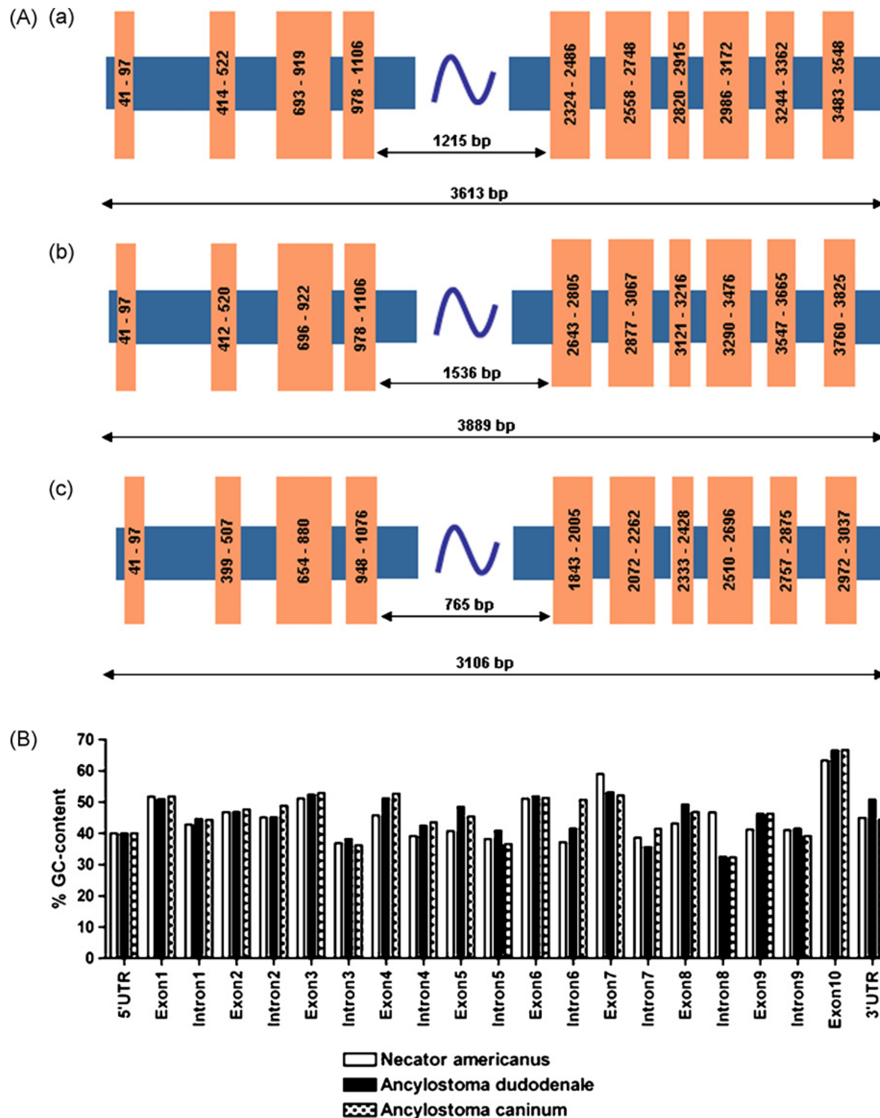

Fig. 1. (A) Genomic organization of *Ancylostoma caninum* (DQ459314), *Ancylostoma duodenale* (EF391850) and *Necator americanus* (EF392851) beta-tubulin isotype-1. The orange boxes present exons and the blue boxes introns. (B) Plot of GC-content for each coding and non-coding region.

pooled stools of several children passing high numbers of eggs after mebendazole treatment. For all samples tested the mean percentage of the susceptible SNP in codon position 200 was at nearly 100%. Values for codon 167 were also nearly 100% for the old DNA samples and DNA from the larval pool, but a slightly lower percentage of 99.54% was recorded for DNA from adult worms. The overall mean percentage of the resistance allele was 0.24% in codon 167 and 0.05% in codon 200 (Table 3). Such a small change from 100% is likely due to experimental variation and not due to the presence of these alleles.

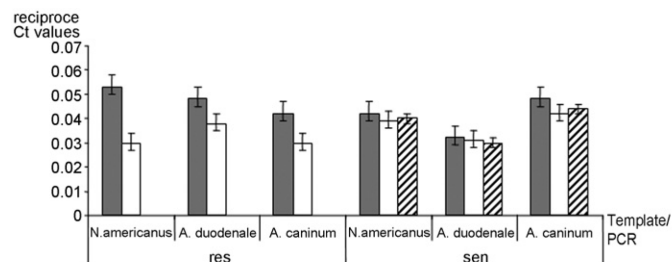

Fig. 2. Controls for detecting resistance-associated SNPs in real-time PCR. DNA of each hookworm species was used in reactions specific for the resistance allele (res) and specific for the sensitive allele (sen), with control clones containing the resistance-associated SNP in concentrations of 1:100 (full grey) and 1:1000 (white), and no control clone (diagonal stripes).

4. Discussion

Beta-tubulin genes are conserved over a wide range of species. Most previous investigations of individual nematode genes examined cDNA sequences while only few studies of genomic structures exist. In phylogenetic aspects, the extremely high identity of the *A. duodenale* and *A. caninum* genes down to the intronic sequences confirms the evolutionary proximity of the two species [26]. In contrast, the *N. americanus* sequence is less similar, suggesting a more distant relationship as might be expected by its designation in a different genus. The large intron

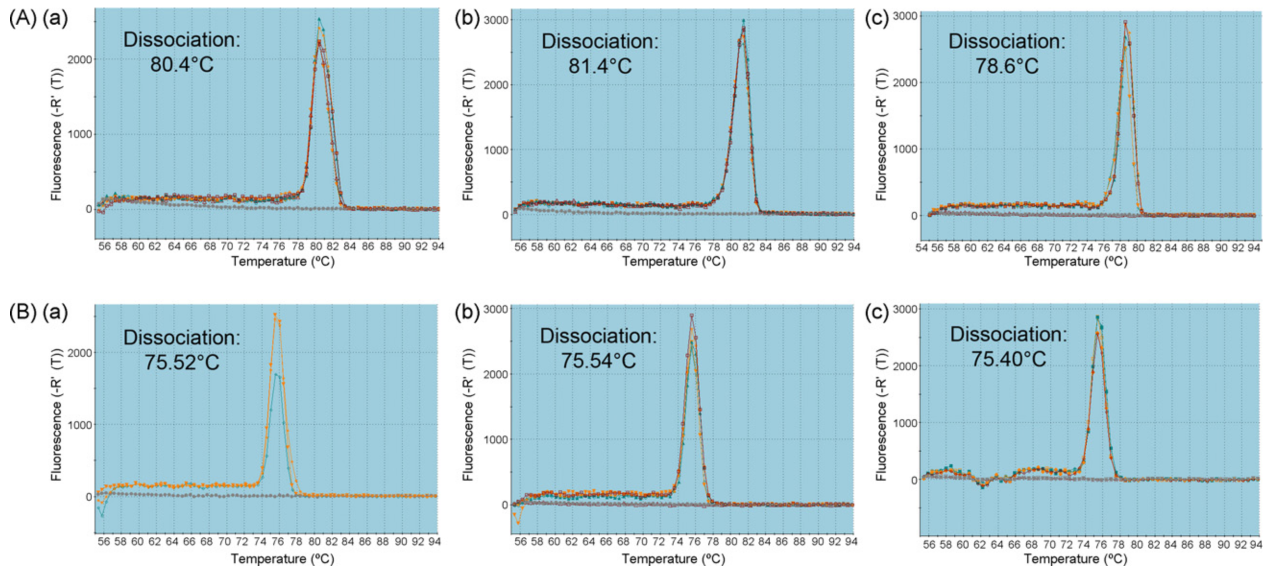

Fig. 3. Melting point plots to identify products of real-time PCR. (A) codon 167 and (B) codon 200: (a) *A. caninum*, (b) *A. duodenale*, (c) *N. americanus*.

4 appears to be a conserved structure in hookworm beta-tubulin, but the genomic structure appears not to be a general feature of nematodes. Nine introns were also found in the sheep nematode *Haemonchus contortus* [27]; however, eight introns were found in the horse nematode *Cylicocyclus nassatus* [21,28] and the dog filariid nematode *Brugia pahangi* [29], three in the free-living nematode *Caenorhabditis elegans* [30] and just one in the beta-tubulin genes of two *Strongyloides* species [31].

The GC content of the coding sequences of the beta-tubulin genes was similar to those of other nematodes [21,27,32], but is higher than in the average for coding sequences of hookworms [33]. The fact that the sequence of *N. americanus* is more AT-rich that the other two species investigated here is in accordance with other genes [33]. The difference in the GC-content between exons and non-coding sequences in all three species appears to be a feature of higher eukaryotes [34].

Selection of optimal codons can occur for translation efficiency and accuracy and favors similar sets of codons [35], which is also evident in the genes investigated here. The closely related species *A. duodenale* and *A. caninum* differ only slightly in codon usage, while differences of both species to *N. americanus* are obvious. This can be interpreted as a function of genetic drift based on local base composition [36], because the used codons are biased by a higher GC-content in the *Ancylostoma* species. In general, the codon usage pattern was in accordance with the pattern estimated for these species on expressed sequence tags [33,35]. However, clear differences were apparent for the codons of arginine, histidine, and serine, which were biased towards a higher GC-content in the beta-tubulin genes compared to the majority of coding sequences.

Selection of the resistance SNP at codon 200 (from phenylalanine to tyrosine) has been demonstrated in many nematode parasites of veterinary importance and, recently, in the human filarial nematode parasite *W. bancrofti* after only two rounds of treatment with albendazole [16]. However, sequencing of a small DNA fragment of the beta-tubulin gene which included the region of codon 200 in 71 *N. americanus* isolates did not reveal mutations in codon 200, even though these worms were recovered from children on Pemba Island in whom lower mebendazole efficacy was observed as compared to previous treatments [7,17]. Knowledge of the whole genomic sequence enabled us to develop a real-time PCR assay, which is a fast and reliable tool for analysis of hookworm populations for the presence of particular alleles. In this study, we confirmed the absence of

Table 3
Beta-tubulin allele frequencies at the 167 and 200 loci as determined by allele-specific real-time PCR (all samples of *N. americanus* were obtained from children on Pemba Island in an area where 13 rounds of mass mebendazole treatment had been administered)

Specimen	Mean allele frequency			
	167sen[a]	167res[b]	200sen	200res
14 DNA samples[c]	99.75 ± 0.25	0.25 ± 0.25	99.88 ± 0.12	0.12 ± 0.12
16 adult worms	99.54 ± 0.40	0.46 ± 0.40	99.99 ± 0.01	0.01 ± 0.01
400 larvae	99.99 ± 0.01	0.01 ± 0.01	99.99 ± 0.01	0.01 ± 0.01
Overall	99.76	0.24	99.95	0.05

[a] sen, PCR specific for sensitive allele.
[b] res, PCR specific for resistance allele.
[c] From a previous study.

the resistance-associated SNP in position 200. Previous studies performed in our laboratory and those of our collaborators have demonstrated that mutations in codon 167 are the principal beta-tubulin mutation found in BZ-resistant cyathostomin nematodes of horses, which are very closely related to hookworms [39]. The percentage of resistance alleles in codon position 167 in hookworm samples was slightly elevated compared to codon 200, but still very low. The values of the larval pool were close to 100% for the susceptible SNPs in both codons which suggests that any selection for mutations associated with BZ resistance may occur in either other parts of the beta-tubulin isotype I gene or within other, as yet undetermined, proteins. Another explanation of the very low level of resistance SNPs in codons 167 and 200 in hookworms could be that the mebendazole treatments, being focused only on children, confer a the level of treatment coverage sufficiently low so that adequate levels of refugia are being maintained in the hookworm population. This in turn may have prevented the accumulation of resistance alleles.

Recently, a BZ resistance-associated SNP in codon position 198 of beta-tubulin isotype-1 resulting in an amico acid change from glutamate to alanine was observed in *H. contortus* [20]. Within the region of codons 190–210 the nucleotide sequence of 71 worms from Pemba Island, DNA of which was partly used in this study, did not reveal nucleotide differences in codon position 198 [17]. At stringent annealing temperatures as used in this study mismatches in proximity of the 3′-end of the primer would have hampered the real-time PCR specific for the susceptible SNP in codon 200 and resulted in no or lesser product. We therefore think that we did not miss a mutation in codon 198 that could cause BZ resistance. In future studies, however, allele-specific real-time PCR assays should be used for surveillance of codon 198 and other positions which have proven to be involved in BZ resistance in fungi [37].

The anthelmintic drug ivermectin has been observed to select for particular alleles resulting in three amino acid changes in the H3 helix and deletions in the adjacent intron of the beta-tubulin gene of *Onchocerca volvulus*, and for a TTC → TAC change in codon 200 of the beta-tubulin gene of *H. contortus* [38]. Ivermectin has been widely applied in onchocerciasis elimination programs as well as in the GPELF. Ivermectin has a good efficacy against some human intestinal helminthes, such as *Ascaris* however, this drug has poor efficacy against human hookworms. Therefore, use of ivermectin for treatment of other parasites is unlikely to select for resistance alleles in hookworm beta-tubulin.

Drug resistance in parasitic nematodes of livestock is reaching alarming levels and subsequently is gaining increasing importance as an issue in animal health and well-being. This phenomenon should not be ignored in human parasites, particularly considering that mass drug administration programs, which are being used with increasing frequency, apply the type of selective pressure on nematode populations that veterinary parasitologists now strongly discourage in parasite control programs of livestock. Our initial investigations using a small number of clinical hookworm samples failed to demonstrate any polymorphisms in sites of the beta-tubulin gene known to be important in other parasitic nematodes. However, the genomic sequence data presented here provide the basis for investigation of the entire beta-tubulin isotype-1 genomic sequence, which is the only gene in which genetic polymorphisms associated with BZ resistance have so far been documented.

Acknowledgement

We thank Dr. Thomas Nolan from the University of Pennsylvania for providing samples of *A. duodenale*.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2007.07.019.

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