

CHARACTERIZATION OF β -TUBULIN cDNA FROM A BENZIMIDAZOLE RESISTANT *STRONGYLOIDES STERCORALIS* ISOLATE

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Abstract. Previous reports have indicated that benzimidazole resistance in gastrointestinal nematodes is linked to a mutation in the β -tubulin codons 167 and 200. In our study, total RNA was isolated from an albendazole-resistant (ABZR) *Strongyloides stercoralis* filariform larval isolate, followed by reverse transcription PCR that was amplified using primers designed according to the alignment of the β -tubulin mRNA of *S. stercoralis* (GenBank accession No. AY 898942). The cDNA sequence of the β -tubulin gene of ABZR *S. stercoralis* larvae revealed 100% identity with the sequence of *S. stercoralis* (AY 898942). The polymorphisms at codons 167 and 200 encoded phenylalanine (Phe). The resistance mechanisms for benzimidazole in *S. stercoralis* were discussed in the light of these results.

INTRODUCTION

Strongyloidiasis is a serious threat to public health in tropical and subtropical areas (Grove, 1996). The disease is caused by infection with *Strongyloides stercoralis*, a nematode that infects several million people worldwide (Genta, 1989). The clinical spectrum of strongyloidiasis varies from asymptomatic infection, to mild symptomatic abdominal and skin diseases, to fatal disseminated infection in immune-suppressed patients (Grove, 1996; Pearson, 2002; Lewthwaite *et al*, 2005). Effective treatment is therefore important to prevent severe infection. Albendazole is the principal drug used to treat human strongyloidiasis (Horton, 2000). However, the efficacy of the treatment of strongyloidiasis with albendazole, a benzimidazole derivative, has been inconsistent and may be never complete (Horton, 2000; Nontasut *et al*, 2005; Singthong *et al*, 2006). Benzimidazoles inhibit the polymerization of the tubulin dimers that comprise microtubules, and as such obstruct vital cellular functions, including cell division (Dumontet and Sikic, 1999).

Many investigators have reported that

nematodes that have infected humans may be resistant to benzimidazoles (Hoti *et al*, 2003; Albonico *et al*, 2004; Schwab *et al*, 2005), and the resistance has been associated with mutations in the β -tubulin (Elard *et al*, 1999). These mutations are supposed to change the structure of the β -tubulin protein, decreasing the β -tubulin-benzimidazole interaction that causes the antimitotic effect of the drug (Robinson *et al*, 2004). One point mutation in position 200 of the amino acid sequence is a phenylalanine (Phe) to tyrosine (Tyr) transition (Prichard, 2001). Another mutation in position 167 is a Phe-Tyr substitution (Silvestre and Cabaret, 2002). Robinson *et al* (2004) hypothesized that a number of mutations that caused substitution in a protein at a crucial junction between its N-terminal and intermediate domains may also confer resistance to benzimidazoles. Recently, the β -tubulin cDNA and genomic DNA of *S. stercoralis* have been cloned and sequenced (Melville *et al*, 2006). Here, we have sequenced β -tubulin cDNA from a benzimidazole resistant *S. stercoralis* isolate. This sequenced data were compared with the β -tubulin mRNA of *S. stercoralis* (GenBank accession No. AY 898942) in order to determine whether the genetic change causing the resistance would be present in other nematodes.

MATERIALS AND METHODS

Preparation of parasite RNA

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An albendazole-resistant isolate of *Strongyloides stercoralis* infective-stage larvae was harvested from stool samples of a hyperinfected strongyloidiasis patient who did not respond to repeated treatment with albendazole. The subject received 800 mg albendazole (Zentel, GlaxoSmithkline Australia) per day, orally after meals, twice daily for three consecutive days; the same dose was repeated again seven days later. The drug efficacy was evaluated by parasitological examination using filter paper culture technique (Beaver *et al*, 1984) on day 14, after the second round of treatment. The surviving larvae were used for molecular analysis of β -tubulin cDNA. Approximately 30,000 worms were pooled and extracted for RNA in Trizol reagent (Invitrogen, Carlsbad, CA) using the manufacturer's protocol.

RT-PCR and sequencing

Complementary DNA was generated using two pairs of gene-specific primers (Table 1) with the Robust II RT-PCR Kit (Finnzymes, Keilaranta, Espoo, Finland) following the manufacturer's instructions. All PCR products were sequenced in both directions by the dideoxynucleotide chain termination method, using the DYEnamic ET Dye terminator cycle sequencing Kit (Amersham Biosciences, Piscataway, NJ) and the MegaBACE DNA Analysis system (Amersham Biosciences). RNA sequenced data were searched against the GenBank data base using NCBI and BLASTN algorithms to assess their similarity to previously characterized β -tubulin mRNA sequences. The alignment of the sequences was carried out

using the ClustalW program (<http://www.ebi.ac.uk/clustalW>).

RESULTS

Four *S. stercoralis* β -tubulin primers (Ss22F1, Ss662F2, Ss703R1 and Ss1329R2) were designed from the *S. stercoralis* β -tubulin mRNA sequence (AY 898942) (Table 1). Primers Ss22F1 and Ss703R1, as well as primers Ss662F2 and Ss1329R2, were used as pairs in a one-step

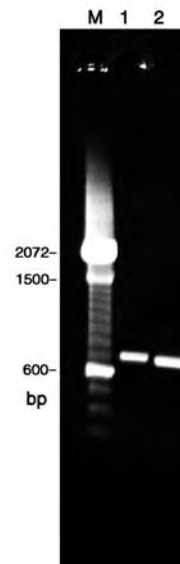


Fig 1- Agarose gel electrophoresis of products from *S. stercoralis* β -tubulin PCRs. Lane 1, RT-PCR with primers Ss22 F1 and Ss703R1; lane 2, RT-PCR with primers Ss662 F2 and Ss1329R2; lane M, 100 bp ladder.

Table 1
Primers employed in characterizing *S. stercoralis* β -tubulin cDNA.

Forward primers	Position (first)	Sequence
Ss22 F1	Ss cDNA Forward strand 1	ATGAGAGAAATTGTTACAGTCC
Ss662 F2	Ss cDNA Forward strand 641	GAACACTCAAGCTTAGTTACACC
Reverse primers	Position (last)	Sequence
Ss703R1	Ss cDNA Reverse strand 723	GAGGCATGTAGTTACACCAGA
Ss1329R2	Ss cDNA Reverse strand 1350	CTTCAGCTATTCTCTTCAGCA

Ss = *S. stercoralis*

reverse transcription PCR to specifically generate and amplify *S. stercoralis* β -tubulin cDNA from a total parasite RNA. The PCR reactions yielded a visible product by agarose gel electrophoresis (Fig 1). An amplicon cDNA of 723 bp was obtained in the Ss22F1-Ss703R1 cycle, and an amplicon cDNA of 710 bp was obtained by Ss662F2-Ss1329R2. The combined coding sequence from the two amplicons, the DNA sequence, and the deduced amino acid sequence revealed 100% identity with the *S. stercoralis* β -tubulin mRNA sequence (AY 898942) (Fig 2). The codons 167 and 200 encoded Phe.

DISCUSSION

The β -tubulin is the target of the benzimidazoles, which are broad-spectrum anthelmintics used to control parasitic helminthes in ruminants (Pape *et al.*, 1999). Here, we described the sequenced data of β -tubulin cDNA of a benzimidazole-resistant *S. stercoralis* isolate by direct sequencing of the PCR products. The hypothesis was that this could help to detect resistance because *Strongyloides* also likely has a single β -tubulin gene in the *S. ratti* genome (Melville *et al.*, 2006). However, the present molecular analysis indicated that codons 167 and 200 encode Phe. This evidence suggests that this polymorphism is not the only reason for the development of benzimidazole resistance in *S. stercoralis*. The development of resistance also possibly evolved through other mechanisms. Different modes of inheritance are probably responsible for the development of resistance in different populations (Prichard, 2001). The low cure rate of *S. stercoralis* may be attributable to the habitat of these worms, the gastrointestinal tract, where the active drug concentration is low (Melville *et al.*, 2006). Other reasons for treatment failures are autoinfection, dissemination, and a variation in the activity of the drug efflux. Several mechanisms of drug resistance are reported in veterinary helminths, such as (1) a change in the molecular structure of the target molecule of the drug such that the drug ineffectively recognizes the target, (2) modification of metabolism resulting in inactivated or completely removed drugs, (3) changes in the drug distribution to the target, and

(4) an extension of the target genes to overcome the drug action (Wolstenholme *et al.*, 2004).

We will continue this investigation with a single worm PCR, genotyping the site that is associated with benzimidazole-susceptibility and resistance in different worm populations. This method can be used to document the resistance status of parasite populations. This result possibly leads to a better understanding of the biology of the parasite: why they become resistant and how the development of resistance could be controlled.

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