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

Wittaya Luadthong^{1,2,3}, Pewpan M Intapan^{1,3}, Thidarat K Prasongdee¹, Chaisiri Wongkham¹, Viraphong Lulitanond^{1,3}, Kanongwan J Imtawil¹ and Wanchai Maleewong^{1,3}

¹Faculty of Medicine, ²Graduate School, ³Research and Diagnostic Center for Emerging Infectious Diseases, Khon Kaen University, Khon Kaen, Thailand

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 immunesuppressed 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

Correspondence: Pewpan M Intapan, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand. Tel: 66-043-348387; Fax 66-043-202475 E-mail: pewpan@kku.ac.th 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 B-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

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 -tubulin cDNA. Approximate 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 -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.

Forward primers	Position (first)	Sequence
Ss22 F1	Ss cDNA Forward strand 1	ATGAGAGAAATTGTTCACGTCC
Ss662 F2	Ss cDNA Forward strand 641	GAACACTCAAGCTTAGTTCACC
Reverse primers	Position (last)	Sequence
Ss703R1	Ss cDNA Reverse strand 723	GAGGCATGTAGTTACACCAGA
Ss1329R2	Ss cDNA Reverse strand 1350	CTTCAGCTATTCCTCTTCAGCA

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

Ss = S. stercoralis

Characterization Of S. stercoralis β -tubulin cDNA

	WEETVUV CTG OFS BOTS LTF
A7898542	ATGRIADARS PROFESSION CLARENCES: CANTERING A CLARENCES RECTARTER OF ATTRIBUTED TOPOLOGY CLARENCE: CANTERING A TRANSPORT RECTARTER OF
AT191242	W E N I S E E H G I V S 2 6 E F V G D W
A32-208	TOGUAAGTTA TETENGANGA ACACOGTATE CANAGTGACG GATEATITGE TOGAGACHAE 110
	I B R C S B R I B V Y Y Y R A S G R Y.
AXII10342	TETHATSAAT GERVERATAD RATADACOTE TACTACAATS ARGETAATES ADGRARATAT 190
And here	TETHATAAAT GELYCHATAN AATAJACSTE TACTAEAATS AASETAATSI ASEAAAATAT ING
	VPA AVHV DLE POT KDET KEE
ATR92942	STEELACOTE CEGTATORE TEATTERIAS COMPARAMENTE TOGETCOTES 240
HDA-BEN	HALLAND CONTRACT TO A CONTRACT OF A CONTRACT
	TTO DLFR PDH FYF GDB 3 A GH
A12-246	ACATATORIAS ASCINCTURE ACCTORCARE THEOTOTTE SACANCING DEMONANCE 300
7.7865647	N W A K G H Y T E D A E L V E S V M D V
A32-2x3	ANTTOGOCTA ANOSTCACTA CACCOANSOT GOODAATTAS TEGRAAACOT TATGGATOTT J60

AVESTORY	TATALANAN AND COLOR L 2 G F 2 L T H 3 L
Abz-teb	STINIANANG ANGCOMANG ATSTINTTOC CTOCANGET TOCANCYTAC ACACTUTETY 420
AY895912	SUBSTOUTS CONSTITUTE TATCOGRACT CIDETTRITT COMMATICS TOMMANING AND
ABZ-GeB	GEADSTREET CTGGATCTOG TATGOGAACT CIACTTATTI CCAAGAITOS TGAAGAATAG 480
47891947	CERGRERGER TERMOVATE TIMECTORE VIEWEATER: CREAKINGTE TERMACRITE SAG
A32-1143	CLAURERGRA TERMONORY TERMETERSTC PERCERTICAL CRAREGEPTS TERCHCRETY SEE
	VERVER LEVERTERT
A7890352	SPINARCAT REARACCAE TENDETUTE CACCARITYS TEGRAPHERS CONTRARGES FOR
A#2-282.	UTURACUNT KIRRIGCON: TITUICIUTI CASCANITIS TIGAAANINI CONTURAAN NOV
	FCIDREALVDICE KTLK 199
SPEDGOXA	TRUBUTATUS ACAATGANGE TUTATACGAT ATAUGETEA GAACACICAA GEVINGITEA 660
ADTINE	TREPUTATES ADAMSAGE TERMERGER ADATGETTER GRACACTERS SETTIOTES AND
	PET GOLS HLV SRT RESV TTC
AV891542	DERECTIVE GAGATERIAA TOATERINE PERATURGA TOTERCHET ARCTREATOR 720
AFZ-DER.	CARCERES SAGALESAN TENETTYPE TEARTUREAN TYLEBORNE ANTACANSE 725
	SKI PGQS HAD LAK LAVH NVF
AY898342	CTEDOTITOL CROGACANTT ANACOCIDAL CITABLARAGE TROUTOTTAN CATOUTACCT THE
N22-1228	COLONING CARDENET AND COLORE CONTRACTOR PROVIDE CONTRACTOR
Quere a la	FFR LHTE HPG FAF LAAL CVE
57898842 55745-3	TROUGACITE TRACTICIT TATGOCAGIA TITOCTECTE TROCTOCECE TOPTOTETET \$40
and the second	COLUMN ADDRESS COLUMN ADDRESS ADDRESS COLUMN
*****	AVE BLTVPRLTOCHTEANNH
ANS-ISIN	RETRICATE CECTAGATE CONSTANT CONSTANT ACCOUNT ACTIVATION CANADATATE SO
	********** ****************************
AVERENTS.	NAACDPENERGARG ACCORDENTS TREPORTATIONS
大百二十四日	MEGGETGETE GEGATECANE ACATOGANEA TATCTIACTE TECCTUTAT CITCACAGEA 940
	mannar mannar mannar mannar mannar manna
AV898947	CARACTERCTA TEAGAGAAGT TEACGAACAA ATGATETCTA TICAACAGAA AAATTCTCCA 1970
ABD-Reh	CAANTUTCTE TEAGAGAAGE TUACGAAGAA ATGATGTOTE TTOAACAGAA AAATTCTUCA 1939
	V N N E N F D N N V P N N P P N N P N N N N N N N N N
AX895312	TACTITUTSE ANTOGATICE ANATALISTI MAGACISCIE TOTOTGACAT ISCHOCAMAA 1940
ANI-SNA	TREFFICITS ARTOSATICE ARMERATOTI ANGRETSETS TETERERAT TOCHCERARA 1980
	ULE NJAT FIG NTT ALGE CER
234: 32UNA	INFACTION OF THE CONTRACTOR ANCACTACAS CTATTENANA ADJUTICANA 2140
あたビージャボ	GGACTIAAGA TOTCIGCIAE CUTVATIGGA AACACTACAS CIAITCAAGA ATOCITCAAA 1140
	NEB BOFT ART FRA AFRINTT
AT096941	OFFATTETS ANDARTTIAC TOCTATOTY ADAMBADAD CITICATION TEGETACACT 1200
N82-183	CENTITUTE ANCANTTAL DECEMBER, ASAASAAAS CITICATACA TROUBLEST 1200
	VICERBERTERESEN
AY89E941	OUTUARDUTA TEGRTURIAT UGANTITACT GARGTURGE CTARCHIGHA TERICTIVIT 12:0
NUS-SER.	ALTERNATION CONCERNMENT SUBALITURES INFORMATION TRACKING THE STREET LINE
100 million (1990)	AFT QUYU KATADA TOBATA
81090942	GUTIAUTAVE AACAAVATUR RUARECEACE GUTUAVEAUT RUAREATED TERUVAVEAR 1320
Non-July	PROFESSION PROPERTY AND
	H. T. V. A. T. T. T.
AY892.942 AV1-645	DIMERANTIN CTURMERINE ATMOSTING AND
Section of the sectio	101111111 111111111 111111111
AVIENERS	ATOPIATAAT GENETAAITA TITCATAAAT OTTATTTCC TAATATZAAA CTATTATCIT 1440
104-148	
A7096942	GEARAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AU2+220	

Fig 2- Alignment of a benzimidazole-resistant *S. stercoralis* β-tubulin sequence (ABZ-SsR) and mRNA of a *S. stercoralis* sequence (GenBank accession No. AY 898942). Codons corresponding to amino acids 167 and 200 are emphasized with bold and italic text. Identity between the two sequences is indicated by asterisks. Stop codon is indicated by underlined text.

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.

ACKNOWLEDGEMENTS

The study was supported by grants from Khon Kaen University, and the Research and Diagnostic Center for Emerging Infectious Diseases, Khon Kaen University. We thank Markus Roselieb for improving the English-language presentation of the manuscript.

REFERENCES

- Albonico M, Wright V, Bickle Q. Molecular analysis of the beta-tubulin gene of human hookworms as a basis for possible benzimidazole resistance on Pemba Island. *Mol Biochem Parasitol* 2004;134:281-4.
- Beaver PC, Jung RC, Cupp EW. Clinical parasitology. 9th ed. Philadelphia: Lea and Febiger, 1984:825pp.
- Dumontet C, Sikic BI. Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. *J Clin Oncol* 1999;17:1061-70.
- Elard L, Cabaret J, Humbert JF. PCR diagnosis of benzimidazole-susceptibility or -resistance in natural populations of the small ruminant parasite, *Teladorsagia circumcincta*. *Vet Parasitol* 1999;80:231-7.
- Genta RM. Global prevalence of strongyloidiasis: critical review with epidemiologic insights into the prevention of disseminated disease. *Rev Infect Dis* 1989; 11: 755-67.
- Grove DI. Human strongyloidiasis. *Adv Parasitol* 1996;38:251-309.

- Horton J. Albendazole: a review of anthelmintic efficacy and safety in humans. *Parasitology* 2000;121 (suppl): S113-32.
- Hoti SL, Subramaniyan K, Das PK. Detection of codon for amino acid 200 in isotype 1 betatubulin gene of *Wuchereria bancrofti* isolates, implicated in resistance to benzimidazoles in other nematodes. *Acta Trop* 2003;88:77-81.
- Lewthwaite P, Gill GV, Hart CA, Beeching NJ. Gastrointestinal parasites in the immunocompromised. *Curr Opin Infect Dis* 2005;18:427-35.
- Melville LA, Sykes AM, McCarthy JS. The betatubulin genes of two *Strongyloides* species. *Exp Parasitol* 2006;112:144-51.
- Nontasut P, Muennoo C, Sa-nguankiat S, Fongsri S, Vichit A. Prevalence of *Strongyloides* in northern Thailand and treatment with ivermectin vs albendazole. *Southeast Asian J Trop Med Public Health* 2005;36:442-4.
- Pape M, von Samson-Himmelstjerna G, Schnieder T. Characterisation of the beta-tubulin gene of Cylicocyclus nassatus? *Int J Parasitol* 1999; 29:1941-7.
- Pearson RD. An update on the geohelminths: Ascaris lumbricoides, hookworms, Trichuris trichiura, and Strongyloides stercoralis. Curr Infect Dis Rep 2002;4:59-64.

Prichard R. Genetic variability following selection

of Haemonchus contortus with anthelmintics. *Trends Parasitol* 2001;17:445-53.

- Robinson MW, McFerran N, Trudgett A, Hoey L, Fairweather I. A possible model of benzimidazole binding to beta-tubulin disclosed by invoking an inter-domain movement. J Mol Graph Model 2004;23: 275-84.
- Schwab AE, Boakye DA, Kyelem D, Prichard RK. Detection of benzimidazole resistanceassociated mutations in the filarial nematode *Wuchereria bancrofti* and evidence for selection by albendazole and ivermectin combination treatment. Am J Trop Med Hyg 2005;73:234-8.
- Singthong S, Intapan MP, Wongsaroj T, Maleewong W. Randomized comparative trial of two high dose albendazole regimens for uncomplicated human strongyloidiasis. *Southeast Asian J Trop Med Public Health* 2006;37 (suppl 3):32-4.
- Silvestre A, Cabaret J. Mutation in position 167 of isotype 1 beta-tubulin gene of Trichostrongylid nematodes: role in benzimidazole resistance? *Mol Biochem Parasitol* 2002;120:297-300.
- Wolstenholme AJ, Fairweather I, Prichard R, von Samson-Himmelstjerna G, Sangster NC. Drug resistance in veterinary helminths. *Trends Parasitol* 2004;20:469-76.