

## Analysis of the $\beta$ -Tubulin Genes from *Enterocytozoon bienersi* Isolates from a Human and Rhesus Macaque

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**ABSTRACT.** *Enterocytozoon bienersi* is the most common and clinically significant microsporidium associated with chronic diarrhea and wasting in immunocompromised humans. Albendazole, which is effective against several helminths, protozoa, and microsporidia, is relatively ineffective against infections due to *E. bienersi*. A likely explanation for the observed clinical resistance to albendazole was discovered from sequence analysis of the *E. bienersi*  $\beta$ -tubulin from isolates from an infected human and a naturally infected rhesus macaque. The  $\beta$ -tubulin of *E. bienersi* has a substitution at Glu<sub>198</sub>, which is one of six amino acids reported to be associated with benzimidazole sensitivity.

**Key Words.** Albendazole resistance, beta-tubulin gene, microsporidia.

THE microsporidia represent a large and diverse group of obligate intracellular eukaryotic parasites and to date, 1,200 species have been identified. Of these, *Enterocytozoon bienersi* is the most clinically significant species associated with AIDS-related human microsporidiosis (reviewed in Cali 1991; Curry and Canning 1993; Didier et al. 2004; Keeling and Fast 2002; Mathis, Weber, and Deplazes 2005; Wittner 1999), with symptoms including chronic diarrhea, wasting, and malabsorption. *Enterocytozoon bienersi* has also been identified in immunocompetent patients (Albrecht and Sobottka 1997; Gainzarain et al. 1998; Sandfort et al. 1994), in individuals receiving immunosuppressive therapy (Guerard et al. 1999; Rabodonirina et al. 1996), and in macaques, both immunocompetent and those infected with simian immunodeficiency virus (SIV) (Green et al. 2004; Mansfield et al. 1997).

Microtubules, which are formed by polymerization of the  $\alpha$ - and  $\beta$ -tubulin subunits, are a major component of the mitotic spindle. The benzimidazoles have been found to prevent both the polymerization of the tubulin subunits by binding to the  $\beta$ -tubulin subunit, thus preventing elongation of the microtubules, and depolymerization of the two subunits. The benzimidazoles are toxic to fungi and helminths (Davidse 1986; Lacey 1988), and have been used to effectively treat both microsporidiosis due to *Encephalitozoon* spp. (De Groote et al. 1995; Katiyar and Edlind 1997; Molina et al. 1998; Ridoux and Drancourt 1998), and some protozoan infections including *Giardia intestinalis* (Katiyar et al. 1994; Lemee et al. 2000). However, these benzimidazole derivatives, including albendazole, are relatively ineffective against *E. bienersi* infections (Blanshard et al. 1992; Contreas et al. 2000; Dieterich et al. 1994).

In this communication, we report the analysis of the  $\beta$ -tubulin gene from *E. bienersi* isolated from an HIV-infected human and an SIV-infected rhesus macaque (*Macaca mulatta*). The sequence data provide an explanation for the reported resistance of *E. bienersi* to albendazole.

### MATERIALS AND METHODS

**Parasite strains.** An HIV-positive adult patient admitted to the Mulago Hospital in Kampala, Uganda, and an SIV-infected rhesus macaque (*Macaca mulatta*), housed at the New England Regional Primate Research Center (Southborough, MA), were found to be

positive for *Enterocytozoon bienersi*. Spores were purified from the human and rhesus macaque stool samples (Sheoran et al. 2005; Zhang et al. 2005), and were confirmed to be *E. bienersi* by electron microscopy and sequencing of the internal transcribed spacer (ITS) of the small subunit rRNA gene. The spores isolated from the human patient and rhesus macaque will be referred to as the H206 isolate and M231 isolate, respectively.

**Cloning and sequencing of the  $\beta$ -tubulin genes.** The  $\beta$ -tubulin gene from the M231 isolate was first isolated from a whole genome amplified (Molecular Staging Inc., New Haven, CT) library cloned into the pHCsmart-Kan vector (Lucigen Corp., Middleton, WI). The *E. bienersi*  $\beta$ -tubulin genes from the H206 and M231 isolates were then amplified from purified genomic DNA (1 ng; DNeasy Tissue Kit; Qiagen Inc., Valencia, CA) using primers, MEB btub-I (5'-AACGGGCAGCTGAGTAGTTTAAGTGATT-3') and MEB btub-J (5'-AATAATCATAACATGACTGGAACCGTGCTAA-3') using the Expand High Fidelity PCR System (Roche Diagnostics Corp., Indianapolis, IN). The PCR products were cloned into pCR4TOPO (Invitrogen Corp., Carlsbad, CA) and used to transform *E. coli* TOP10 cells. Inserts from four independent clones were double-strand sequenced for each isolate.

### RESULTS AND DISCUSSION

**Analysis of the  $\beta$ -tubulin of *Enterocytozoon bienersi*.** The *E. bienersi*  $\beta$ -tubulin gene was first identified in the M231 amplified genomic library. One clone, with an open reading frame of 1,314 bp, had significant similarity ( $E$ -value =  $e^{-177}$ ) to other  $\beta$ -tubulin sequences in the NCBI database using the BLASTp program (Altschul et al. 1990; McGinnis and Madden 2004). PCR primers, MEB btub-I and MEB-btub-J, were designed to amplify the  $\beta$ -tubulin gene from purified spores isolated from a human patient (H206) and a rhesus macaque (M231). These genes were compared using the ClustalW algorithm (Thompson, Higgins, and Gibson 1994). Both genes were 1,314 bp and were identical except for five synonymous transitions (99.62% sequence identity). The A-T content was 55.4% and 55.6% for the M231 and H206  $\beta$ -tubulin genes, respectively. Analysis of the 5'—and 3'—flanking regions revealed no obvious canonical elements, such as promoter elements or polyadenylation sequences.

The *E. bienersi*  $\beta$ -tubulin gene encoded a 438-amino acid polypeptide with a molecular weight of 49,116 daltons. A high degree of sequence identity (68%–73%) was observed between the *E. bienersi*  $\beta$ -tubulin and  $\beta$ -tubulins from the family Encephalitozoonidae, *Antonospora locustae*, *Trachipleistophora hominis*, *Saccharomyces cerevisiae*, and *Homo sapiens*. However, the

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sensitivity, and changes of one or both of these amino acids results in resistance to benzimidazole. The *E. bienersi*  $\beta$ -tubulin data are consistent with these observations.

The microsporidia have been placed within the fungal clade based on phylogenetic analyses of the  $\alpha$ - and  $\beta$ -tubulin genes (Edlind et al. 1996; Keeling and Doolittle 1996), sequencing of the *E. cuniculi* genome (Katinka et al. 2001; Vivares et al. 2002), the existence of relic mitochondrial genes in the nuclear genome, and the presence of mitochondria and Golgi-like membranes. In addition, morphological and life cycle data are consistent with their placement within the fungal clade (reviewed in Germot, Philippe, and Le Guyader 1997; Keeling and Doolittle 1996). We attempted to align the *E. bienersi*  $\beta$ -tubulin to 67 other  $\beta$ -tubulin sequences representative of the major eukaryotic groups, and then use maximum likelihood methods (TREE-PUZZLE 5.2 and PHYML; Guindon and Gascuel 2003; Schmidt et al. 2002) to determine the phylogenetic position of *E. bienersi*. Unfortunately, the *E. bienersi*  $\beta$ -tubulin sequence was found to be highly divergent and its position within the tree was not well resolved (data not shown).

While *E. bienersi* is clinically the most significant human microsporidium, it is also the least understood with respect to its biology and epidemiology. Greater than 50 genotypes have been identified using the ITS sequence (Mathias et al. 2005; Sulaiman et al. 2004). A recent study characterized *E. bienersi* isolates from cattle and found some genotypes were host-adapted while others were identical to the human genotype K, suggesting that these isolates might have the potential to infect humans (Sulaiman et al. 2004). Additional independent genetic markers are needed to provide more genotyping tools, which would also aid in clarifying the genetic structure of *E. bienersi*. The determination of the sequence of the  $\beta$ -tubulin gene provides the first such independent marker for studies on the heterogeneity of *E. bienersi* populations. But more importantly, the significant finding of this study was the correlation between the observed clinical resistance of *E. bienersi* to benzimidazoles and its  $\beta$ -tubulin sequence.

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#### LITERATURE CITED

- Albrecht, H. & Sobottka, I. 1997. *Enterocytozoon bienersi* infection in patients who are not infected with human immunodeficiency virus. *Clin. Infect. Dis.*, **25**:344.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.*, **215**:403–410.
- Blanshard, C., Ellis, D. S., Tovey, D. G., Dowell, S. & Gazzard, B. G. 1992. Treatment of intestinal microsporidiosis with albendazole in patients with AIDS. *AIDS*, **6**:311–313.
- Cali, A. 1991. General microsporidian features and recent findings on AIDS isolates. *J. Protozool.*, **38**:625–630.
- Conteas, C. N., Berlin, O. G., Ash, L. R. & Pruthi, J. S. 2000. Therapy for human gastrointestinal microsporidiosis. *Am. J. Trop. Med. Hyg.*, **63**:121–127.
- Cruz, M. C., Bartlett, M. S. & Edlind, T. D. 1994. In vitro susceptibility of the opportunistic fungus *Cryptococcus neoformans* to anthelmintic benzimidazoles. *Antimicrob. Agents Chemother.*, **38**:378–380.
- Curry, A. & Canning, E. U. 1993. Human microsporidiosis. *J. Infect.*, **27**:229–236.
- Davidse, L. C. 1986. Benzimidazole fungicides: mechanism of action and biological impact. *Ann. Rev. Phytopathol.*, **24**:43–65.
- De Groote, M. A., Visvesvara, G., Wilson, M. L., Pieniazek, N. J., Siemenda, S. B., deSilva, A. J., Leitch, G. J., Bryan, R. T. & Reves, R. 1995. Polymerase chain reaction and culture confirmation of disseminated *Encephalitozoon cuniculi* in a patient with AIDS: successful therapy. *J. Infect. Dis.*, **171**:1375–1378.
- Didier, E. S., Stovall, M. E., Green, L. C., Brindley, P. J., Sestak, K. & Didier, P. J. 2004. Epidemiology of microsporidiosis: sources and modes of transmission. *Vet. Parasitol.*, **126**:145–166.
- Dieterich, D., Lew, E., Kotler, D., Poles, M. & Orenstein, J. 1994. Treatment with albendazole for intestinal disease due to *Enterocytozoon bienersi* in patients with AIDS. *J. Infect. Dis.*, **169**:178–183.
- Edlind, T. D., Li, J., Visvesvara, G. S., Vodkin, M. H., McLaughlin, G. L. & Katiyar, S. K. 1996. Phylogenetic analysis of  $\beta$ -tubulin sequences from amitochondrial protozoa. *Mol. Phylogenet. Evol.*, **5**:359–367.
- Gainzarain, J. C., Canut, A., Lozano, M., Labora, A., Carreras, F., Fenoy, S., Navajas, R., Pieniazek, N. J., da Silva, A. J. & del Aguila, C. 1998. Detection of *Enterocytozoon bienersi* in two human immunodeficiency virus-negative patients with chronic diarrhea by polymerase chain reaction in duodenal biopsy specimens and review. *Clin. Infect. Dis.*, **27**:394–398.
- Germot, A., Philippe, H. & Le Guyader, H. 1997. Evidence for loss of mitochondria in Microsporidia from a mitochondrial-type HSP70 in *Nosema locustae*. *Mol. Biochem. Parasitol.*, **87**:159–168.
- Green, L. C., Didier, P. J., Bowers, L. C. & Didier, E. S. 2004. Natural and experimental infection of immunocompromised rhesus macaques (*Macaca mulatta*) with the microsporidian *Enterocytozoon bienersi* genotype D. *Microbes Infect.*, **6**:996–1002.
- Guerard, A., Rabodonirina, M., Cotte, M. L., Liguory, O., Piens, M. A., Daoud, S., Picot, S. & Touraine, J. L. 1999. Intestinal microsporidiosis occurring in two renal transplant recipients treated with mycophenolate mofetil. *Transplantation*, **68**:699–707.
- Guindon, S. & Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.*, **52**:696–704.
- Jung, M. K., Wilder, I. B. & Oakley, B. R. 1992. Amino acid alterations in the *benA* ( $\beta$ -tubulin) of *Aspergillus nidulans* that confer benomyl resistance. *Cell Motil. Cytoskeleton*, **22**:170–174.
- Katinka, M. D., Duprat, S., Cornillot, E., Méténier, G., Thomarat, F., Prensier, G., Bare, V., Peyretailade, E., Brottier, P., Wincker, P., Delbac, F., El Alaoui, H., Peyret, P., Saurin, W., Gouy, M., Weissenbach, J. & Vivares, C. P. 2001. Genome sequence and gene compaction of the eukaryote parasite *Encephalitozoon cuniculi*. *Nature*, **414**:450–453.
- Katiyar, S. K. & Edlind, T. D. 1997. In vitro susceptibilities of the AIDS-associated microsporidian *Encephalitozoon intestinalis* to albendazole, its sulfoxide metabolite, and 12 additional benzimidazole derivatives. *Antimicrob. Agents Chemother.*, **41**:2729–2732.
- Katiyar, S. K., Gordon, V. R., McLaughlin, G. L. & Edlind, T. D. 1994. Antiprotozoal activities of benzimidazoles and correlations with  $\beta$ -tubulin sequence. *Antimicrob. Agents Chemother.*, **38**:2086–2090.
- Keeling, P. J. & Doolittle, W. F. 1996. Alpha-tubulin from early diverging eukaryotic lineages and the evolution of the tubulin family. *Mol. Biol. Evol.*, **13**:1297–1305.
- Keeling, P. J. & Fast, N. M. 2002. Microsporidia: biology and evolution of highly reduced intracellular parasites. *Ann. Rev. Microbiol.*, **56**:93–116.
- Kirk-Mason, K. E., Turner, M. J. & Chakraborty, P. R. 1988. Cloning and sequence of  $\beta$ -tubulin cDNA from *Giardia lamblia*. *Nucleic Acids Res.*, **16**:2733.
- Kwa, M. S., Veenstra, J. G. & Roos, M. H. 1994. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. *Mol. Biochem. Parasitol.*, **63**:299–303.
- Lacey, E. 1988. The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. *Int. J. Parasitol.*, **18**:885–936.
- Lemee, V., Zaharia, I., Nevez, G., Rabodonirina, M., Brasseur, P., Ballet, J. J. & Favennec, L. 2000. Metronidazole and albendazole susceptibility of 11 clinical isolates of *Giardia duodenalis* from France. *J. Antimicrob. Chemother.*, **46**:819–821.
- Li, J., Katiyar, S. K., Hamelin, A., Visvesvara, G. S. & Edlind, T. D. 1996. Tubulin genes from AIDS-associated microsporidia and implications for phylogeny and benzimidazole sensitivity. *Mol. Biochem. Parasitol.*, **78**:289–295.
- Mansfield, K. G., Carville, A., Shvets, D., MacKey, J., Tzipori, S. & Lackner, A. A. 1997. Identification of an *Enterocytozoon bienersi*-like

- microsporidian parasite in simian-immunodeficiency-virus-inoculated macaques with hepatobiliary disease. *Am. J. Pathol.*, **150**:1395–1405.
- Mathias, A., Weber, R. & Deplazes, P. 2005. Zoonotic potential of the microsporidia. *Clin. Microbiol. Rev.*, **18**:423–445.
- McGinnis, S. & Madden, T. L. 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. *Nucleic Acids Res.*, **32**:W20–W25.
- Molina, J. M., Chastang, C., Goguel, J., Michiels, J. F., Sarfati, C., Desportes-Livage, I., Horton, J., Derouin, F. & Modai, J. 1998. Albendazole for treatment and prophylaxis of microsporidiosis due to *Encephalitozoon intestinalis* in patients with AIDS. *J. Infect. Dis.*, **177**:1373–1377.
- Orbach, M. J., Porro, E. B. & Yanofsky, C. 1986. Cloning and characterization of the gene for  $\beta$ -tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as dominant selectable marker. *Mol. Cell. Biol.*, **6**:2452–2461.
- Rabodonirina, M., Bertocchi, M., Desportes-Livage, I., Cotte, L., Levrey, H., Piens, M. A., Monneret, G., Celard, M., Mornex, J. F. & Mojon, M. 1996. *Enterocytozoon bieneusi* as a cause of chronic diarrhea in a heart-lung transplant recipient who was seronegative for human immunodeficiency virus. *Clin. Infect. Dis.*, **23**:114–117.
- Ridoux, O. & Drancourt, M. 1998. In vitro susceptibilities of the microsporidia *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, and *Encephalitozoon intestinalis* to albendazole and its sulfoxide and sulfone metabolites. *Antimicrob. Agents Chemother.*, **42**:3301–3303.
- Sandfort, J., Hannemann, A., Gelderblom, H., Stark, K., Owen, R. L. & Ruf, B. 1994. *Enterocytozoon bieneusi* infection in an immunocompetent patient who had acute diarrhea and who was not infected with the human immunodeficiency virus. *Clin. Infect. Dis.*, **19**:514–516.
- Schmidt, H. A., Strimmer, K., Vingron, M. & von Haeseler, A. 2002. TREE-PUZZLE: maximum-likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics*, **18**:502–504.
- Sheoran, A. S., Feng, X., Kitaka, S., Green, L., Pearson, C., Didier, E. S., Chapman, S., Tumwine, J. K. & Tzipori, S. 2005. Purification of *Enterocytozoon bieneusi* from stools and production of specific antibodies. *J. Clin. Microbiol.*, **43**:387–392.
- Sulaiman, I. M., Fayer, R., Yang, C., Santin, M., Matos, O. & Xiao, L. 2004. Molecular characterization of *Enterocytozoon bieneusi* in cattle indicates that only some isolates have zoonotic potential. *Parasitol. Res.*, **92**:328–334.
- Thomas, J. H., Neff, N. F. & Botstein, D. 1985. Isolation and characterization of mutations in the  $\beta$ -tubulin gene of *Saccharomyces cerevisiae*. *Genetics*, **112**:715–734.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**:4673–4680.
- Vivarès, C. P., Gouy, M., Thomarat, F. & Méténier, G. 2002. Functional and evolutionary analysis of a eukaryotic parasitic genome. *Curr. Opin. Microbiol.*, **5**:499–505.
- Wittner, M. (ed.). 1999. The Microsporidia and Microsporidiosis. ASM Press, Washington, D.C.
- Zhang, Q., Singh, I., Sheoran, A., Feng, X., Nunnari, J., Carville, A. & Tzipori, S. 2005. Production and characterization of monoclonal antibodies against *Enterocytozoon bieneusi* purified from rhesus macaques. *Infect. Immun.*, **73**:5166–5172.

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