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Analysis of the β-Tubulin Genes from *Enterocytozoon bieneusi* Isolates from a Human and Rhesus Macaque

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ABSTRACT. *Enterocytozoon bieneusi* 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. bieneusi*. A likely explanation for the observed clinical resistance to albendazole was discovered from sequence analysis of the *E. bieneusi* β-tubulin from isolates from an infected human and a naturally infected rhesus macaque. The β-tubulin of *E. bieneusi* has a substitution at Glu_{198}, 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 bieneusi* 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 bieneusi* 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 α- and β-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 β-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. bieneusi* infections (Blanshard et al. 1992; Conteas et al. 2000; Dieterich et al. 1994).

In this communication, we report the analysis of the β-tubulin gene from *E. bieneusi* 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. bieneusi* 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 bieneusi*. 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. bieneusi* 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 β-tubulin genes. The β-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. bieneusi* β-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′-AATAATCATACATGACTGGAACCGTGCTAA-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 β-tubulin of *Enterocytozoon bieneusi*. The *E. bieneusi* β-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 β-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 β-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 β-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. bieneusi* β-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. bieneusi* β-tubulin and β-tubulins from the family Encephalitozoonidae, *Antonospora locustae*, *Trachipleistophora hominis*, *Saccharomyces cerevisiae*, and *Homo sapiens*. However, the

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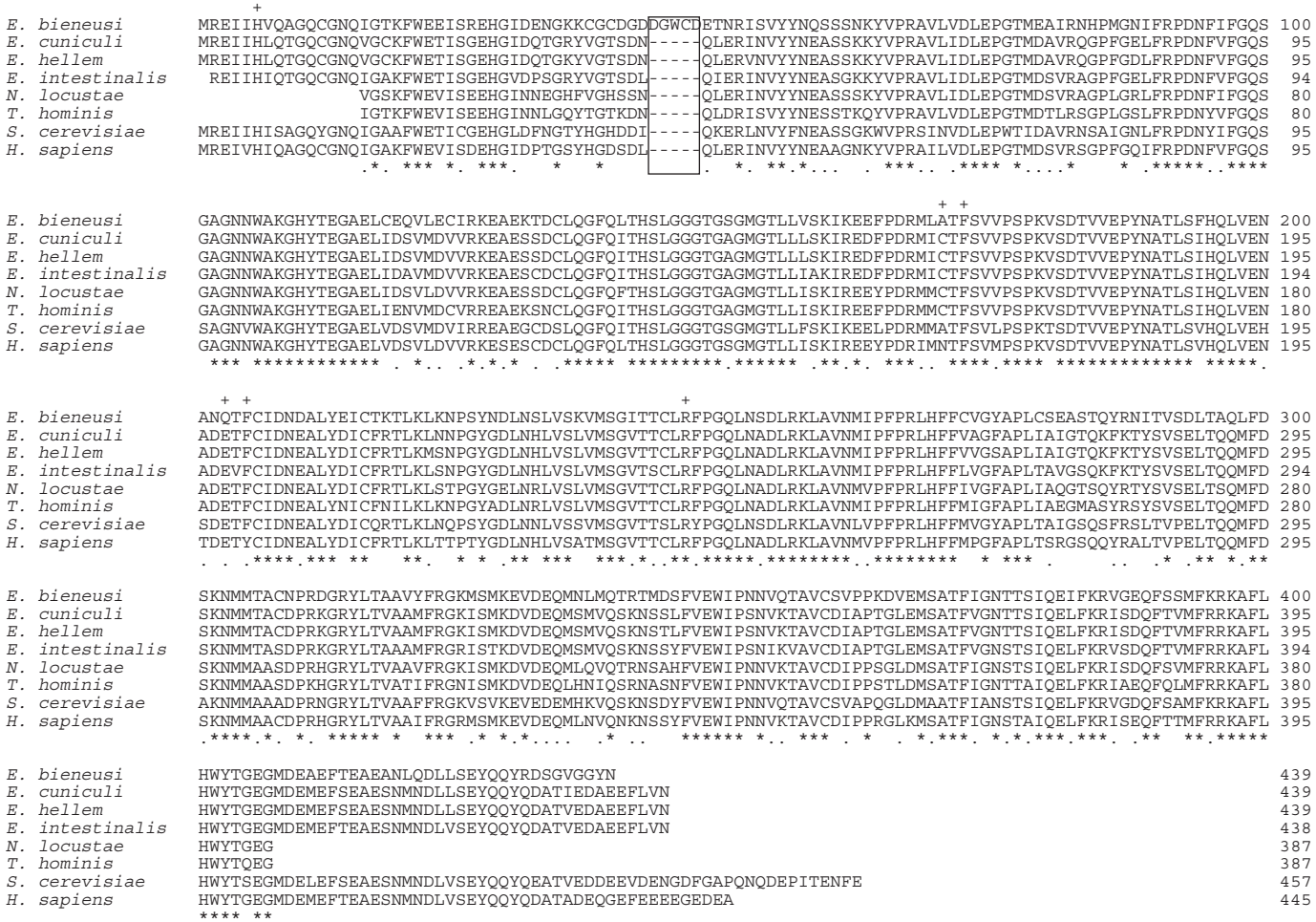

Fig. **1.** ClustalW alignment of the β-tubulin amino acid sequences from *Enterocytozoon bieneusi*, *Encephalitozoon cuniculi* (GenBank Accession no. NP_597591), *Encephalitozoon hellem* (GenBank Accession no. AAB12034), *Encephalitozoon intestinalis* (GenBank Accession no. AAN78302), *Nosema locustae* (GenBank accession no. AAN35161), *Trachipleistophora hominis* (GenBank Accession no. AAF31660), *Saccharomyces cerevisiae* (GenBank Accession no. CAA24603), and *Homo sapiens* (GenBank Accession no. T08726). Positions with identical residues in all eight sequences are indicated by asterisks (below). Conserved changes are indicated by a dot (below). The β-tubulin sequence from *E. bieneusi* had an apparent five amino acid insertion (boxed), which was not found in other fungal, microsporidial or mammalian sequences. Changes in the six amino acids (His_6, Phe_{167}, Glu_{198}, Phe_{200}, Arg_{241}; numbering based on *S. cerevisiae*), which are associated with benzimidazole resistance, are indicated by a cross (+) above. Glu_{198}, which has a high association with benzimidazole sensitivity, is changed to a glutamine in *E. bieneusi*. Nucleotide sequences of the β-tubulin genes are available in the GenBank database under Accession numbers, DQ242639 (human) and DQ242640 (rhesus macaque).

E. bieneusi β-tubulin has near its amino terminus an additional five residues (DGWCD) that are unlikely to represent an intron because of the absence of a canonical GT-AG splice boundary, as well as, an AT-AC boundary (Fig. 1).

The most significant finding was a molecular explanation for the observed clinical resistance of *E. bieneusi* to benzimidazoles. The *E. bieneusi* β-tubulin has only five of the six amino acids that have been reported to be associated with benzimidazole activity (His_6, Phe_{167}, Glu_{198}, Phe_{200} or Arg_{241}; numbering based on the *S. cerevisiae* sequence, Fig. 1) (Jung, Wilder, and Oakley 1992; Ohrbach, Porro, and Yanofsky 1986; Thomas, Neff, and Botstein 1985). The one residue difference occurred at position 198 in which glutamine was substituted for glutamic acid.

Organisms resistant to benzimidazole lack one or both of Glu_{198} or Phe_{200}. Both *E. bieneusi* and *Entamoeba histolytica* have changes at Glu_{198}, and both are relatively resistant to albendazole. *Cryptosporidium parvum* and *Acanthamoeba polyphaga* have changes at both Glu_{198} and Phe_{200}, and both are resistant to benzimidazole. The helminths, fungi, and humans have changes at Phe_{200}, which confer resistance to benzimidazole (Katiyar et al. 1994; Kwa, Veenstra, and Roos 1994). With respect to Ala_{165}, the β-tubulins of the Encephalitozoonidae have five of the six residues, but with Ala_{165} changed to a Cys_{165}. Yet, they are highly sensitive to albendazole (Cruz, Bartlett, and Edlind 1994; Katiyar et al. 1994). This residue is also a cysteine in *Giardia lamblia* (Kirk-Mason, Turner, and Chakrabory 1988) and *Cryptococcus neoformans* (Li et al. 1996), which are also sensitive to albendazole. Contrary to this, Ala_{165} is replaced by a glycine in thiabendazole-resistant strains of *Aspergillus nidulans*. Of these six amino acids, His_6 and Arg_{241}, are not highly predictive of benzimidazole sensitivity because these two residues are conserved in both benzimidazole-resistant and benzimidazole-sensitive organisms. Phe_{167} is also not highly predictive of benzimidazole sensitivity because the *Trichomonas vaginalis* β-tubulins have tyrosine instead of phenylalanine at this position (Katiyar and Edlind 1997), yet the organism is sensitive to the benzimidazoles. Overall, the data for these six amino acids show that substitutions of either Glu_{198} or Phe_{200} are highly associated with benzimidazole

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

The microsporidia have been placed within the fungal clade based on phylogenetic analyses of the α- and β-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 mitosomes 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 Guyander 1997; Keeling and Doolittle 1996). We attempted to align the *E. bieneusi* β-tubulin to 67 other β-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. bieneusi*. Unfortunately, the *E. bieneusi* β-tubulin sequence was found to be highly divergent and its position within the tree was not well resolved (data not shown).

While *E. bieneusi* 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. bieneusi* 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. bieneusi*. The determination of the sequence of the β-tubulin gene provides the first such independent marker for studies on the heterogeneity of *E. bieneusi* populations. But more importantly, the significant finding of this study was the correlation between the observed clinical resistance of *E. bieneusi* to benzimidazoles and its β-tubulin sequence.

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