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Characterization of *Entercytozoon bieneusi* and Drug Resistance-Associated Mutations Using the β-Tubulin Gene

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ABSTRACT

OBJECTIVE: In the β-tubulin gene of *Enterocytozoon bieneusi*, five mutations: His_6, Phe_{167}, Glu_{198}, Phe_{200}, and Arg_{241} have been implicated in reducing albendazole efficacy, with Glu_{198} and Phe_{200} being particularly significant. The primary objective of this study was to characterize mutations in the β-tubulin gene of *E. bieneusi* associated with albendazole resistance. Specifically, the study focused on mutations at codons Glu_{198} and Phe_{200} using newly developed primers (EbBtu198/200) with DNA extracted from fecal samples of pigs and humans.

METHODS: A total of 38 stored DNA samples, comprising 30 from different pigs and eight from different humans, were analyzed to evaluate the sensitivity of the newly designed primers and optimize the polymerase chain reaction (PCR) conditions. The encoded amino acid sequences were examined to identify the mutations at codons 198 and 200 in the β-tubulin gene of *E. bieneusi*. Additionally, a phylogenetic analysis was performed using the β-tubulin nucleotide sequences to determine the genetic relationships among different isolates.

RESULTS: PCR-amplification of the β-tubulin gene yielded a 427 bp product, with a primer sensitivity rate of 94.74%. Sequencing of 18 gene products revealed that ten sequences from pigs corresponded to Haplotype A, while human samples showed four haplotypes: A, B, C, and D. Notably, a mutation resulting in the substitution of glutamic acid with glutamine at codon 198 (E198Q) was identified, uncovering a potential mechanism for albendazole resistance. Phylogenetic analysis applying the maximum likelihood method demonstrated that all β-tubulin sequences formed a monophyletic group, indicating low genetic diversity among the *E. bieneusi* isolates.

CONCLUSION: This study underscores the significance of mutations in the β-tubulin gene, particularly at codon Glu_{198}, as key factors potentially contributing to albendazole resistance in *E. bieneusi*. These findings may offer valuable insights for improving treatment strategies in patients harboring isolates with such mutations. Furthermore, the β-tubulin gene analysis revealed limited genetic diversity among *E. bieneusi* isolates, with distinct haplotypes detected in pig and human samples, suggesting possible host-specific adaptations or transmission patterns.

KEYWORDS: albendazole resistance, *Enterocytozoon bieneusi*, haplotype, β-tubulin gene

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INTRODUCTION

Enterocytozoon bieneusi, an obligate intracellular parasite from the group microsporidia, has been reported in various mammalian hosts, including humans globally. Intestinal infection of *E. bieneusi* can cause self-limiting watery diarrhea in immunocompetent persons and severe chronic diarrhea in immunocompromised persons, especially in HIV/AIDS patients. The drug of choice for the therapy of microsporidiosis caused by *Encephalitozoon* spp. is albendazole, a benzimidazole derivative, which inhibits microtubule assembly. This drug is also effective against diarrhea caused by *E. intestinalis* and disseminated infection by *E. hellem* and *E. cuniculi*[1,2]. However, it is ineffective against *E. bieneusi* which is possibly linked to mutations in the β-tubulin gene[3-6]. Several human- and animal-derived genotypes of *E. bieneusi* have been identified from the stools of infected humans proving the occurrence of cross-species transmission. The β-tubulin gene is one of the targets utilized for the detection and genotype characterization of albendazole resistance-associated mutations[2]. Mutations in β-tubulin in *E. bieneusi* at five amino acids, His_6, Phe_{167}, Glu_{198}, Phe_{200}, and Arg_{241}, have been reported to relatively reduce albendazole activity[1,2]. In particular, mutations at the codons of Glu_{198} and Phe_{200} are potentially implicated in albendazole resistance. Thus, this study investigated the characterization of β-tubulin gene of *E. bieneusi* using a polymerase chain reaction (PCR) assay, which provides beneficial for examining the genotype and can be employed to identify mutations associated with albendazole resistance. Albendazole resistance in *E. bieneusi* has been reported widely, but evidence in Thailand is still lacking. Several human and animal-derived genotypes of *E. bieneusi* have been discovered from the stools of infected humans, demonstrating the possibility of cross-species transmission. Furthermore, the findings of this study will provide valuable information on genotype characteristics and assist in identifying mutations linked to albendazole resistance, thereby contributing to a better understanding of the molecular epidemiology and transmission patterns of infections, which is essential for effective management and control.

METHODS

A total of 38 stored *E. bieneusi* DNA samples extracted from stool samples, were confirmed by PCR assay using the internal transcribed spacer (ITS) of the small subunit rRNA gene (ITS-PCR), following the protocol established by Thathaisong et al[7]. The DNA samples were obtained from the Department of Parasitology, Phramongkutklao College of Medicine, Thailand. Of them, 30 were from different pigs, and eight were from different humans.

Primers targeting the β-tubulin gene of *E. bieneusi*, specifically designed to amplify regions containing mutations at codons Glu_{198} and Phe_{200}, were designed using the Primer3 software (https://primer3.org/)[8]. The β-tubulin gene sequence of *E. bieneusi* (GenBank accession number: DQ242640) was used as a reference template. The forward primer (EbBtu198/200-F: 5′-TGGAAATAACTGGGCCAAAG-3′) spans 20 base pairs starting at position 817, while the reverse primer (EbBtu198/200-R: 5′-ACACGTTGTGATCCCACTCA-3′) spans 20 base pairs starting at position 1245. Both primers were optimized for an annealing temperature of 59°C and a GC content of 45–50%. The resulting PCR product is 427 base pairs in length, covering the target regions of interest. In addition, the specificity of the primers to the β-tubulin gene and the absence of off-target amplification were verified using NCBI Primer-BLAST, and the primers were synthesized by Macrogen, Seoul, Republic of Korea. The PCR assay optimal for amplifying the gene of interest was developed and evaluated for sensitivity and specificity. PCR amplifications were carried out in a reaction mix with a final volume of 50 µL,

consisting of the 2 µL of DNA template (~21ng/µL), 10 pmol of each primer, 200 µM dNTP, 2 mM of $MgCl_2$, 1X PCR buffer, and 1 unit of KAPA *Taq* HotStart DNA Polymerase (5 U/µL) (Merck, USA). The reactions were performed using a Mastercycler personal thermal cycler (Bio-Rad Laboratories, California, USA). The PCR program consisted of: denaturation at 94°C for 5 min; followed by 35 cycles at 94°C for 30 sec, 59°C for 30 sec, and 72°C for 30 sec; final extension at 72°C for 10 min; and a holding temperature of 12°C. The PCR products were run for 30 min on a 2% agarose gel in 1X Tris/borate/EDTA buffer at 100V. A 100 bp DNA ladder (Vivantis Technologies, Selangor Darul Ehsan, Malaysia) was used. DNA was stained with SYBR Safe DNA Gel (Invitrogen, USA) by mixing 1 µL with 10 mL of agarose gel. Finally, the PCR products were detected by the Molecular Imager® Gel Doc XR+ Imaging System (Bio-Rad Laboratories, CA, USA).

To evaluate the specificity of the Eb198/200 primers for amplifying the β-tubulin gene of *E. bieneusi*, DNA samples from a variety of common intestinal pathogens that may co-infect with *E. bieneusi* intestinal parasites were selected, including *Trichuris trichiura*, *Ascaris lumbricoides*, hookworm, *Blastocystis* sp., *Opisthorchis viverrini*, and *Haplorchis taichui*, allowing for a comprehensive assessment of primer specificity.

For DNA sequencing, 18 positive samples, accounting for 50% of the total, were randomly chosen from the pool of samples exhibiting high-intensity PCR-positive bands (28/34 samples). This method ensured a sufficient concentration of target DNA, minimizing the likelihood of sequencing errors or low-quality data and ensuring reliable and accurate results. The sequences were subjected to the Basic Local Alignment Search Tool (BLAST) to examine the similarity of nucleotide sequences and species identity. The DNA sequences were aligned utilizing the BioEdit software, version 7.0.9 and then translated to amino acid sequences using the nucleotide sequence translation tool Transeq (EMBOSS) (https://www.ebi.ac.uk/Tools/st/). The β-tubulin DNA and amino acid sequences have been submitted to the GenBank database for accession numbers.

For nucleotide sequence alignment, 18 nucleotide and amino acid sequences were analyzed using the ClustralW program (BioEdit software, version 7.0.9 and Seaview 5.0.5). The phylogenetic tree was constructed by applying the maximum likelihood tree based on the kimura-2 parameter method (+G+I) using Molecular Evolutionary Genetics Analysis Version X (MEGA version X)[9]. The clade stability of β-tubulin topologies was assessed with a bootstrap analysis consisting of 1,000 replicates of bootstrap values. The reference sequences of the β-tubulin gene of *E. bieneusi* (AB472273), *E. bieneusi* isolate M231 (DQ242640), *E. bieneusi isolate H206 (DQ242639), Encephalitozoon cuniculi* (KC513611), *Enterocytozoon hepatopenaei* (KY593130), *Encephalitozoon hellem* (L47271), *Nosema locustae* (AF190772), *Trachipleistophora hominis* (AF162081), and *Vittaforma corneae* (EU031749) as the outgroup, were retrieved from GenBank.

The research protocol was approved by the Institutional Review Board of the Faculty of Medicine at Vajira Hospital in Bangkok, Thailand. This approval confirms adherence to international standards for human research protection, such as the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines, and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP). The study was approved under the approval number COE 008/2020.

RESULTS

PCR-amplification of the β-tubulin gene, using the Eb198/200 primers produced a single 427 bp DNA fragment (Figure 1). The left primer initiated amplification at position 817 bp, while the right primer extended to position 1,243 bp. The optimal annealing temperature for Eb198/200 primers, demonstrating nonspecific bands was 59°C. The PCR-based amplification of the β-tubulin gene of *E. bieneusi* took ~1 h and 45 min to complete 35 cycles. It showed a detection sensitivity of 94.74% (36/38) compared with the ITS-PCR assay. In addition, the Eb198/200 primers did not cross-react with other intestinal parasites, including *T. trichiura*, *A. lumbricoides*, hookworm, *Blastocystis* sp., *O. viverrini*, and *H. taichui* (Figure 2). The selected β-tubulin PCR products positive for *E. bieneusi*, ten from pigs and eight from humans were sequenced. The blast results of sequencing ten nucleotide sequences: isolates ChB36, ChB79, ChB83, ChB92, ChB95, ChB199, ChB200, ChB215, ChB239, and ChB243 (accession number: ON939626-ON939635) obtained from pigs revealed 100% identity to the β-tubulin gene of *E. bieneusi*, genotype K isolated from a cat (accession number: AB472273), 99.8% with *E. bieneusi* isolate M231, obtained from a rhesus macaque, (accession number: DQ242640) and 99.2% with *E. bieneusi* isolate H206, isolated from a patient (accession number: DQ242639). Moreover, eight nucleotide sequences (isolates NMU1–NMU8; accession number: ON939636-ON939643) of the *E. bieneusi* β-tubulin were isolated from humans; four sequences (isolates NMU1–NMU4,) were 100% identical to *E. bieneusi* genotype K (AB472273), 99.8% to *E. bieneusi* isolate M231 (DQ242640), and 99.2% to *E. bieneusi* isolate H206 (DQ242639). In addition, the other four sequences (isolates NMU5–NMU8) were 99.8% similar to the *E. bieneusi* genotype K (AB472273), 99.5% to the *E. bieneusi* isolate M231 (DQ242640), and 99.1% to the *E. bieneusi* isolate H206 (DQ242639). All 18 β-tubulin nucleotide sequences of *E. bieneusi*, accession numbers ON939626–ON939643, were submitted to GenBank.

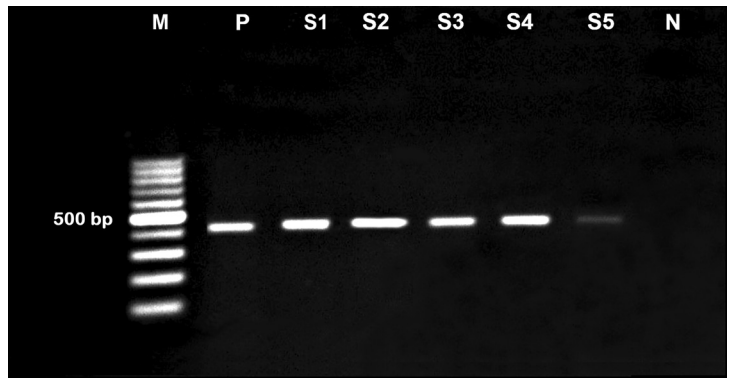

Figure 1 The PCR products of the β-tubulin gene in *Enterocytozoon bieneusi*
Note: Amplicons of β-tubulin products of *E. bieneusi* are 427 bp.
Lane M: a 100 bp DNA ladder; Lane P: positive control; Lanes S1 to S5 refer to five separate fecal samples obtained from individual pigs; Lane N: negative control
Abbreviations: bp, base pairs; PCR, polymerase chain reaction

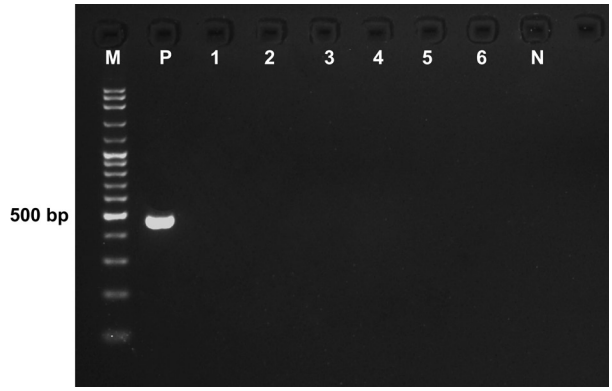

Figure 2 Agarose gel electrophoresis of DNA samples from intestinal parasites were used to evaluate the specificity of primers designed for the detection of *Enterocytozoon bieneusi*.
Lane M: a 100 bp DNA ladder; Lane P: positive control for *E. bieneusi* (427 bp); Lane 1: *T. trichiura* DNA, Lane 2: *A. lumbricoides* DNA, Lane 3: Hookworm DNA, Lane 4: *Blastocystis* sp. DNA, Lane 5: *O. viverrini* DNA, and Lane 6: *H. taichui* DNA and Lane N: negative control.
Abbreviation: bp, base pairs

The alignment of 18 nucleotide sequences of β-tubulin of *E. bieneusi* were classified into Haplotypes A, B, C, and D. All ten isolates: ChB36, ChB79, ChB83, ChB92, ChB95, ChB199, ChB200, ChB215, ChB239, and ChB243 from pigs were identified as Haplotype A. The eight isolates, NMU1–NMU8 from humans were recognized as Haplotypes A, B, C, and D.

A comparison of the β-tubulin nucleotide sequence of *E. bieneusi* from Haplotype A with the *E. bieneusi* isolate M231 (DQ242640), revealed a single nucleotide polymorphism (SNP) at the 1,066 bp position. Moreover, when comparing Haplotype A with the *E. bieneusi* isolate H206 (DQ242639) or the human isolate, three heterogenous SNPs were also detected at positions 1,036, 1,051, and 1,108 bp[2]. Haplotypes B (isolates NMU5 and NMU6) and D (NMU8) revealed four heterogenous SNPs at positions 850, 1,036, 1,051, and 1,108 bp compared with the *E. bieneusi* isolate H206 (DQ242639). Haplotype C showed five heterogenous SNPs at positions 850, 852, 1,036, 1,051, and 1,108 compared with H206 (DQ242639). The 18 β-tubulin nucleotide sequences of *E. bieneusi* were translated to 139 amino acids which revealed 100% identity to reference isolates (*E. bieneusi* genotype K: isolates M231 and H206) with no polymorphisms[2]. Interestingly, 18 amino acid sequences, including three reference sequences (genotype K: isolates M231 and H206), revealed a point mutation at codon Glu_{198} to glutamine (E198Q); however, no mutation at codon Phe_{200} was found (Figure 3).

The maximum likelihood tree was constructed with 1,000 bootstrapping values using an alignment of 416 β-tubulin nucleotide sequences with no gaps based on the kimura-2 parameter method (+G+I). The accession number lists display the selected β-tubulin reference sequences. The topologies of ten β-tubulin *E.bieneusi* isolates in the tree showed a monophyletic group with references including *E.bieneusi* accession numbers: DQ242640, DQ242639, and AB472273, with a bootstrapping value of 99% and was paraphyletic with *E. hepatopenaei* (KY593130); bootstrapping values of 100% (Figure 4). In addition, ten isolates of *E. bieneusi* obtained in this study diverged from the *E. cuniculi*, *E. hellem*, *N. locustae*, and *T. hominis* clade, with bootstrapping values at 97%.

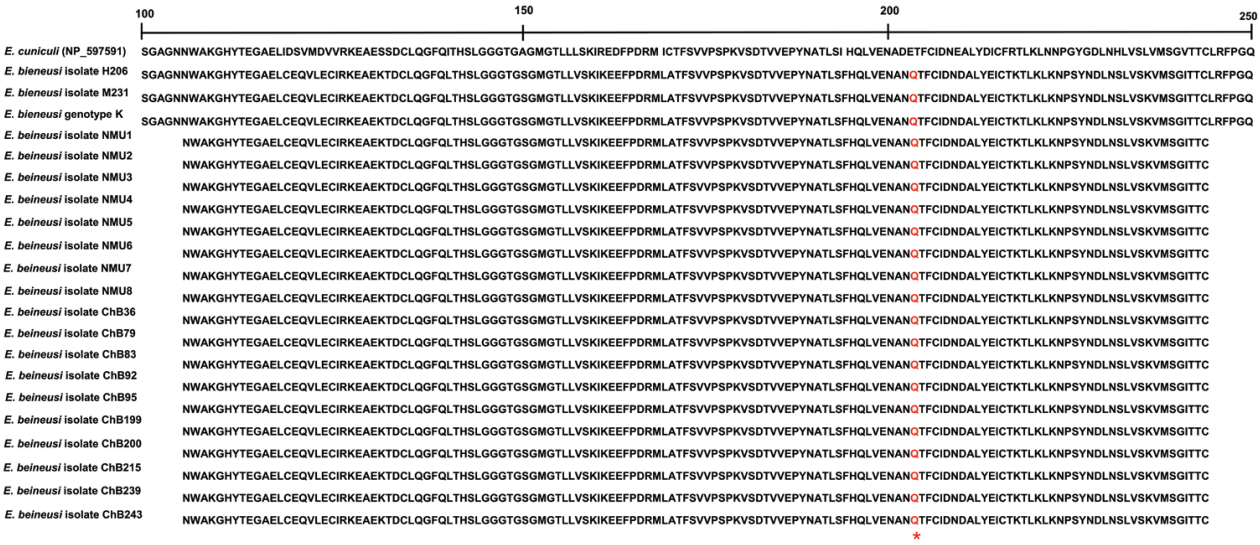

Figure 3 Amino acid alignment of β-tubulin in *Enterocytozoon bieneusi*

Note: The amino acid sequences consist of three reference sequences (isolates H206, M231, and genotype K), eight sequences from humans (isolates NMU1 to NMU8), and ten sequences from pigs (ChB36, ChB79, ChB83, ChB92, ChB95, ChB199, ChB200, ChB215, ChB239, and ChB243), demonstrating a length of 139 amino acids.

*a point mutation at the codon Glu_{198} with glutamine (E198Q). Numbering is based on *Saccharomyces cerevisiae* (Akiyoshi et al, 2007).

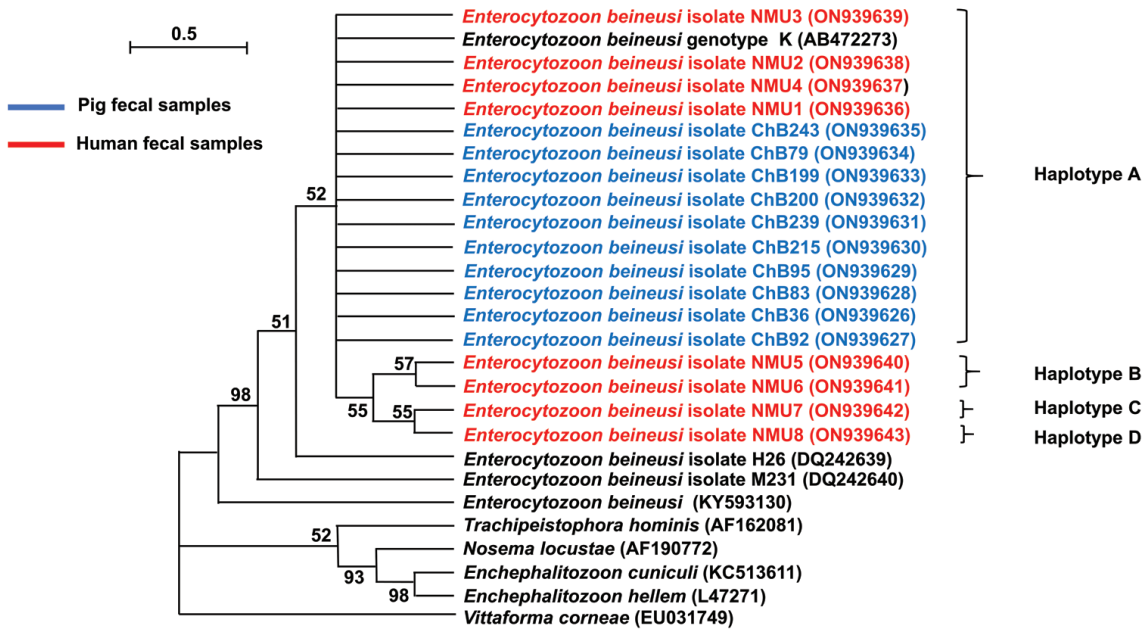

Figure 4 Maximum likelihood tree based on kimura-2 parameter method (+G+I) model of microsporidia species using β-tubulin gene sequences

A maximum likelihood tree was performed on an alignment of 401 nucleotide sequences with no gaps, and 27 β-tubulin sequences from microsporidia species were subjected to tree construction. The GenBank accession numbers are shown in this order after the species name, and the percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values of > 50 are shown, and the branch lengths are measured in the number of substitutions per site.

DISCUSSION

This study developed a highly sensitive β-tubulin-PCR assay to amplify 427 bp that could be an alternative method for *E. bieneusi* characterization. The sensitivity of the primers EbBtu198/200 was 94.74% compared with the ITS-PCR assay for detecting *E. bieneusi* and did not cross-react with other intestinal parasites detected Haplotypes A, B, C, and D from pig and human stool samples. Furthermore, the specificity of PCR assay for accurately detecting mutations in the β-tubulin gene associated with albendazole resistance in *E. bieneusi* can be significantly enhanced through the use of advanced techniques such as nested PCR, real-time PCR, and allele-specific PCR. Haplotype A was identified in both pigs and humans; thus, it might be potentially zoonotic with cross-species transmission. Moreover, β-tubulin in *E. bieneusi* from this study showed a few SNPs, indicating a highly conserved region.

Transmission of *E. bieneusi* can occur from person to person or from animals to humans via a fecal-oral route by ingesting food and water contaminated with infective spores. Previous genotype identification of *E. bieneusi* from infected humans revealed that they were animal-derived, implying potential zoonotic and cross-species transmission[10,11]. In addition, asymptomatic persons infected with *E. bieneusi* possibly highlight the epidemiological relevance and play a role in the transmission of this organism. In a previous study, *E. bieneusi* genotype PigEBITS was identified in HIV patients in Thailand, supporting potential zoonotic transmission to humans. The PigEBITS genotype exists in many hosts that could infect both humans and pigs[10]. Moreover, other animal-derived genotypes were also identified in infected persons[12]. Pigs are considered one of the main reservoir hosts of *E. bieneusi*. Due to the ability to infect multiple host species, this finding supports the zoonotic transmission of *E. bieneusi* Haplotype A (genotype K in cats) from pigs to humans. Additionally, Haplotypes A, B, C, and D containing albendazole resistance-associated mutations were first described in Thailand. The β-tubulin nucleotide sequences of these haplotypes obtained from humans demonstrated a few SNPs compared with those of a rhesus macaque (DQ242639) and an HIV patient (DQ242640), suggesting high conservation of the β-tubulin gene among different host species. Using phylogenetic analysis, *E. bieneusi* isolated from ten individual pigs, one human, one rhesus macaque, and one cat were clustered into the same clade (99% of bootstrap values) or were monophyletic, demonstrating non-significant genetic variation of the β-tubulin gene among *E. bieneusi* genotypes.

Akiyoshi et al. analyzed the amino acids encoded at six codons of the β-tubulin gene, His_{6}, Ala_{165}, Phe_{167}, Glu_{198}, Phe_{200}, and Arg_{241}, to study the sensitivity or resistance of *E. bieneusi* to albendazole[2]. Of these six amino acids, those highly associated with albendazole sensitivity were substituted with either Glu_{198} or Phe_{200}, and changes in one or both of these resulted in albendazole resistance. While fungi and helminths carrying Glu_{198} and Phe_{200} genotypes are albendazole susceptible[1,2,13]. Albendazole has been widely used to treat microsporidiosis caused by *Encephalitozoon* spp. However, albendazole is quite ineffective in the treatment of *E. bieneusi* infection[6]. Mutations in the β-tubulin gene, particularly at key codons like Glu_{198} and Phe_{200}, pose a significant challenge in the treatment of *E. bieneusi* infections. These mutations reduce the binding affinity of albendazole to β-tubulin, its primary molecular target[14-17], which in turn diminishes its ability to inhibit microtubule assembly. As microtubule assembly is essential for parasite growth, survival, and reproduction, this reduction in albendazole's effectiveness leads to decreased therapeutic efficacy and an increased risk of treatment failure.

In those for whom albendazole derivative treatment failed, nitazoxanide has been successfully employed to treat *E. bieneusi*

infection in two case reports[18,19]. Although the exact mechanism of action of nitazoxanide remains unclear, it is believed to interfere with anaerobic energy production by inhibiting the enzyme pyruvate-ferredoxin oxidoreductase (PFOR)[20,21], with another target in microsporidia being protein disulfide isomerase[22]. Recently, nitazoxanide has shown effectiveness against various parasitic infections, including *G. intestinalis* and *Entamoeba histolytica*, positioning it as a promising alternative when albendazole is ineffective due to resistance[23]. Another alternative medicine for *E. bieneusi* infection is fumagillin, which inhibits methionine aminopeptidase type 2 (MetAP2) an essential enzyme for protein synthesis in *E. bieneusi* [24,25]. Fumagillin has completely eradicated *E. bieneusi* infections at 60 mg/day for two weeks[26]. However, more trials using nitazoxanide and fumagillin are required to evaluate their possible role in treating *E. bieneusi* infection.

Analysis of ten β-tubulin nucleotide sequences obtained from individual pigs revealed 100% identity with previously reported amino acid sequences (accession numbers BAH02265, ABB72136, and ABB72137), with no polymorphisms detected. Interestingly, a mutation at codon Glu_{198} to glutamine (E198Q) of the β-tubulin gene of *E. bieneusi* was observed among the ten amino acid sequences from ten different pigs that could be related to albendazole resistance. However, the limitation of the study includes the type of samples employed, as they lacked demographic data for a more comprehensive analysis and interpretation. Furthermore, alternative treatments for *E. bieneusi* infection need to be evaluated and validated to ensure proper management in patients with albendazole-resistant genotypes. A previous study demonstrated a point mutation of the β-tubulin gene of *G. intestinalis* at the codon Glu_{198} to lysine (E198K), showing albendazole resistance[27]. Additionally, a study in Sudan reported that *Haemonchus contortus*, a goat nematode, showed resistance to albendazole due to β-tubulin polymorphism at codon 198[14]. The eight amino acid sequences encoded by the *E. bieneusi* β-tubulin gene isolated from humans was also 100% identical to the reference amino acid sequences: BAH02265, ABB72136, and ABB72136, as well as the pig isolates that had the E198Q mutation in the β-tubulin gene. Other organisms also showed that mutations of one or both amino acids at codons 198 and 200 are potentially related to resistance to albendazole and its derivatives. These included the fungi: *Fusarium graminearum*, *Penicillium digitatum*, *Aspergillus* spp., *Saccharomyces cerevisiae*, *V. corneae*, *E. hellem*, *E. cuniculi*, *E. bieneusi* [1,28-31]; protozoa: *G. intestinalis*, *Acanthamoeba polyphaga*, *Cryptosporidium parvum*, *Entamoeba histolytica*, *Leishmania major* [1]; and nematodes: *A. lumbricoides*, *T. trichiura*, *H. contortus*, *Necator americanus*, and *Teladorsagia circumcincta* [32-36].

To circumvent drug resistance against *E. bieneusi*, information obtained from this study can be used in the rational design of new microsporidia therapeutic targets such as tubulin, type 2 methionine aminopeptidase polyamines, chitin synthases, topoisomerase IV, triosephosphate isomerase, and lipase or enzymes involved in spore germination and invasion processes[37]. Novel inhibitors and specific targets of *E. bieneusi* can be searched by screening compound libraries, and computational molecular modeling techniques, such as molecular dynamics and docking. The application of these computational methods can identify the action of different ligands on unique therapeutic targets, which can economize the drug discovery process[38]. Studies on therapeutic targets of these drugs in microsporidia are needed to further their development as an effective treatment against *E. bieneusi*.

CONCLUSION

In conclusion, a new PCR assay for the detection of β-tubulin in *E. bieneusi* was developed which showed 94.74% sensitivity. An analysis of the nucleotide sequence of

the β-tubulin in *E. bieneusi* provided one haplotype from pig stool samples and four haplotypes from human samples, revealing a few SNPs among host types. An analysis of the β-tubulin nucleotide sequence in *E. bieneusi* revealed one haplotype in pig feces samples, four haplotypes in human samples, and a few SNPs among host types. Examination of the amino acid sequences revealed mutations at codon Glu_{198} (E198Q); thus, *E. bieneusi* isolated in this study might have been involved in resistance to benzimidazole and its derivatives. Thus, further epidemiological investigation of β-tubulin in *E. bieneusi* in local animals and farms is required, which may play a significant role in disseminating benzimidazole resistance. The findings of this study emphasize the significance of genetic epidemiology, prevention, control, and dynamic transmission, particularly animal–human transmission.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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DATA AVAILABILITY STATEMENT

All data are available in the GenBank database with the following accession numbers, β-tubulin sequences: ON939626-ON939643, AB472273, DQ242640, DQ242639, L47271, AF190772, AF162081, EU031749, KY593130, KC513611 and NP_597591.

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