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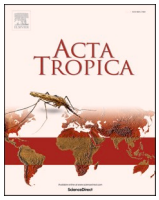

High genotype diversity and zoonotic potential of *Enterocytozoon bieneusi* in laboratory mice from two medical experimental animal centers

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ABSTRACT

Enterocytozoon bieneusi is a common zoonotic pathogen in wild, farmed and pet rodents worldwide. Recently, one study reported *E. bieneusi* infection in laboratory mice. To investigate the positive rate and genotype distribution of *E. bieneusi* in laboratory mice and assess the zoonotic potential of *E. bieneusi* isolates, 390 mixed mouse fecal specimens were collected from two medical experimental animal centers in Heilongjiang Province, China. *E. bieneusi* was identified and genotyped by nested PCR amplification and sequence analysis of the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene. Seventy-one specimens (18.2%) were positive for *E. bieneusi*, and 38 genotypes were identified, including eight known genotypes (EbpC, D, Peru8, CS-4, Henan-III, CHC5, ETMK4 and SHWR14) and 30 novel genotypes (HLJLM1 to HLJLM30). Genotypes CHC5 and ETMK4 were firstly detected in rodents. Genotype EbpC showed a dominance (50.7%, 36/71) in positive specimens. Zoonotic genotypes (EbpC, D, Peru8, Henan-III and CS-4) accounted for 64.8% (46/71) of *E. bieneusi*-positive specimens. In phylogenetic analysis, the novel genotypes fell into three subgroups (1a, 1d, 1e) of Group 1. The identification of known zoonotic genotypes and the phylogenetic result of novel genotypes indicate the potential of laboratory mice in the transmission of *E. bieneusi* to humans. The routine detection of *E. bieneusi* should be a recommended practice in laboratory animals to ensure the accuracy of the experimental results. Meanwhile, health education of the potential zoonotic transmission of *E. bieneusi* should be provided to those people having close contact with laboratory mice and their feces.

1. Introduction

Enterocytozoon bieneusi is the most common microsporidian species worldwide and is a causative agent in more than 90% of human microsporidiosis cases (Wang et al., 2024). To date, *E. bieneusi* has been recorded in humans in at least 34 countries distributing in Africa, Europe, Asia, South America and Oceania, with an overall prevalence of 6.6% (Wang et al., 2024). *E. bieneusi* is an obligate intracellular intestinal pathogen, and mainly causes disease characterized by diarrhea. The occurrence of *E. bieneusi* and the severity of related diarrhea are mainly associated with the human immune status (Nourrisson et al., 2024). Immunocompromised/immunodeficient individuals usually have a higher infection rate, such as HIV-positive patients (14.4%), and microsporidiosis caused by *E. bieneusi* can progress to life-threatening diarrhea in this population (Wang et al., 2024; Zhang et al., 2021). Epidemiological data have demonstrated that in addition to humans, *E. bieneusi* has also been detected in at least 210 animal species, including livestock, companion and wild animals, and even some laboratory animals in more than 50 countries distributing on all continents except Antarctica (Koehler et al., 2022). The findings of the same genotypes in humans and animals support the presumption of zoonotic or cross-species transmission potential. Due to zoonotic nature and broad host range and geographical distribution of *E. bieneusi*, microsporidiosis related to *E. bieneusi* has raised a widespread public health concern.

E. bieneusi is a complex species with multiple genotypes. Due to a hypervariable sequence (243 bp) in the internal transcribed spacer (ITS)

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region of the ribosomal RNA (rRNA) gene, sequence analysis of the ITS region is regarded as the standard method for identification and genotyping of *E. bieneusi* (Santin and Fayer, 2009). With the accumulation of genotyping data, the number of *E. bieneusi* genotypes has reached more than 900 (Jiang et al., 2024b). They are genetically divided into 15 different phylogenetic groups with varying degrees of host specificity and zoonotic potential (Jiang et al., 2024b). Group 1 is the largest and the most complicated and is thought to be zoonotic. This group contains almost all the genotypes from humans and the majority of genotypes from animal hosts, with some of them (mainly including genotypes D, EbpC and Type IV) being found in both humans and animals (Zhang et al., 2021). Group 2 is the second largest group previously considered adapted to ruminants. However, the fact of some genotypes (notably BEB4, BEB6, CHN3, I and J) in humans and other animals raises public health importance of this group (Li and Xiao, 2021). The remaining 13 groups (Groups 3 to 15) seem to be composed of host-adapted genotypes, indicating minimal or unknown zoonotic implications of these groups (Nourrisson et al., 2024).

Rodents are the most abundant mammalian group and are widely distributed worldwide. They are composed of 2552 rodent species, accounting for nearly 40% of the total number of mammals (Islam et al., 2021). They can facilitate the transmission of at least 84 zoonotic diseases as carriers or reservoirs, posing a serious threat to human and animal populations (Islam et al., 2023). *E. bieneusi* has also been found in numerous rodent species living in different habitats, including wild, farmed and pet rodents, observing overall prevalence of 13.6% (Jiang et al., 2024a; Taghipour et al., 2022). To date, 238 genotypes have been identified in rodents. Among these genotypes, there have been 40 genotypes found in humans: 30 in Group 1 (mainly including genotypes D, EbpA, EbpC and A); six in Group 2 (including genotypes BEB4, BEB6, CHG3, CHG5, I and J); one in Group 6 (genotype Nig7); one in Group 10 (genotype S7); two in Group 13 (including genotypes HNH7 and HNP-II) (Table S1 and Fig. S1). The role that rodents play has attracted more and more attention in the epidemiology of *E. bieneusi* as reservation hosts and vectors.

Rodents are also the main laboratory animals in teaching and scientific research studies. Based on the fact that mice share high genetic similarity with humans, they are used in laboratory tests in a huge number worldwide (Dutta and Sengupta, 2016). According to the annual reports of the United States Department of Agriculture, mice constituted the largest share (96.6%) of laboratory mammals annually (Carbone, 2021). Recently, one study reported *E. bieneusi* infection in laboratory mice (Wang et al., 2022). To determine the positive rate of *E. bieneusi* and analyze its genetic characterization at the genotype level, a molecular investigation of *E. bieneusi* was carried out in laboratory mice from two medical experimental animal centers by PCR amplification and sequence analysis of the ITS region of the rRNA gene. Meanwhile, the zoonotic potential of the novel *E. bieneusi* genotypes was assessed by phylogenetic and homologous analyses.

2. Methods

2.1. Ethics statements

The present study was strictly performed in accordance with the guidelines of the Chinese Laboratory Animal Administration Act (2017). The protocol was reviewed and approved by the Animal Ethics Committee of Harbin Medical University and the Laboratory Animal Welfare & Ethics Committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, China (reference no. IPD-2021–21). No animals were injured during fecal specimen collection.

2.2. Collection and processing of fecal specimens

From April to August 2023, a total of 390 mixed mouse fecal specimens were collected from mouse cages (one specimen each cage) involving approximately 2000 laboratory mice in two medical experimental animal centers (respectively named Center A and Center B for convenient description) in Heilongjiang Province in the northeast of China. All the mice were fed with sterilized water and pelleted chow and each cage containing 5–7 mice was individually ventilated. Mouse feces with bedding materials were caged-removed regularly and then handled with harmless treatment. In the present study, all mixed fecal specimens were cage-sampled by randomly selecting approximately 120 mouse fecal pellets (approximately 2 g) from one cage. Each mixed specimen was homogenized with distilled water and then centrifuged at 1500 g for 10 min. Each obtained sediment was stored in a refrigerator at –20 °C for subsequent molecular analysis. At the time of sampling, we did not record animal age, gender and strain.

2.3. DNA extraction and PCR amplification

Genomic DNA was extracted from 180 to 200 mg of each processed fecal specimen using the QIAamp Fast DNA Stool Mini Kit (QIAgen, Hilden, Germany) according to the manufacturer's recommended procedures. The procedure is comprised of two major steps. In the first steps, fecal specimens are lysed in InhibitEX Buffer and during lysis, DNA-degrading substances and PCR inhibitors present in the fecal specimens are separated from the DNA by the InhibitEX buffer. In the second steps, the QIAamp DNA purification procedure involves digestion of proteins, binding DNA to the QIAamp silica membrane, washing away impurities and eluting pure DNA from the spin column. The extracted DNA was stored at –20 °C until PCR analysis.

E. bieneusi was identified and genotyped by nested PCR amplification of an approximately 410 bp fragment of the rRNA gene including the ITS region, as previously designed by Mirjalali et al. (2015). Two pairs of primers were used: forward primer EbGeno-fe (5′-TTC AGA TGG TCA TAG GGA TG-3′) and reverse primer EbGeno-re (5′-ATT AGA GCA TTC CGT GAG G-3′) used in the first PCR, forward primer EbGeno-fi (5′-TCG GCT CTG AAT ATC TAT GG-3′) and reverse primer EbGeno-ri (5′-ATT CTT TCG CGC TCG TC-3′) in the second PCR. Each specimen was subjected to at least two parallel PCR reactions. A positive control (DNA of human-derived genotype Type IV) and a negative control (DNase-free water) were included in each PCR test. 2 × TransTaq®-T PCR SuperMix (+dye) (TransGen Biotech Co., Beijing, China) was used for all the PCR reactions. All secondary PCR products were subjected to electrophoresis in 1.5% agarose gel dyed with GelStrain (TransGen Biotech, Beijing, China) and visualized under UV by the Gel Doc™ EZ Gel Documentation System (Bio-Rad, USA).

2.4. Nucleotide sequencing and analyzing

All expected size secondary PCR products were sent to Comate Bioscience Company Limited (Jilin, China) for nucleotide sequencing using the secondary PCR primers on an ABI PRISM 3730XL DNA Analyzer, using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). Sequence accuracy was assured by bi-directional sequencing. To determine *E. bieneusi* genotypes, nucleotide sequences obtained in the present study were manually assembled, aligned and analyzed with each other and reference sequences deposited in the GenBank database by using Clustal X 1.81 (http://www.clustal.org/) and Basic Local Alignment Search Tool (BLAST). A site was identified as ambiguous when double peaks occurred in the same position in both nucleotide strands. For the DNA specimens suffering from sequence chromatograms with double peaks, each primary PCR product was diluted 1:100 with sterile deionized water, followed by secondary PCR amplification (five parallel reactions per sample) and sequencing. For the DNA specimens with only one PCR reaction positive for *E. bieneusi,* each of them was subjected to two parallel PCR reactions again to avoid missing co-infection cases.

All the genotypes obtained in the present study were identified only

based on nucleotide sequence of the ITS region of the rRNA gene of *E. bieneusi*, according to the established nomenclature system (Santin and Fayer, 2009). Those sequences identical to published ones were determined as known genotypes and given the first published names. In contrast, the sequences different from published ones were considered novel genotypes and given their names by adding the Arabic numbers behind abbreviation HLJLM (Heilongjiang laboratory mouse). All novel genotypes were confirmed by sequencing another two separate PCR products.

2.5. Phylogenetic analysis

The ITS sequences of the selected novel genotypes of *E. bieneusi* obtained in the present study and known ones downloaded in GenBank database were implemented into Mega 11 software (http://www.megasoftware.net/) to analyze their genetic relationship. A maximum likelihood (ML) tree was constructed based on the substitution rates calculated by the Tamura-Nei model. Bootstrap analysis with 1000 replicates was used to assess the reliability of the phylogenetic tree. A nucleotide sequence of *E. bieneusi* from a kangaroo (GenBank: KY706128) was used as an outgroup in phylogenetic analysis.

2.6. Nucleotide sequence accession numbers

Representative nucleotide sequences of the novel ITS genotypes of *E. bieneusi* obtained in the present study were deposited in the GenBank database under the following accession numbers of PQ570018–PQ570047.

3. Results

3.1. Positive rate of E. bieneusi

A total of 390 mixed fecal specimens from laboratory mice were screened for the presence of *E. bieneusi* by nested PCR amplification of the ITS region of the rRNA gene (Fig. 1). Seventy-one PCR-positive specimens (18.2%, 71/390) were successfully sequenced and confirmed positive for *E. bieneusi* (Table 1). This pathogen was detected in both medical experimental animal centers.

3.2. Genetic characterization of E. bieneusi and genotype distribution by sampling site

In total, 177 clean sequences of the ITS region of the rRNA gene were obtained out of 71 *E. bieneusi*-positive specimens. By aligning all the sequences (at least two) from each positive specimen, 55 (77.5%) and 16 (22.5%) of 71 positive specimens belonged to mono-infection and co-infection of *E. bieneusi* genotypes, respectively. Detailed flow chart of strategy for PCR amplification was shown in Fig. S2.

Sequence analysis identified 38 genotypes and 49 polymorphic sites were observed among them (Fig. 2). These identified genotypes were composed of eight known genotypes—EbpC ($n = 36$), D ($n = 6$), Peru8 ($n = 5$), Henan-III, CS-4, CHC5, ETMK4 and SHWR14 (one each) and 30 novel genotypes namely HLJLM7 ($n = 5$), HLJLM2 ($n = 3$), HLJLM1, HLJLM3 to HLJLM6 and HLJLM8 to HLJLM30 (one each) (Table 1). Genotype EbpC showed an absolute dominance in positive specimens from two centers investigated (50.7%, 36/71). The zoonotic genotypes (EbpC, D, Peru8, Henan-III and CS-4) were found in 64.8% (46/71) of the positive specimens.

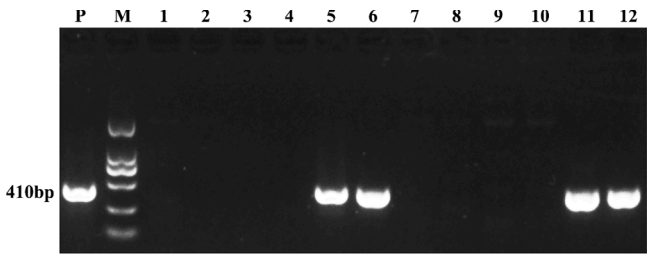

Fig. 1. Nested PCR amplification of the partial rRNA gene of *E. bieneusi* in laboratory mice. P: Positive control. M: DL 2000 DNA marker. Lanes 1–12: Mixed fecal specimens.

The difference in genotype constitution was found between two medical experimental animal centers: six known genotypes (EbpC, D, Peru8, CS-4, Henan-III and CHC5) and 14 novel genotypes (HLJLM1 to HLJLM9, HLJLM19, HLJLM20, HLJLM22, HLJLM24 and HLJLM25) in Center A; three known genotypes (EbpC, ETMK4 and SHWR14) and 17 novel genotypes (HLJLM7, HLJLM10 to HLJLM18, HLJLM21, HLJLM23 and HLJLM26 to HLJLM30) in Center B (Table 1). It was observed that only genotypes EbpC and HLJLM7 appeared in both two sampling sites.

Among 30 novel genotypes, the vast majority (86.7%, 26/30) of them had the largest similarities to four zoonotic genotypes EbpC (98.4−99.6%), D (98.8−99.6%), Henan-III (98.3−99.6%) and EbpD (99.6%). Detailed results of homology analysis of the ITS region of the rRNA gene of the novel *E. bieneusi* genotypes were summarized in Table 2.

3.3. Phylogenetic relationship of E. bieneusi genotypes

Based on the fact that some novel genotypes had the same similarities to known genotypes, only the ITS sequences of 13 novel genotypes were used to phylogenetic analysis. Among them, genotypes HLJLM2, HLJLM17 and HLJLM6 represented 15, two and three genotypes, respectively.

All novel genotypes fell into Group 1, and they were further classified into three different subgroups. Six of them (HLJLM6, HLJLM9, HLJLM13, HLJLM14, HLJLM23 and HLJLM30) were in subgroup 1a together with four known genotypes (D, Peru8, ETMK4 and SHWR14). Twenty-three of them belonged to subgroup 1d together with four known genotypes (EbpC, Henan-III, CS-4 and CHC5), and the remaining one (HLJLM8) fell into subgroup 1e. Meanwhile, it was also observed that the sequences from different rodent hosts including those obtained in the present study were classified into five different subgroups (1a, 1d, 1e, 1 h, 1i) (Fig. 3).

4. Discussion

Epidemiological data demonstrate *E. bieneusi* is a common intestinal pathogen infecting rodents. However, limited information is available regarding *E. bieneusi* infection in laboratory rodents. For mice used widely in laboratories, to date, there has been only one study identifying and genotyping *E. bieneusi*. In the present study, *E. bieneusi* was found in laboratory mice in two medical experimental animal centers, China. Mice were found to have a higher positive rate of *E. bieneusi* (71/390) compared to the only one investigation of *E. bieneusi* conducted in laboratory mice from four cities, China (1.8%, 18/1027) (Wang et al., 2022). The higher positive rate of *E. bieneusi* might be due to the experiment design (cage-sampled). If one mouse was infected with *E. bieneusi* in one cage, the mixed specimen could be positive. In fact, immune status of hosts seems to be the key factor for *E. bieneusi* infection, and it is closely related to host ages. Usually, younger animals are susceptible to *E. bieneusi* infection due to their underdeveloped immune systems. In two recent studies of molecular identification of *E. bieneusi* conducted in rodents, China, juvenile animals were observed to have higher infection rates than adult ones: 13.6% aged 1–3 months vs 9.0% aged 4–10 months for hamsters; 23.8% aged 1–3 months vs 11.8% aged 4–8 months for guinea pigs; 12.4% aged 4–6 weeks vs 8.5% aged 7–10 weeks for fancy rats (Lv et al., 2022; Wang et al., 2020). Actually, the infection rate of *E. bieneusi* in laboratory animals is a complicated issue. Except for the factors mentioned above, there are some other factors possibly influencing the infection rate of *E. bieneusi*, such as sample size,

Table 1
Infection rate and genotype distribution of *E. bieneusi* in laboratory mice.

Location	Examined no.	Positive specimens				
		Number (%)	Zoonotic[a] (n)		Non-zoonotic (n)	
			Mono-genotype	Co-infection genotype	Mono-genotype	Co-infection genotype
Center A	146	47 (32.2)	EbpC (19); D (4); Peru8 (2)	EbpC + CHC5 (1); EbpC + CS-4 (1); EbpC + Henan-III (1); EbpC + HLJLM7 (1); EbpC + HLJLM8 (1); EbpC + HLJLM20 (1); D + Peru8 (1); D + HLJLM7 (1); Peru8 + HLJLM2 (1); Peru8 + HLJLM22 (1)	HLJLM1 (1); HLJLM2 (2); HLJLM3–HLJLM7 (one each); HLJLM9 (1); HLJLM19 (1); HLJLM24 (1); HLJLM25 (1)	–
Center B	244	24 (9.8)	EbpC (7)	EbpC + HLJLM16 (1); EbpC + HLJLM18 (1); EbpC + HLJLM21 (1); EbpC + HLJLM27 + HLJLM28 (1)	ETMK4 (1); SHWR14 (1); HLJLM7 (2); HLJLM10 (1); HLJLM11 (1); HLJLM15 (1); HLJLM17 (1); HLJLM23 (1); HLJLM29 (1); HLJLM30 (1)	HLJLM12 + HLJLM26 (1); HLJLM13 + HLJLM14 (1)
Total	390	71 (18.2)	EbpC (26); D (4); Peru8 (2)	EbpC + CHC5 (1); EbpC + CS-4 (1); EbpC + Henan-III (1); EbpC + HLJLM7 (1); EbpC + HLJLM8 (1); EbpC + HLJLM16 (1); EbpC + HLJLM18 (1); EbpC + HLJLM20 (1); EbpC + HLJLM21 (1); D + Peru8 (1); D + HLJLM7 (1); Peru8 + HLJLM2 (1); Peru8 + HLJLM22 (1); EbpC + HLJLM27 + HLJLM28 (1)	ETMK4 (1); SHWR14 (1); HLJLM1 (1); HLJLM2 (2); HLJLM3–HLJLM6 (one each); HLJLM7 (3); HLJLM9–HLJLM11 (one each); HLJLM15 (1); HLJLM17 (1); HLJLM19 (1); HLJLM23–HLJLM25 (one each); HLJLM29 (1); HLJLM30 (1)	HLJLM12 + HLJLM26 (1); HLJLM13 + HLJLM14 (1)

The bars denote negative results at the locus.
[a] Among fecal specimens positive for *E. bieneusi*, those specimens in which at least one zoonotic genotype was identified are considered zoonotic ones.

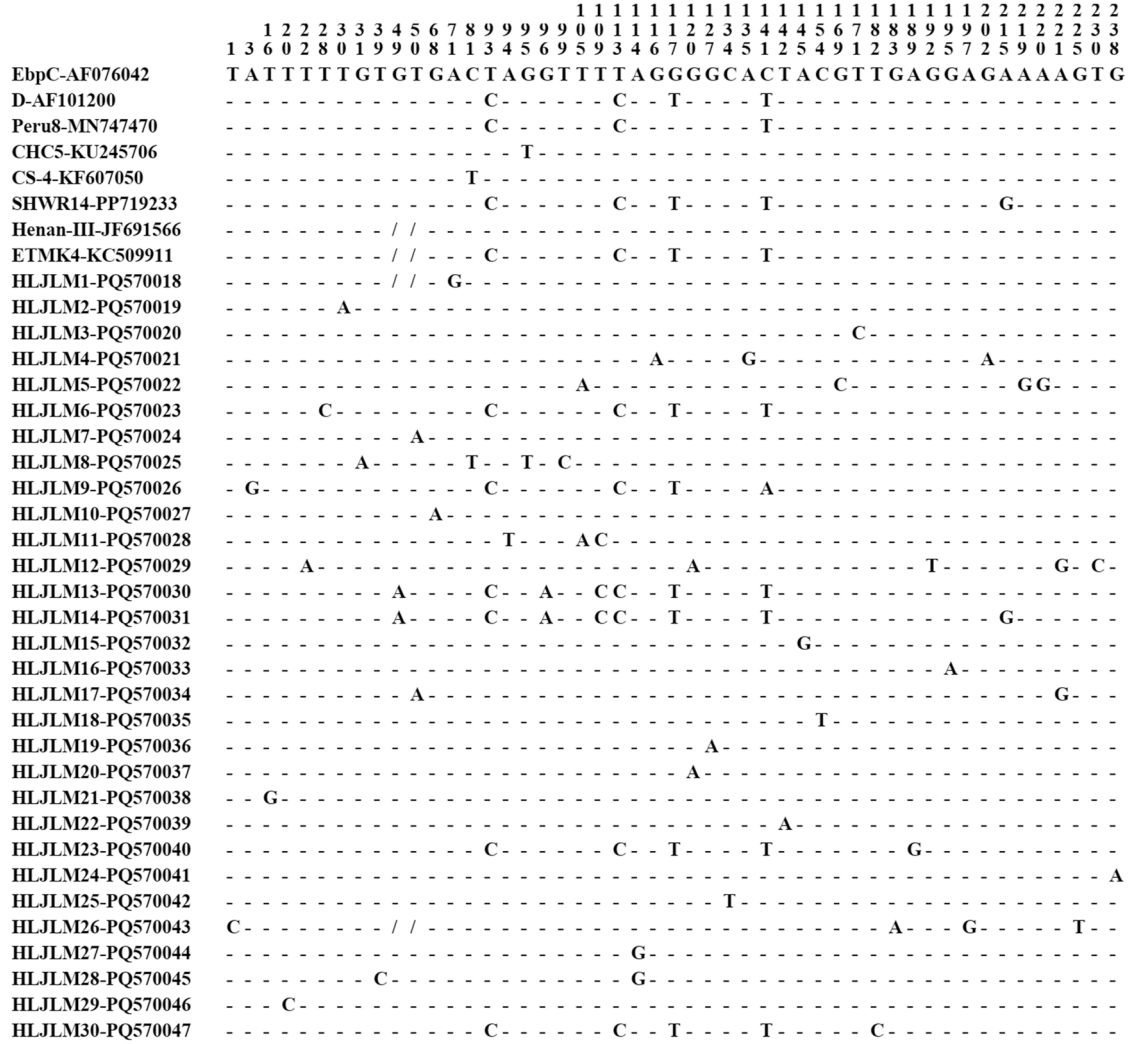

Fig. 2. Nucleotide variation in the ITS region of the rRNA gene of *E. bieneusi* genotypes identified in the present study. Dashes indicate the same base identity as the ITS gene sequence of genotype EbpC.

Table 2
Homology analysis of the novel *E. bieneusi* genotypes in the ITS region of the rRNA gene.

Genotype (Accession No.[a])	Homology (%)	Genotype (Accession No.[b]): Nucleotide (position)
EbpC (AF076042)	99.6	**HLJLM2 (PQ570019)**: T to A (30); HLJLM3 (PQ570020): T to C (171); HLJLM7 (PQ570024): T to A (50); HLJLM10 (PQ570027): G to A (68); HLJLM15 (PQ570032): A to G (145); HLJLM16 (PQ570033): G to A (195); HLJLM18 (PQ570035): C to T (154); HLJLM19 (PQ570036): G to A (127); HLJLM20 (PQ570037): G to A (120); HLJLM21 (PQ570038): T to G (16); HLJLM22 (PQ570039): T to A (142); HLJLM24 (PQ570041): G to A (238); HLJLM25 (PQ570042): C to T (134); HLJLM27 (PQ570044): A to G (114); HLJLM29 (PQ570046): T to C (20)
	99.2	**HLJLM17 (PQ570034)**: T to A (50); A to G (221); HLJLM28 (PQ570045): T to C (39); A to G (114)
	98.4	**HLJLM5 (PQ570022)**: T to A (105); G to C (169); A to G (219); A to G (220)
D (AF101200)	99.6	**HLJLM6 (PQ570023)**: T to C (28); HLJLM23 (PQ570040): A to G (189); HLJLM30 (PQ570047): T to C (182)
	99.2	**HLJLM9 (PQ570026)**: A to G (3); T to A (141)
	98.8	**HLJLM13 (PQ570030)**: G to A (49); G to A (96); T to C (109)
Henan-III (JF691566)	99.6	**HLJLM1 (PQ570018)**: A to G (69)
	98.3	**HLJLM26 (PQ570043)**: T to C (1); G to A (181); A to G (195); G to T (223)
EbpD (AF076043)	99.6	**HLJLM8 (PQ570025)**: T to C (99)
BLC10 (MN758749)	99.2	**HLJLM4 (PQ570021)**: G to A (116); A to G (135)
XZP-I (MG729499)	99.2	**HLJLM11 (PQ570028)**: A to T (94); T to A (105)
SHWR14 (PP719233)	98.8	**HLJLM14 (PQ570031)**: G to A (49); G to A (96); T to C (109)
BLC3 (MN758742)	98.4	**HLJLM12 (PQ570029)**: T to A (22); G to A (120); G to T (192); A to G (221)

Note: The genotypes in bold are novel genotypes occurred in the phylogenetic tree.

[a] Accession Nos. indicate the published sequences with the largest similarity to the sequences obtained in the present study.

[b] Accession Nos. indicate sequences obtained in the present study for the first time.

management system, population density and so on.

Increasing ITS genotyping data has identified over 900 *E. bieneusi* genotypes with intraspecific variability and provides deeper understanding of the zoonotic nature of the pathogen. The present sequence analysis revealed a high genotype diversity of *E. bieneusi* in laboratory mice investigated in the present study. Eight known and 30 novel genotypes were identified. Among them, five known genotypes (D, EbpC, Peru8, Henan-III and CS-4) have been documented in humans (Koehler et al., 2022). Genotypes D and EbpC are the two most common genotypes in human cases of microsporidiosis caused by *E. bieneusi*, constituting the two largest shares (29.1% and 14.5%), respectively (Wang et al., 2024). Both of them have a broad host range and geographical distribution. To date, genotype D has been identified in 93 host species (including 75 mammal and 18 bird species) distributing in 41 countries on all continents except Antarctica (Koehler et al., 2022; Zhao et al., 2022; Liu et al., 2024; Naguib et al., 2022). Genotype EbpC has been found in 46 host species (including 41 mammal, one bird, two reptile and one amphibian species as well as one insect species) in 16 countries distributing in Europe, Asia, North America and South America (Koehler et al., 2022; Dashti et al., 2022; Zhao et al., 2023; An et al., 2024; Chen et al., 2024). Epidemiological studies of *E. bieneusi* revealed that these two genotypes were also frequently identified in multiple rodent species from different sources, such as wild rats and beavers, farmed porcupines, pet squirrels as well as laboratory rodents (mice and rats) (Table S1). Compared with genotypes D and EbpC, the other three zoonotic genotypes (Peru8, Henan-III and CS-4) were observed to have a relatively narrow host range and a limited geographical distribution. To date, genotype Peru8 has only been detected in 15 host species (14 mammal and one bird species) in eight countries distributing in Europe, Asia, Africa and South America (Koehler et al., 2022; Naguib et al., 2022), including wild rats (China) and wild house mice (Germany) (Sak et al., 2011; Gui et al., 2020; Zhao et al., 2020; Feng et al., 2024). Genotype Henan-III has only been found in eight host species in two countries (China and Malaysia) while CS-4 in six host species only in China (Jiang et al., 2015; Zhou et al., 2020; Koehler et al., 2022; Jiang et al., 2024b; Zhang et al., 2024; Jian et al., 2024). Meanwhile, these two genotypes were also identified in some rodent species: Henan-III in wild house rats and farmed bamboo rats (Zhao et al., 2023; Zhang et al., 2024) and CS-4 in wild brown rats (Jiang et al., 2024b). These observations above suggested that rodents may play an important role in the epidemiology of *E. bieneusi* as reservoir hosts or vectors. Our finding of a higher positive rate (18.2%) of *E. bieneusi* in laboratory mice and a larger proportion (64.8%) of zoonotic genotypes in positive specimens indicated that laboratory mice infected with *E. bieneusi* may pose a zoonotic threat to individuals working in the medical experimental animal centers, especially those having close contact with these infected animals. The remaining three known genotypes (SHWR14, ETMK4 and CHC5) have only been found in animals till now: SHWR14 in brown rats (Jiang et al., 2024b); ETMK4 in cats (Mori et al., 2013); CHC5 in pigs, cattle, wild boars and masked palm civets (Koehler et al., 2022; Zhao et al., 2021). The present study identified genotypes ETMK4 and CHC5 in rodents for the first time, indicating that these two genotypes might have more extensive host range than excepted.

A phylogenetical analysis based on a ML tree of *E. bieneusi* ITS gene sequences showed the genetic relationship between the novel genotypes and the known ones. Thirty novel genotypes identified in the present study fell into zoonotic Group 1. Moreover, 86.7% (26/30) of the novel genotypes exhibited the largest similarities to zoonotic genotypes (EbpC, D, Henan-III and EbpD). Currently, Group 1 genotypes are further subdivided into ten subgroups designated as 1a to 1j and zoonotic genotypes could be found in eight subgroups (1a–1h), mainly in subgroups 1a–1e (Li et al., 2019; Koehler et al., 2022; Ni et al., 2021; Fan et al., 2021). In subgroup analysis, all the novel genotypes obtained in the present study fell into three subgroups (1a, 1d, 1e), further indicating their large zoonotic potential. However, because some genotypes in these three subgroups were only detected in animals, their host range and zoonotic potential need to be assessed by more molecular epidemiological investigations of *E. bieneusi* in humans and animals in the future. In phylogenetic analysis, there were no relationships between geographical locations or rodent hosts and genotypes due to the genotypes of different rodent hosts appearing in the five subgroups (1a, 1d, 1e, 1 h, 1i).

The genetic variations of the ITS region of mouse-derived *E. bieneusi* were observed in the present study. By sequence analysis, 49 polymorphic sites (1–100: 19 sites; 101–200: 22 sites; 201–243: 8 sites) were observed among 38 genotypes obtained in the present study, beginning at the first position and ending at the 238th position in the ITS region of the rRNA gene (Fig. 2). The genotyping data further confirmed a high degree of genetic diversity in the ITS region within *E. bieneusi*. Early in 2002, Buckholt et al. proposed that all the 243 nucleotides in the ITS region could be found to be polymorphic if enough *E. bieneusi* isolates were sequenced (Buckholt et al., 2002). It is known that the *E. bieneusi* genotypes usually have 243 bp in the ITS region. However, with the increasing genotyping date of *E. bieneusi*, the length variations were noted for the genotypes in this region from 240 bp to 245 bp. For example, the ITS region is 240 bp for genotype EbCar1 (MG458706), 241 bp for genotypes Henan-III (JF691566) and WL6 (OP103977), 242 bp for genotypes CAF4 (DQ683749) and SYSWR28 (PP719273), 244 bp

Fig. 3. Phylogenetic relationships among various *E. bieneusi* genotypes are inferred using a maximum likelihood analysis of the ITS rRNA gene sequences based on substitution rates calculated by the Tamura-Nei model. Bootstrap values derived from 1000 replicates and displayed on the branches. Each sequence is identified by its accession number, host origin and genotype designation. The group terminology for the clusters is based on the work of Li et al. (2019). Black circles and black triangles indicate known and novel genotypes identified in the present study, respectively. We selected only one of those novel genotypes having the same similarity to some known genotype. Finally, 13 novel genotypes which represented all novel ones were shown in the ML tree.

for genotypes HNM-V (MK139698) and HNP-I (MN630621), 245 bp for genotype Q (AF267147). In the present study, we obtained two novel genotypes HLJLM1 (PQ570018) and HLJLM26 (PQ570043) with 241 bp in this region.

Medical experimental animal centers generally have high sanitary conditions and standards. According to the Chinese national standard: Laboratory animal–Environment and housing facilities (GB 14925–2023), laboratory animals including mice are all provided with sterilized water and pelleted chow. However, even under this condition, the present study detected *E. bieneusi* in laboratory mice in two medical experimental animal centers. Currently, the infection or contamination source of *E. bieneusi* is still unclear and confusing. Considering that the infection of *E. bieneusi* is most likely from living environments, further work should focus on the epidemiological investigations of *E. bieneusi* in environmental specimens. To date, besides our study, there have been two studies identifying *E. bieneusi* in laboratory rats, mice and guinea pigs (Li et al., 2020; Wang et al., 2022). Although no studies mentioned whether *E. bieneusi* affected the experimental results, we might as well advice using laboratory animals negative for *E. bieneusi* when conducting scientific experiments.

5. Conclusion

The present study demonstrated a high positive rate and genotype diversity of *E. bieneusi* in laboratory mice in two medical experimental animal centers in Heilongjiang Province, China. Eight known and 30 novel genotypes were identified in these mice, with EbpC being predominant. We found genotypes CHC5 and ETMK4 in rodents for the first time, revealing their potential on expanding host range. The identification of five zoonotic genotypes accounting for 64.8% of positive specimens and the fact of all 30 novel genotypes falling into three subgroups (1a, 1d, 1e) of Group 1 indicated the potential of laboratory mice in the transmission of *E. bieneusi* to humans. Health education should be carried out to make people having close contact with laboratory mice and their feces aware of the risk of zoonotic transmission of *E. bieneusi* and the importance of public health. Meanwhile, further efforts should be made to seek for the sources of infection or contamination and get rid of them to ensure the accuracy of the experimental results as well as human and animal health within laboratories.

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CRediT authorship contribution statement

Lan Yao: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis. **He Li:** Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis. **Xinyu Hu:** Formal analysis. **Zhen Li:** Formal analysis. **Haowen Dong:** Validation. **Yanyan Jiang:** Writing – review & editing, Supervision, Resources, Project administration. **Jianping Cao:** Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2025.107585.

Data availability

Data will be made available on request.

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