



Enterocytozoon bieneusi in Diarrheal and Asymptomatic Children in Southern Zhejiang Province, China: Prevalence and Genetic Diversity

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Received: 26 November 2025 / Accepted: 9 April 2026

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Abstract

Purpose *Enterocytozoon bieneusi* (*E. bieneusi*) is a globally prevalent enteric pathogen that poses a major threat to children's health. This study aimed to investigate the prevalence, genotypic characteristics and zoonotic transmission potential of *E. bieneusi* in children under 6 years of age in Wenzhou, Zhejiang, China, where pediatric infection data of this pathogen remains absent.

Methods A total of 1,032 stool samples were collected from children at Wenzhou Yuying Children's Hospital, including 684 from diarrheal individuals and 348 from asymptomatic controls. Nested polymerase chain reaction (PCR) and sequencing, targeting the internal transcribed spacer (ITS) region of the *E. bieneusi* small subunit (SSU) ribosomal RNA (rRNA) gene, were conducted for pathogen identification and genotyping.

Results The overall prevalence of *E. bieneusi* was 1.4% (14/1032), with 1.6% in diarrheal children and 0.9% in asymptomatic children. Two zoonotic *E. bieneusi* genotypes were identified, namely D (n=5) and Peru11 (n=9).

Conclusion This study provides the first molecular confirmation of *E. bieneusi* infection in children in Zhejiang Province, China, despite a relatively low infection rate. The detection of zoonotic genotypes D and Peru11 indicates potential animal to-human transmission of the pathogen, highlighting the need for further epidemiological investigation and strengthened preventive measures.

Keywords *Enterocytozoon bieneusi* · children · ITS region · Genotyping · Zoonotic · China

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Introduction

Enterocytozoon bieneusi (*E. bieneusi*) is a widely recognized intestinal pathogen that frequently causes diarrhea in both humans and animals [7, 14]. In humans, the clinical manifestations associated with *E. bieneusi* infection include diarrhea, fatigue, abdominal pain, and other symptoms. Nourrisson et al., [14]. Globally, the estimated prevalence of *E. bieneusi* infection in humans is approximately 6.59% [21]. Human infections with *E. bieneusi* can occur via multiple routes, including direct contact with infected individuals (human-to-human transmission), exposure to animals (animal-to-human transmission), and the ingestion of contaminated water and food sources [9]. These aspects have attracted substantial attention in public health and veterinary medicine research. Moreover, *E. bieneusi* has been classified as a Category B biodefense pathogen by the National Institutes of Health (NIH), which emphasizes its potential menace to public health. Additionally, it is included in the Environmental Protection Agency's catalog of microbial contaminants, which accentuates the risk of waterborne transmission related to this organism [28].

Children exhibit heightened susceptibility to infection by *E. bieneusi*, and asymptomatic infections may occur with relative frequency [12, 21]. Empirical data indicate that *E. bieneusi* has been linked to substantial declines in linear anthropometric z-scores, suggesting its detrimental influence on the growth and development of children [4, 16]. Nevertheless, in specific countries and regions, the prevalence and transmission patterns of this pathogen among children are inadequately documented, resulting in an underestimation of its adverse effects. Monitoring the infection status of *E. bieneusi* in vulnerable populations is of utmost importance for effectively alleviating the associated health burden and minimizing potential public health risks.

The transmission routes and potential sources of *E. bieneusi* infections have been ascertained and traced via molecular techniques through the comparison of genotypes across diverse hosts and geographical areas [9]. By conducting PCR analysis on the internal transcribed spacer (ITS) region within the small subunit of the ribosomal RNA (*SSU rRNA*) gene of *E. bieneusi*, researchers have identified over 860 distinct ITS genotypes [14, 28]. Subsequently, based on comprehensive analyses, these genotypes have been classified into 13 unique phylogenetic groups [9, 28]. Notably, a minimum of 56 genotypes have been identified in both human and animal populations, and they are predominantly concentrated within groups 1 or 2, which further substantiates the hypothesis of zoonotic potential [28].

In recent years, *E. bieneusi* has been detected in human populations across various provinces and municipalities in China, with reported prevalence ranging from 0.2% to

22.5% [20, 27]. Nevertheless, the prevalence and transmission patterns of *E. bieneusi* in localized settings are poorly understood. Wenzhou, a coastal city in southern Zhejiang with intensive livestock production, high population density and close human-animal contact, is a high-priority area for surveillance. Previous investigations have confirmed *E. bieneusi* infection in diverse animal hosts in Wenzhou, with multiple genotypes of zoonotic potential ([30]; Zhang et al., 2024; [22]). Also, *E. bieneusi* has been detected in diarrheal adults in this region [6]. Notably, data on *E. bieneusi* infection in children in Zhejiang are still lacking. Therefore, this study was designed to investigate the prevalence of *E. bieneusi* in children from Wenzhou City, southern Zhejiang Province, China, and to characterize its genetic features at the genotype level using molecular techniques.

Materials and Methods

Sample Collection

This study adopted a cross-sectional approach utilizing convenience sampling methodology to determine the occurrence of *E. bieneusi* in children residing in southern Zhejiang Province, China. The sample size was determined utilizing the formula introduced by Thrusfield (Thrusfield et al., 1997), taking into account 95% confidence intervals and a 5% precision value. Drawing from previously documented prevalence rates of *E. bieneusi* in children in China, an anticipated maximum prevalence of 22.5% was presumed [27]. Applying this formula, we have computed a minimum sample size of 268 children for our study area.

Between March 2021 and January 2022, a total of 1032 fecal samples from children were collected at the Laboratory Department of Yuying Children's Hospital in Wenzhou, China. The parents/guardians of these children were instructed on the appropriate technique for collecting fecal samples using a plastic fecal collector. Based on the distinctive characteristics of the feces, we can assess whether these children are experiencing diarrhea. Diarrhea was defined as watery stools or loose stools at the sampling time. Among these, 348 children were asymptomatic, whereas 684 presented with diarrhea. All stool collection containers were labeled with the date of collection, the age and sex of the child. To minimize selection bias, samples were randomly collected from eligible children. No demographic or clinical data were collected except age and gender. All participants were under six years of age. Children receiving antibiotic treatment or with immunodeficiency were excluded. Samples were stored at 4 °C and processed within 24 h.

DNA Extraction

Genomic DNA was extracted from all the 1032 stool samples with a weight ranging from 180 to 200 mg using the QIAamp DNA Stool Mini Kit provided by QIAgen (QIAgen, Hilden, Germany) (Zhao et al. [29]). Throughout the entire extraction procedure, strict compliance with the manufacturer's instructions was maintained. After extraction, DNA concentration and purity were measured with a NanoDrop spectrophotometer to ensure sufficient quality and quantity for downstream PCR amplification. The extracted DNA was then stored at -20 °C for subsequent utilization.

PCR Amplification

Enterocytozoon bieneusi was detected through nested PCR amplification of an approximately 389-bp fragment that encompasses 76 bp of the 3' end of the *SSU rRNA* gene, 243 bp of the internal region, and 70 bp of the 5' region of the large-subunit (*LSU rRNA* gene). The primers and PCR cycle settings for this PCR assay were previously reported [2]. The external primers utilized were EBIT3: (5'-GGTCATAGGGATGAAGAG) and EBIT4: (5'-TTCGAGTTCTTCGCGCTC), whereas the internal primers were EBIT1: (5'-GCTCTGAATATCTATGGCT) and EBIT2: (5'-ATCGCCGACGGATCCAAGTG). The two sets of cycling parameters consisted of 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 40 s, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s, with both sets including a final extension step at 72 °C for 10 min. All PCRs were performed in a 25 µL reaction mixture using TaKaRa Taq DNA Polymerase (TaKaRa Bio Inc., Tokyo, Japan). Double-distilled water without DNA template was used as the no-template negative control, and genomic DNA of *E. bieneusi* genotype BEB6 was used as the positive control. Both positive and negative controls were included in every PCR assay to ensure the accuracy and reproducibility of the results. After the secondary PCR amplification, the amplified products were electrophoresed by loading them onto a 1.5% agarose gel stained with GelRed (Biotium Inc., CA, USA).

Sequence Analyses

All the PCR-positive samples were promptly dispatched to Sangon Biotech Co., Ltd., situated in Shanghai, China, for bidirectional sequencing. The obtained nucleotide sequences were subsequently edited using DNASTAR Lasergene v7.10 (<http://www.dnastar.com/>). Furthermore, we utilized Clustal X v2.1 (<http://www.clustal.org/>) to accurately determine the genotypes of *E. bieneusi* by aligning them with reference sequences obtained from the National

Center for Biotechnology Information (NCBI) through the Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Phylogenetic Analysis

The Neighbor-joining (NJ) approach coupled with the Kimura-2-parameter model in MEGA X (<http://www.megasoftware.net/>) [8] was employed to definitively classify the genogroups and establish the genetic relationships among the ITS genotypes of *E. bieneusi*. The robustness of the constructed trees was rigorously assessed through bootstrap analysis involving 1000 replications, ensuring their reliability.

Statistical Analysis

A chi-square (χ^2) test was performed in SPSS version 22.0 (SPSS Inc, Chicago, IL, USA) to analyze the prevalence of *E. bieneusi* in the sex and symptom groups, respectively. A *P* value threshold of less than 0.05 was used to determine statistical significance.

Results

Prevalence of *E. bieneusi*

After conducting an analysis of the ITS region of the *SSU rRNA* gene, *E. bieneusi* was identified in 1.4% of all the samples (14/1032). Notably, *E. bieneusi* was more common in children with diarrhea (1.6%, 11/684) than in asymptomatic children (0.9%, 3/348), but the difference was not statistically significant ($\chi^2=0.96$, $P=0.327$). Additionally, the prevalence of *E. bieneusi* was slightly greater in males (1.6%, 9/571) than in females (1.1%, 5/461), but this difference was not statistically significant ($\chi^2=0.667$, $P=0.414$).

Genetic Characterization and Distribution of *E. bieneusi* Genotypes

Sequencing analysis revealed the presence of two distinct genotypes of *E. bieneusi*, designated Peru11 ($n=9$) and D ($n=5$), based on ITS sequence consistency. Specifically, the genotype Peru11 was found in six children with diarrhea and three asymptomatic children, whereas genotype D was exclusively detected in five children with diarrhea. The genotypes were uniformly distributed across both male and female populations. Specifically, within the male and female groups, six instances of genotype Peru11 and three instances of genotype D were identified in males, while

three instances of genotype Peru11 and two instances of genotype D were identified in females (Table 1).

Phylogenetic Analysis

Both genotypes D and Peru11 clustered into Group 1, the main zoonotic group of *E. bieneusi*, which has a wide host range and strong cross-species transmission potential. This result confirms the zoonotic risk of isolates in this study. The low genetic diversity suggests limited local transmission sources and relatively stable population structure of *E. bieneusi* in Wenzhou (Fig. 1).

Nucleotide Sequence Accession Numbers

The nucleotide sequences obtained from our current research endeavors have been duly registered in the GenBank database with the specific accession numbers PQ404861 assigned to genotype D and PQ404862 designated for genotype Peru11.

Discussion

This study presents the initial data regarding the prevalence and genetic diversity of *E. bieneusi* isolates obtained from children in Wenzhou City, Southern Zhejiang Province, China. The prevalence of *E. bieneusi* infection among children stood at 1.4% (14/1032). Remarkably, in comparison with asymptomatic children (0.9%), those with diarrhea exhibited a higher infection rate of 1.6%. These findings are consistent with those reported from Henan, where the infection rate of *E. bieneusi* among children was 1.2% [24]. Nevertheless, certain regions in China have documented relatively elevated infection rates of *E. bieneusi* among children. These regions encompass Hainan (3.7%), Xinjiang (5.9%), Heilongjiang (7.5%), Chongqing (11.8%), and Jilin (22.5%) [3, 15, 23, 25, 27]. Conversely, a research carried out in Hubei Province reported a comparatively low infection rate of 0.2% [20]. Similar to the results observed

in China, studies conducted in various other countries also show considerable disparities. Specifically, in Uganda, studies have reported infection rates of *E. bieneusi* among children ranging from 6.6 to 76.9% [18]. Likewise, in Nigeria, three independent studies respectively reported that the infection rates of *E. bieneusi* among children were 6.4%, 9.3%, and 39.6% [1, 5, 11]. The above facts fully reveal the complexity of comparing infection rates between different countries or regions and the challenges they face. In addition to geographical location, the infection rate is also affected by many other variables, such as the accuracy of the detection method, the sample size, the differences in the living environment of children, and the health status of children [25]. These factors interact with each other, making it extremely difficult to determine the true infection rates of children, other populations, and even other hosts in a specific country or region. Therefore, to more accurately understand the real situation of the infection rate, larger, more in-depth, and continuous studies are needed.

Similar to other opportunistic pathogens, immune status is one of the primary factors influencing the prevalence of *E. bieneusi*. Consequently, it is postulated that individuals infected with HIV exhibit higher infection rates compared to HIV-negative individuals [21]. In the study, none of the children exhibited immunodeficiency, which might serve as one of the factors contributing to the low infection rate.

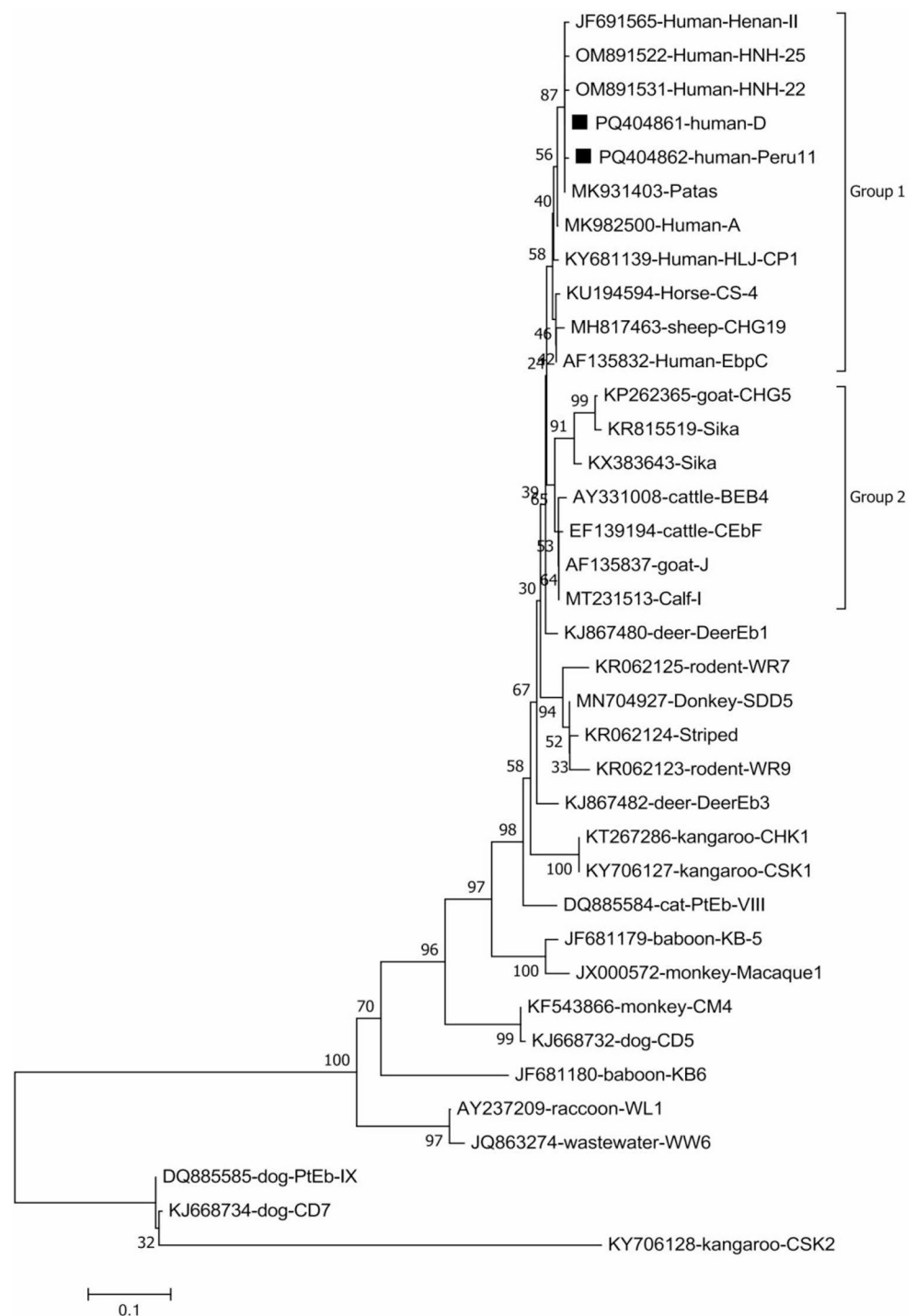
Moreover, children demonstrate a greater susceptibility to infection than adults, and contact with animals serves as a notable factor contributing to the elevation of infection rates [21]. In China, animals are commonly infected with *E. bieneusi*, which poses a potential zoonotic risk [9, 19, 21]. In the present study area, the documentation indicates the presence of *E. bieneusi* in multiple animal hosts, encompassing pigs, cattle, goats, and wild rodents [22, 26, 30]. This situation establishes conditions conducive to zoonotic transmission. Although the infection rate of *E. bieneusi* among children in this study was comparatively low, it is of great significance to conduct continuous surveillance on *E. bieneusi* infections in children, while implementing proper hygiene measures and minimizing contact with animals.

Two well-known zoonotic *E. bieneusi* genotypes, D and Peru11, were identified in this study. Genotype D is the most reported and widely distributed genotype both in humans and animals [21, 28]. According to statistics, this genotype has been identified in humans in more than 40 countries and in more than 80 species of animals [28]. In China, this genotype is also widely distributed, and there are reports of these genotypes infecting wild rats and diarrhea patients in the province where this study was conducted (Liu et al., [10, 13, 26]). Therefore, zoonotic transmission of this genotype is possible. However, due to the lack of environmental information, such as information about water sources, it is

Table 1 Prevalence and genotype distribution of *E. bieneusi* in diarrheic and asymptomatic children from Wenzhou city China

Groups	<i>E. bieneusi</i> No. Positive/ No. examined (%)	P-value	Genotype(s) (n)
Gender			
Males	9/571 (1.6)	P=0.414	Peru11 (6) D (3)
Females	5/461 (1.1)		Peru11 (3) D (2)
Symptom			
Diarrhea	11/684 (1.6)	P=0.327	Peru11 (6) D (5)
Non-Diarrhea	3/348 (0.9)		Peru11 (3)
Total	14/1032 (1.4)		Peru11 (9) D (5)

Fig. 1 The phylogenetic tree of *E. bieneusi* constructed based on ITS sequences implements the Neighbor-Joining method rooted in the Kimura 2-parameter model. For validating the tree's reliability bootstrap values were acquired through 1000 iterations. In this tree the genotypes uncovered in our study are highlighted with black squares



impossible to accurately determine the specific transmission mode of this species.

Genotype Peru11 is also prevalent in humans, with a diverse array of animal hosts. Moreover, it has been detected in wild rats within this region [26]. This phenomenon may signify a potential transmission route of genotype Peru11 from wild rats to children, and further research is required to validate this hypothesis. In this area, social awareness of

this latent threat should be enhanced, encompassing measures such as refraining from contact with rats and their feces and upholding good hygiene practices.

This study has several limitations. Firstly, it relies on convenience sampling conducted at a single hospital, which may not represent the broader pediatric population in the region. Additionally, the infection rate might be underestimated because only one fecal sample was collected from

each child, which might not have captured intermittent spore discharge. Another limitation is that diarrhea status was determined based only on stool consistency at sampling, without detailed information on defecation frequency, duration of diarrhea, abdominal symptoms, or reason for hospitalization. In addition, we did not analyze co-infections with other common enteropathogens (e.g., *Cryptosporidium*, *Giardia*, bacteria, or viruses), so their potential contributions to diarrhea could not be evaluated. Lastly, the study did not involve sampling of animals or the environment surrounding the children, indicating that it did not offer direct evidence of *E. bienersi* contamination and its transmission from animals from a health perspective. Despite these limitations, the study has successfully identified the presence of *E. bienersi* in children in the surveyed areas and provided initial observations on the genotypic characteristics of their genes.

Conclusions

This study investigated the prevalence of *E. bienersi* infections among children residing in the southern region of Zhejiang, China. The results indicated that the infection rate was 1.6% in children presenting with diarrhea symptoms and 0.9% in those without symptoms. Despite the relatively low prevalence, this study identified the presence of the highly zoonotic genotypes D and Peru11. This finding implies that *E. bienersi* infections may be transmitted via specific potential zoonotic transmission routes within the studied child population.

Author Contributions Jiangqing Ke : investigation, formal analysis, data curation, visualization, writing—original draft preparation, visualization; Wanghang Zhou : investigation, formal analysis, data curation, visualization, writing—original draft preparation, visualization; Xin Peng : investigation, formal analysis; Yanyan Jiang : Conceptualization, validation, resources, writing—review and editing, supervision, funding acquisition; Huicong Huang : Conceptualization, validation, resources, writing—review and editing, supervision, funding acquisition; Wei Zhao : Conceptualization, methodology, software, validation, formal analysis, resources, data curation, writing—original draft preparation, writing—review and editing, supervision, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding This work was supported by the National Natural Science Foundation of China (82273693) (YJ) and the Basic scientific research project of Wenzhou (Y2023070) (WZ). The funding sponsors had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data Availability The data supporting the conclusions of this article are included within the article.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical Approval The study was approved by the Ethics Committees of Wenzhou Medical University under the reference number SCILLSC-2021-01.

Informed Consent Prior to providing consent the parents/guardians of the participants were fully informed about the study objectives and methodologies. Subsequently written informed consent was obtained from the parents or guardians of each participant.

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