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Enterocytozoon bieneusi in Jiangsu Sheep and Goats: 36.51% Prevalence and Six Non-Zoonotic Group 2 Genotypes

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ABSTRACT

This research explored the occurrence and molecular diversity of Enterocytozoon bieneusi in sheep and goats across Jiangsu Province. A total of 786 fresh fecal specimens were gathered from 18 farms in five regions and analyzed by nested PCR amplification and sequencing of the ribosomal internal transcribed spacer (ITS). E. bieneusi DNA was detected in all surveyed areas, with infection levels varying from 23.65% to 42.81%, resulting in an overall positivity rate of 36.51% (287/786). No significant statistical differences were observed between sheep and goats or among different age classes ($p > 0.05$), but infection rates differed notably according to the animals' health status ($p < 0.05$). Sequence and cloning results identified six distinct genotypes—BEB6, CHG2, CHG3, CHC8, CHG14, and COS-I—all belonging to Group 2, a cluster previously regarded as non-zoonotic.

Keywords: Enterocytozoon bieneusi, Genotype, Sheep, Goats, Prevalence

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Introduction

Enterocytozoon bieneusi is a microsporidian parasite that affects more than 200 species of mammals, including humans, companion animals, and wildlife [1, 2]. Infected hosts may develop digestive disturbances such as chronic or acute diarrhea, while individuals with weakened immune systems can experience severe or fatal enteric disease [3]. Because its spores are minute and poorly visualized under a microscope, molecular detection—especially PCR—is the method of choice. Based on the ITS region of rRNA, E. bieneusi shows remarkable genetic variability, encompassing over 800 genotypes worldwide. Among these, 126 have been isolated from humans, 614 from animals, and 58 are shared between them, suggesting zoonotic potential [4]. Genotypes most frequently detected in both humans and animals include D, EbpA, EbpC, Type IV, BEB6, O, J, CM4, Peru6, I, Peru8, Peru11, and BEB4 [5]. Phylogenetically, the species is grouped into nine clusters, with Group 1 primarily containing zoonotic genotypes, while Groups 2–9 are generally host-restricted [6]. However, recent findings indicate that genotypes BEB4, BEB6, and J, although originally host-specific, can occasionally infect humans [7, 8].

In goats, 49 genotypes have been described (with 44 reported in China, including 12 zoonotic types), and the most prevalent are BEB6 and CHG3. In sheep, 79 genotypes are known (of which 61 occur in China and 17 are zoonotic), with BEB6 and CM7 most frequently detected [4].

The parasite's spores serve as the infective stage, and transmission mainly occurs through contaminated feed or water via the fecal–oral route [9]. While infections in immunocompetent hosts typically resolve without treatment, immunocompromised individuals may develop serious symptoms such as vomiting, fever, dysentery, or weight loss. The spores are environmentally resilient, and there are currently no specific chemotherapeutic options, posing health hazards to animal handlers and farm workers.

Sheep and goat farming represent a significant segment of animal production in Jiangsu Province. Driven by agricultural modernization and consumer demand, the small ruminant population reached 6.306 million in 2022 [10]. E. bieneusi infections in these animals can cause poor growth, digestive disorders, or death in severe cases [9, 11, 12].

The objective of the present survey was to determine the infection prevalence and genotype composition of E. bieneusi in small ruminants from 18 farms located in Suzhou, Suqian, Nantong, Huai'an, and Taizhou. The results expand current epidemiological data on this parasite in Jiangsu and offer reference information for prevention and control programs.

Materials and Methods

Sample collection and reagents

Sampling was conducted in August 2022. A total of 786 fecal samples were collected from 18 sheep and goat farms situated in Suzhou, Suqian, Nantong, Huai'an, and Taizhou **(Figure 1 and Table 1)**. Certain farms raised both species in separate enclosures. The five-point sampling strategy was applied: approximately 50 g of fresh feces was taken from each pen (each containing animals of the same age), sealed in sterile bags, and clearly labeled with site, age group, and health status (healthy or diarrheic). A "diarrheic pen" was defined as one containing at least a single sick animal, where only diarrheic feces were sampled. Pens where all animals appeared normal were marked as "healthy." Each collected specimen was assigned a distinct identification code for tracking and subsequent laboratory analysis.

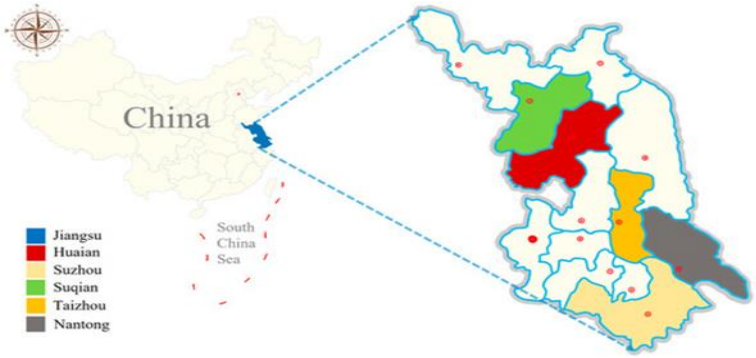

Figure 1. Map illustrating the five sampling regions within Jiangsu Province, China.

Table 1. Summary of sampling information.

Region	0~2 Month	2~6 Month	6~10 Month	Breed
Suzhou	90	90	0	Goats
Nantong	80	80	118	Sheep and goats
Taizhou	0	0	60	Sheep
Suqian	20	40	88	Sheep and goats
Huaian	60	0	60	Sheep and goats
Total	**250**	**210**	**326**	

Reagents used: EasyPure® Quick Gel Extraction Kit (TransGen Biotech, Beijing, China); DL2000 DNA Marker (Takara Bio, Kusatsu, Japan); DH5α Competent Cells (TransGen); Premix Taq™ (TaKaRa Taq™ Version 2.0, Takara); pGEM-T Easy Vector (Promega, Beijing, China); and EasyPure® Stool Genomic DNA Kit (TransGen).

DNA isolation and PCR protocol

For DNA preparation, approximately 200 mg of fecal material was mixed with sterile water in a beaker. A 250 μL aliquot of this suspension was transferred to a 2 mL microcentrifuge tube, and genomic DNA was extracted according to the EasyPure® Stool Genomic DNA Kit protocol.

Nested PCR targeting the Enterocytozoon bieneusi internal transcribed spacer (ITS) gene was conducted using primer sequences described by Buckholt *et al*. [13] **(Table 2)**.

Table 2. Primer details for nested-PCR detection of E. bieneusi.

Gene Locus	Primer	Sequence	Amplified Product
ITS	NEBF1	5′-GGTCATAGGGATGAAGAG-3′	410 bp
	NEBR1	5′-TTCGAGTTCTTTCGCGCTC-3′	
	NEBF2	5′-GCTCTGAATATCTATGGCT-3′	392 bp
	NEBR2	5′-ATCGCCGACGGATCCAAGTG-3′	

The first PCR reaction consisted of a 25 μL total mixture, containing 12.5 μL Premix Taq, 1 μL of each primer (10 mmol/L), 1 μL of DNA template, and sterile double-distilled water to a final volume. The thermal cycle included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min, and a terminal extension of 72 °C for 10 min.

For the second amplification, a similar reaction composition was used, substituting the DNA with 1 μL of the first-round product diluted 1:10. The cycling parameters were 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, followed by a 10 min final extension at 72 °C.

Ten microliters of each second-round amplicon were subjected to electrophoresis on 1% agarose gels in TAE buffer, operated at 120 V for approximately 30 minutes. Visualization was performed using a BIO-RAD ultraviolet imaging system. The DL2000 DNA Marker was used for fragment size reference, and the PCR-positive bands were purified using the EasyPure® Quick Gel Extraction Kit.

Sequence analysis and statistical evaluation

Raw sequences were aligned using Clustal X (version 1.83), and nucleotide identity was confirmed by BLAST analysis against sequences from the GenBank database. Phylogenetic relationships were inferred with MEGA 7 (version 7.0.26) to classify genotypes.

Statistical analyses were conducted using IBM SPSS Statistics 26, with chi-square tests applied to evaluate potential associations between infection prevalence and variables such as host species and health condition.

Results and Discussion

PCR screening outcomes

From the 786 fecal samples tested, 287 were positive for E. bieneusi based on ITS gene amplification, representing an overall prevalence of 36.51%.

Sequence typing and phylogenetic findings

Sequencing of 96 positive products revealed six distinct genotypes—BEB6, CHG2, CHG3, CHC8, CHG14, and COS-I—as determined by BLAST comparison. The phylogenetic reconstruction using MEGA 7 indicated that all identified genotypes were clustered within Group 2, which is recognized for host specificity and not zoonotic potential **(Figure 2)**.

Regional analysis showed genotype diversity varied by location: Nantong exhibited all six genotypes, followed by Suzhou (4), Taizhou (3), Suqian (3), and Huai'an (3). When comparing hosts, BEB6, CHG2, CHG3, CHC8, and COS-I occurred in both sheep and goats, whereas CHG14 was unique to goats.

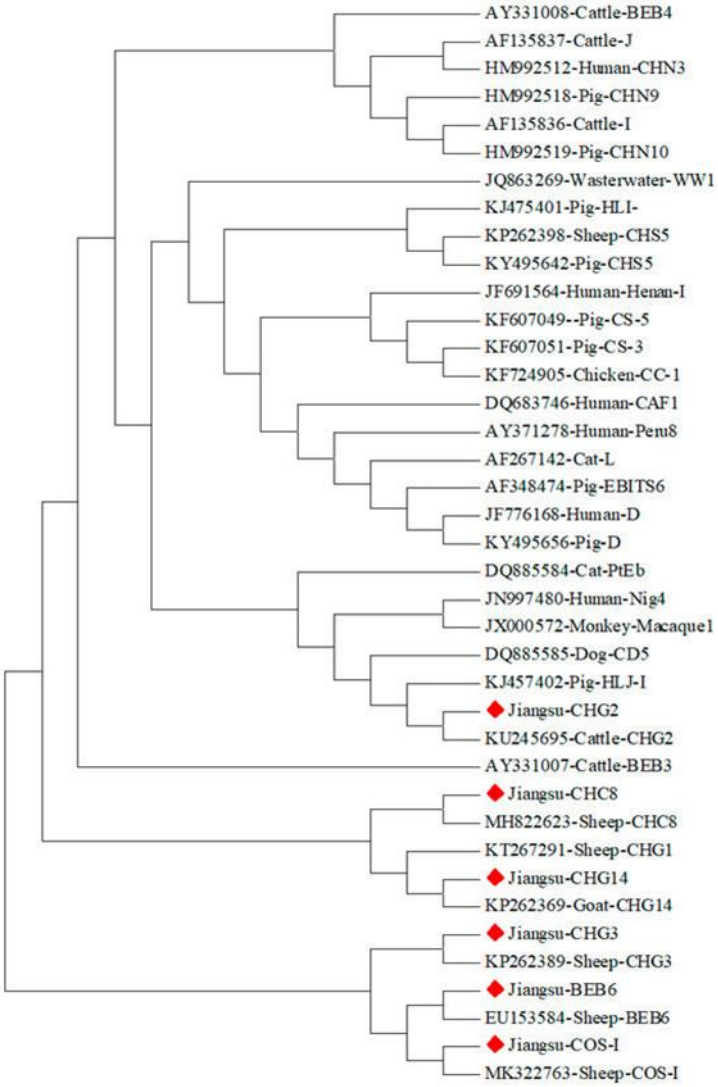

Figure 2. Phylogenetic clustering of E. bieneusi ITS sequences. Red diamonds denote the isolates characterized in the present study.

Prevalence of E. bieneusi among sheep and goats

Data comparison from herds across five locations indicated regional variation in E. bieneusi infection among meat sheep **(Table 3)**. The overall prevalence in different areas ranged from 23.65% to 42.81%, the highest being recorded in Nantong (42.81%), followed by Huai'an (39.17%), Suzhou (38.33%), Taizhou (28.33%), and the lowest in Suqian (23.65%).

When comparing hosts, sheep (38.89%) and goats (35.54%) showed comparable infection frequencies, with no statistically significant difference ($p > 0.05$) **(Table 4)**.

In goats, infection was most common in younger animals aged 0–2 months (37.39%) and 2–6 months (39.26%), while the 6–10-month age group had a markedly lower rate (25%). Among sheep, the greatest incidence occurred in individuals aged 2–6 months (75%), followed by 0–2 months (40%) and 6–10 months (34.18%). However, age-related variation was not statistically significant for either species ($p > 0.05$) **(Table 5)**.

Regarding health status, diarrheic goats showed a higher infection rate than healthy goats, whereas in sheep, the reverse pattern was seen—healthy individuals tested positive more often than diarrheic ones. This difference between health groups was statistically significant ($p < 0.05$) **(Table 6)**.

Table 3. Prevalence of E. bieneusi among mutton sheep across different regions.

Region	Farm	Sample Size	No. of Positive	% of Positive	Average Positive %
Suzhou	1	60	20	33.33	38.33

	2	60	19	31.67	
	3	60	30	50	
Nantong	4	80	24	30	42.81
	5	60	17	28.33	
	6	37	8	21.62	
	7	30	20	66.67	
	8	21	12	57.14	
	9	30	23	76.77	
	10	20	15	75	
Taizhou	11	30	16	53.33	28.33
	12	30	1	3.33	
Suqian	13	48	5	10.42	23.65
	14	40	15	37.5	
	15	60	15	25	
Huaian	16	40	26	65	39.17
	17	40	17	42.5	
	18	40	4	10	
Total		**786**	**287**	**36.51**	

Table 4. Comparison of infection levels in sheep and goats.

Breed	Sample Size	No. of Positive	% of Positive
Goat	588	209	35.54
Sheep	198	77	38.89
Total	**786**	**287**	**36.51**

Table 5. E. bieneusi detection in sheep at various ages.

Breed	Age (Month)	Sample Size	No. of Positive	% of Positive
Goat	0–2	230	86	37.39
	2–6	242	95	39.26
	6–10	116	29	25
Sheep	0–2	20	8	40
	2–6	20	15	75
	6–10	158	54	34.18

Table 6. Association between diarrhea and E. bieneusi positivity in animals.

Breed	Symptom	Sample Size	No. of Positive	% of Positive
Goat	Diarrheic	140	62	44.29
	Healthy	448	148	33.04
Sheep	Diarrheic	38	8	21.05
	Healthy	160	69	43.13

Enterocytozoon bieneusi is a microsporidian parasite capable of infecting humans and numerous mammalian hosts [14]. In recent years, the number of reports describing its presence in sheep and goats has increased globally, particularly in central, eastern, and southwestern China [15–21].

In the present research, the infection rate across Jiangsu Province reached 36.51% (287/786). This is higher than rates previously observed in Xuzhou (2.7%) and Anhui (4.09%) [7] but below those reported from Henan (73.6%), Chongqing (62.5%), and Shanxi (47.8%, 43.5%) [15, 16].

Marked geographic variation was detected, with the Nantong region having the greatest prevalence (42.81%) and Suqian the lowest (23.65%). Substantial differences also occurred among farms within a single area. For instance, Farm 14 in Suqian exhibited an infection rate of 37.5%, approximately fourfold higher than Farm 13 (10.42%); likewise, Farm 11 in Taizhou (53.33%) recorded nearly 20 times the rate observed at Farm 12. These contrasts likely reflect differences in herd management, housing density, sanitation, and feed hygiene.

Analysis by age indicated that lambs aged 0–2 months and sheep aged 6–10 months were more frequently infected than those aged 2–6 months. Similar patterns were documented among Tibetan sheep and yaks, where E. bieneusi occurrence was highest in animals younger than 1 year, decreased in the 1–2-year range, and lowest after 2 years of age [6]. A Brazilian survey also found a higher prevalence in lambs under 6 months (34.1%) than in adults (11.1%) [22]. Broader Chinese surveys suggest infection rates tend to decline with increasing age [23]. The heightened susceptibility of lambs younger than 10 months is probably due to the loss of maternal antibodies, which may leave them immunologically vulnerable. Thus, strengthening husbandry practices—such as providing balanced nutrition and isolating young animals—can reduce infection risk.

In terms of clinical status, goats exhibiting diarrhea were more often positive for E. bieneusi than healthy goats, whereas sheep showed the reverse trend. This discrepancy suggests that diarrhea in sheep might be caused by other enteric organisms, such as Eimeria spp. or Trichostrongylidae spp. Moreover, the limited number of diarrheic samples may have contributed to false-negative results. Future research should therefore include larger sample sizes and test for multiple intestinal pathogens to clarify these relationships.

Currently, 79 distinct genotypes of E. bieneusi have been documented in sheep and 49 in goats, 15 of which are shared: BEB6, CHC8, CHG1, CHG13, CHG2, CHG3, CHS5, CHS7, CM7, COS-I, COS-II, D, EbpA, EbpC, and Peru6. In Gansu, Wu *et al*. identified four genotypes—BEB6, CM7, CHS3, and CGS1—all within Group 2, dominated by BEB6 [24]. From Heilongjiang, Zhao *et al*. reported 14 types, including six known and eight newly discovered (COS-I–COS-VII, COG-I) [25]. In Yunnan, Xie *et al*. found 15 genotypes from 907 black goat samples, consisting of four novel forms (CYG-1–CYG-4) and eleven previously recognized genotypes (CHG1, CHG2, CHG3, CHG5, CHG28, J, D, BEB6, Wildboar3, CD6, SDD1) [21].

A national-scale study by Yang *et al*. identified BEB6 as the predominant genotype across 11 provinces [26].

In our dataset, six genotypes were characterized among 287 positive samples: BEB6, CHG2, CHG3, CHC8, CHG14, and COS-I. The most prevalent was BEB6 (55.21%), followed by CHG3 (25%). Five genotypes (BEB6, CHG2, CHG3, CHC8, and COS-I) occurred in both species, whereas CHG14 was restricted to goats. Phylogenetic reconstruction assigned all genotypes to Group 2, typically regarded as non-zoonotic; however, BEB6 remains notable for its potential zoonotic transmission. In China, this genotype has been found in Bactrian camels, red deer, roe deer, sika deer, and Altai marmots, suggesting it may threaten public health [27–30]. Therefore, systematic monitoring and regular epidemiological assessments are essential to trace its spread and prevent cross-species transmission.

Conclusion

The current investigation revealed a 36.51% prevalence of E. bieneusi among sheep and goats in Jiangsu Province, with six genotypes identified—BEB6 being predominant and of potential zoonotic importance. Strengthening husbandry for animals younger than 10 months and broadening surveillance for co-infecting intestinal pathogens are strongly recommended. Continuous epidemiological observation of E. bieneusi is vital for understanding regional infection dynamics and mitigating risks to public health.

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