

Prevalence and genotypes of *Enterocytozoon bieneusi* in captive wild hedgehogs (*Erinaceus amurensis*) in China

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Abstract

Enterocytozoon bieneusi is now recognised as an emerging pathogen, which can cause a series of intestinal diseases in humans and animals. However, limited information is available about the occurrence of *E. bieneusi* in hedgehogs. In this study, a total of 205 fecal samples of captive wild hedgehogs (*Erinaceus amurensis*) were collected from two wildlife conservation centers in Henan and Anhui provinces, China. Genomic DNA was extracted from the stool samples and screened by nested PCR targeting the internal transcribed spacer (ITS) sequences. The overall prevalence of *E. bieneusi* in captive wild hedgehogs was 44.4% (91/205). Five zoonotic genotypes were identified, including four known genotypes (IV, D, K and WildBoar3) and one new genotype designated (AHCW-1). Of these, genotype IV was the most prevalent in the present study (n = 63; 69.2%). Phylogenetic analysis revealed that all five genotypes clustered within the zoonotic Group 1, suggesting their zoonotic potential. To our knowledge, this study reports the novel genotype AHCW-1 and provides the first epidemiological data on *E. bieneusi* in captive wild hedgehogs from Henan and Anhui Provinces, China. The identification of these zoonotic genotypes in captive wild hedgehogs indicates that these animals may serve as a potential reservoir, highlighting the need for further investigation into zoonotic transmission to humans in the studied regions.

Keywords: captive wild hedgehogs, China, *Enterocytozoon bieneusi*, ITS-1, prevalence, zoonotic genotypes

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Introduction

Microsporidiosis is an important emerging infectious disease (Stentiford *et al.*, 2016). There are approximately 1,500 described species of microsporidia within 200 genera. Currently, 17 microsporidia species are recognised to infect humans, of which *Enterocytozoon bienersi* is most commonly found pathogenic species (Mathis *et al.*, 2005). Humans become infected with *E. bienersi* mainly through the consumption of contaminated food or water (Wang *et al.*, 2018). *E. bienersi* infection may cause many clinical symptoms, such as chronic diarrhea, malabsorption and occasionally growth impairments (Matos *et al.*, 2012). In general, *E. bienersi* usually causes a self-limiting infection in immunocompetent individuals; however, it can cause life-threatening diarrhea in immunocompromised individuals (1.3-78%) (Matos *et al.*, 2012), such as AIDS patients, cancer patients and transplant recipients (Didier and Weiss, 2006).

The accurate identification and classification of *E. bienersi* spores using microscopy is difficult, such that molecular tools need to be employed. PCR-based sequencing of the internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) has been widely used for *E. bienersi* detection and genotyping because it shows high levels of genetic diversity among isolates (Sulaiman *et al.*, 2003). Using this molecular tool, so far, more than 200 genotypes of *E. bienersi* have been identified in human and animal hosts, and in wastewater (Li *et al.*, 2019). Based on phylogenetic analyses of these genotypes, they can be grouped into eleven different large groups (Li *et al.*, 2019). Group 1 is considered a zoonotic group, which has been frequently reported in humans and domestic and wild animals (Thellier and Breton, 2008). In contrast, the remaining groups have been considered host-specific, and have no significant public health importance (Guo *et al.*, 2014; Zhang *et al.*, 2018).

Various studies have assessed the diversity of *E. bienersi* genotypes in a broad range of domestic and wild animals in China, such as cattle, chicken, deer, goat, pig, sheep, and rat (Wang *et al.*, 2018). In China, *E. bienersi* infection has been reported in 93 wild mammal species (Wang *et al.*, 2018). The prevalence of *E. bienersi* (0-83.3%) varied significantly among different wild mammal species, and 84 genotypes were identified from these wild animals with the zoonotic genotype D as the predominant genotype (Wang *et al.*, 2018). These findings suggest that wild mammals may represent a potential zoonotic reservoir.

Although *E. bienersi* has been reported in many wild mammals, data on hedgehogs remain limited. A recent study first reported *E. bienersi* in hedgehogs (*Erinaceus amurensis*) from several provinces in China (Gong *et al.*, 2021), with subsequent reports from other regions (Baz-González *et al.*, 2023; Gomes-Gonçalves *et al.*, 2024). However, epidemiological data on the prevalence and genetic diversity of *E. bienersi* in hedgehog populations from specific regions, such as Henan and Anhui provinces, are still insufficient. The hedgehog is a widely distributed heterothermic mammal belonging to the family Erinaceidae. In Southern China, *E. amurensis* is a common species (Bai *et al.*, 2022). Given its ecological presence and potential

interactions with human environments, understanding its role in the epidemiology of *E. bienersi* is relevant. Therefore, the present study aims to investigate the prevalence and genotypes of *E. bienersi* in captive wild hedgehogs (*E. amurensis*) from Henan and Anhui provinces, China.

Materials and Methods

Collection of specimens: A total of 205 fresh fecal samples were obtained from captive wild hedgehogs at two wildlife conservation centers, which are located in Henan and Anhui provinces of China, between April and December 2018 (Table 1). These wild animals were kept in captive cages. Approximately 5 g of each fresh sample was immediately collected from the floors using sterile gloves and placed on ice in separate containers. Prior to use, samples were preserved in 2.5% potassium dichromate solution at -20 °C until processing. Information about each hedgehog, such as species, gender, weight, region and season, was collected (Table 1). All procedures involving animals in the present study were approved and this study was approved by the Animal Ethics Committee of HuaiHua Vocational & Technical College (No. 2017003).

DNA extraction and PCR amplification: Fecal samples (approximately 3 g) were washed with distilled water three times and then centrifuged at 3,000× g for two minutes to remove the potassium dichromate. Genomic DNA was extracted from these samples using an E.Z.N.A.® Stool DNA Kit (Omega Biotek Inc., Norcross, GA, USA) following the manufacturer's instructions. The identification of *E. bienersi* was achieved by nested PCR based on ITS marker using previously established methods (Sulaiman *et al.*, 2003). PCR reaction (25 µL) was composed of 2 µL each deoxy-ribonucleoside triphosphate (dNTP) (2.5 mM), 15.8 µL of double-distilled water, 1.5 µL of MgCl₂ (25 mM), 2.5 µL of Ex Taq buffer (Mg²⁺ free), 0.25 µL of each primer (50 pmol) (F1: GGTCATAGGGATGAAGAG, R1: TTCGAGTCTTTCGCGCTC; F2: GCTCTGAATATCTATGGCT, R2: ATCGCCGA CGGATCCAAGTG), 0.2 µL of Ex Taq DNA polymerase (5 U) (TAKARA, Kyoto, Japan), and 2.5 µL of DNA template in a thermocycler (Biometra, Göttingen, Germany) under the following conditions: denaturation at 94°C for 5 min, followed by 35 cycles of 45 sec at 94°C, 45 sec at 55°C, and 1 min at 72°C, with a final extension of 72°C for 10 min. Positive and negative controls were included in each PCR reaction systems. All the secondary PCR products were detected under UV light after electrophoresis (125V, 25 min) in 1.0 % agarose gels containing GoldView™ (Solarbio, China), and purified (EasyPure PCR Purification Kit, TransGen Biotech Inc., Beijing, China) positive products were sent to Sangon Biotech (Shanghai, China) for sequencing from both directions.

Sequencing and phylogenetic analyses: Sequence chromatograms from each strand were aligned and inspected using DNASTAR software (DNASTAR, Madison, Wis, USA). All obtained sequences were subjected to Basic Local Alignment Search Tool

(BLAST) searches in the GenBank database to confirm their genotypes. Sequences with mutations (including single nucleotide substitutions, insertions, or deletions) not previously reported were identified as novel genotypes. Each obtained sequence was also aligned individually with sequences downloaded from the GenBank database at the National Center for Biotechnology Information (NCBI) using MAFFT 7.122 (Katoh and Standley, 2013). The neighbor-joining (NJ) were performed using Mega 5.0 (Tamura et al., 2011). The Kimura 2-parameter model was selected as the most suitable model. NJ tree were calculated based on 1,000 bootstrap replicates. A novel genotype was defined as an ITS sequence that contained one or more nucleotide substitutions, insertions, or deletions compared with all existing *E. bieneusi* genotypes deposited in GenBank. The newly identified genotype AHCW-1 was confirmed by BLAST analysis to have no identical match in the database, with the closest known genotype showing three nucleotide differences over the ITS region.

Statistical analysis: The variation in *E. bieneusi* prevalence was analyzed by χ^2 test using SPSS V20.0 (IBM, Chicago, IL, USA). Univariable binary logistic regression was performed for each independent variable separately to calculate odds ratios (OR) and their 95% confidence intervals (95% CI), assessing the strength of association between *E. bieneusi* positivity and each tested condition. When $P < 0.05$, the results were considered statistically significant.

Results

Based on PCR and sequencing, we detected *E. bieneusi* in 91 (44.4%; 95% CI: 37.6-51.2) out of 205 investigated samples. The present study showed that the prevalence of *E. bieneusi* was higher in males than in females (49.0%; 95% CI: 39.3-58.7 vs 39.8%; 95% CI: 30.3-49.3), although these differences were not statistically significant ($P > 0.05$). We also found that there was no significant difference between in the prevalence of *E. bieneusi* in captive wild hedgehogs according to region and season ($P > 0.05$) (Table 1).

Five genotypes of *E. bieneusi* were identified, including four known genotypes (IV, K, WildBoar3, D) and one new genotype (AHCW-1). These obtained sequences have been deposited in the GenBank database under the accession numbers: MK841504–MK841508. All obtained sequences assigned to a given genotype were identical to each other. In the present study, the genotype IV (69.2%; 63/91) was the most predominant *E. bieneusi* genotype in captive wild hedgehogs, which was detected in all weight groups and investigated regions; followed by genotype K (22%; 20/91), which was also present in each weight group and region, genotype WildBoar3 (5.5%; 5/91) and genotype D (2.2%; 2/91). The novel genotype (AHCW-1) (1.1%; 1/91) was found in Anhui province, China.

Phylogenetic analysis indicated that the five *E. bieneusi* genotypes from captive wild hedgehogs and reference genotypes from other hosts clustered within the same clade (Group 1, Fig 1). Groups 1 and 2 were more closely related to each other than to other groups (i.e., Groups 3–8). These results indicate that all *E. bieneusi* isolates identified in the present study belong to Group 1 (Fig. 1).

Table 1 Factors associated with prevalence of *Enterocytozoon bieneusi* in captive wild hedgehog in China.

Factor	Category	No. tested	No. positive	% (95 % CI)	OR (95 % CI)	P-value
Gender	Female	103	41	39.8 (30.3-49.3)	Reference	0.207
	Male	102	50	49.0 (39.3-58.7)	1.5 (0.8-2.5)	
Weight	≥0.5kg	114	48	42.1 (33.0-51.2)	Reference	0.482
	<0.5kg	91	43	47.3 (37.0-57.6)	1.2 (0.7-2.1)	
Region	Anhui	100	42	42.0 (32.3-51.7)	Reference	0.574
	Henan	105	49	46.7 (37.2-56.2)	1.2 (0.7-2.0)	
Season	Winter	100	42	42.0 (32.3-51.7)	Reference	0.574
	Summer	105	49	46.7 (37.2-56.2)	1.2 (0.7-2.0)	

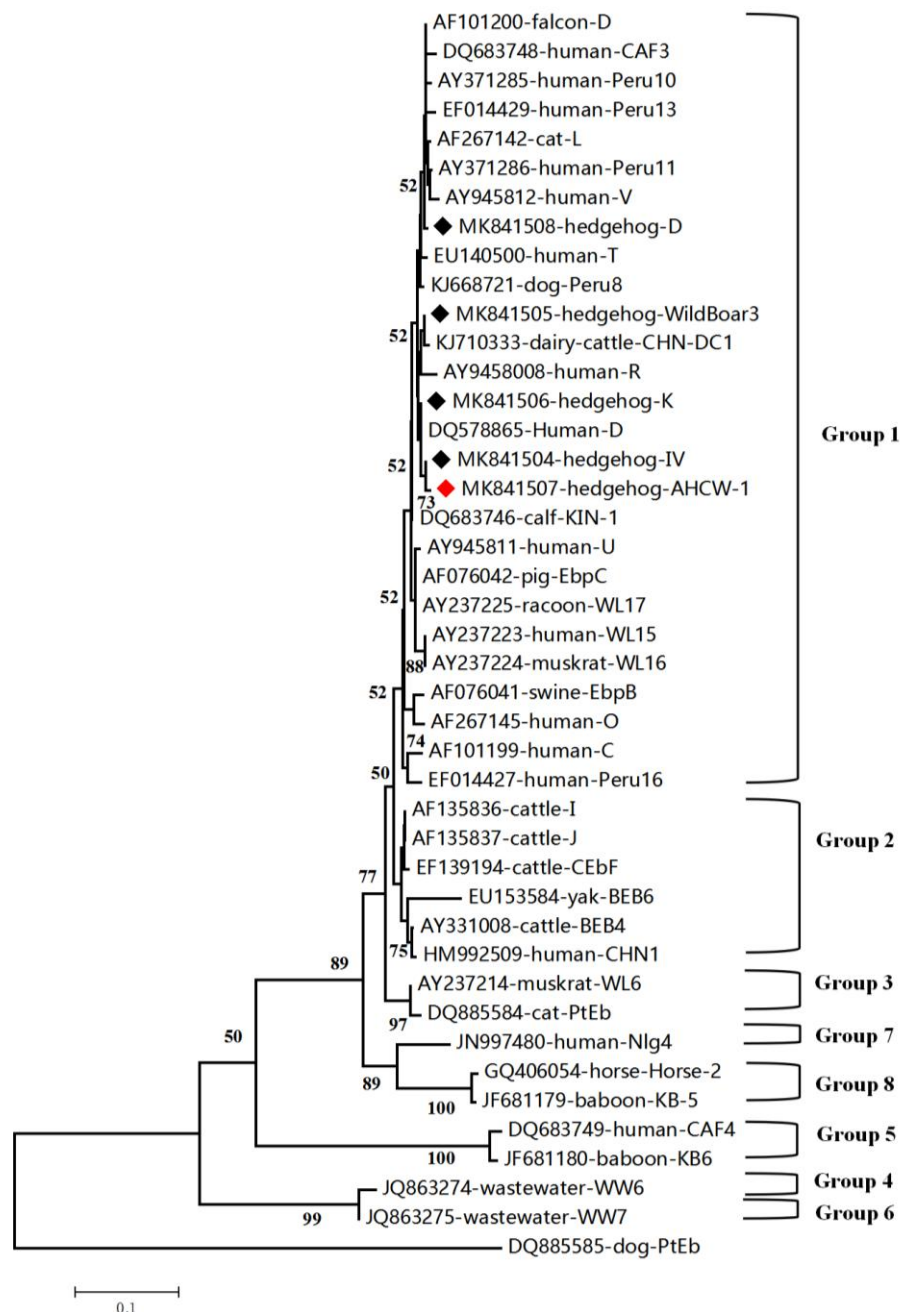


Figure 1 Unrooted phylogenetic tree showed that the *Enterocytozoon bieneusi* genotypes were distributed across eight representative distinct groups. Genotypes marked with red diamond are novel genotypes identified and black diamond are known genotypes identified in the study.

Discussion

The prevalence of *E. bieneusi* in captive wild hedgehogs observed in this study was significantly higher than those previously identified in other animals in China, such as black bears (27.4%; 29/106) (Deng *et al.*, 2017), blue foxes (16.4%; 18/110) (Zhao *et al.*, 2015a), foxes (27.7%; 53/191) (Yang *et al.*, 2015), giant pandas (8.7%; 4/46) (Tian *et al.*, 2015), red pandas (13.9%; 5/36) (Tian *et al.*, 2015), giraffes (33.3%; 1/3) (Du *et al.*, 2016), kangaroos (23.0%; 14/61) (Zhong *et al.*, 2017), golden takin (14.7%; 28/191) (Zhao *et al.*, 2015b), sika deer (35.9%; 221/615) (Huang *et al.*, 2017), and red deer (20%; 1/5) (Zhao *et al.*, 2014). The prevalence of *E. bieneusi* in captive wild hedgehogs observed in this study (44.4%; 91/205) is higher than that reported in

hedgehogs from Portugal (22.7%; 25/110 in *E. europaeus*) (Gomes-Gonçalves *et al.*, 2024), but lower than the extremely high prevalence (61.1%; 22/36) reported in North African hedgehogs (*Atelerix algirus*) in Spain (Baz-González *et al.*, 2023) and in pet African pygmy hedgehogs (*Atelerix albiventris*) from China (70%; 266/380) (Meng *et al.*, 2023). These discrepancies highlight significant variations across hedgehog species, geographical locations, and living conditions (wild, captive, or pet). It was lower than that in golden snub-nosed monkeys (46.2%; 74/160) (Yu *et al.*, 2017), and gorals (83.3%; 5/6) (Du *et al.*, 2016). The discrepancies observed in prevalence may be related to differences in the host animal species surveyed. In the present study, we also found that there was no

significant difference in the prevalence in captive wild hedgehogs according to gender, weight, region and season ($P > 0.05$) (Table 1). In contrast to previous studies on other animals that reported significant associations with host weight (Akinbo *et al.*, 2012), region (Yang *et al.*, 2018; Song *et al.*, 2018) and sampling season (Feng *et al.*, 2019; Cheng *et al.*, 2011), the present study found no significant differences in prevalence by gender, weight, region, or season ($P > 0.05$) (Table 1), suggesting that these factors may have host-specific effects.

In this study, five genotypes were identified (IV, K, WildBoar3, D, and AHCW-1) in captive wild hedgehogs in Henan and Anhui provinces. In China, the genotype IV was detected in humans and other animals (Wang *et al.*, 2018; Zhang *et al.*, 2016; Li *et al.*, 2017; Wan *et al.*, 2016; Li *et al.*, 2014), and the genotype K was found in immunodeficiency patients (Liu *et al.*, 2017). The genotype WildBoar3 was mainly found in foxes (Yang *et al.*, 2015) and raccoon dogs (Yang *et al.*, 2015). The genotypes D has been frequently found in macaques (Yang *et al.*, 2017), horses (Qi *et al.*, 2016), foxes (Zhang *et al.*, 2016), non-human primates (Yu *et al.*, 2017; Ye *et al.*, 2014), dogs and cats (Li *et al.*, 2015; Xu *et al.*, 2016), pet chinchillas (Qi *et al.*, 2015), pet chipmunks (Deng *et al.*, 2018), and humans (Liu *et al.*, 2017; Wang *et al.*, 2013; Wang *et al.*, 2017).

Phylogenetic analysis indicated that the five *E. bieneusi* genotypes from captive wild hedgehogs were considered to belong to a zoonotic group with moderate support (Fig. 1) (Yang *et al.*, 2018), suggesting that these genotypes (IV, K, D, WildBoar3 and AHCW-1) identified in the present study have the potential for zoonotic transmission. However, because some nodes in the phylogenetic tree had relatively low bootstrap support, the zoonotic interpretation should remain cautious. In this tree, *E. bieneusi* genotypes were classified into eight different groups. These findings raised the possibility that captive wild hedgehogs may carry zoonotic genotypes of *E. bieneusi*; however, direct evidence of transmission from hedgehogs to humans is lacking. Therefore, further work is required to confirm this hypothesis using larger numbers of specimens from more host species (such as *E. amurensis* and *E. concolor*) and broader geographical regions.

The genotype IV identified here as the dominant type has also been found in captive wild hedgehogs from Portugal (Gomes-Gonçalves *et al.*, 2024) and in other Chinese hedgehogs (Gong *et al.*, 2021), reinforcing its wide host range and potential zoonotic significance. The novel genotype AHCW-1 adds to the genetic diversity of *E. bieneusi* in captive wild hedgehogs, which also includes genotypes like Peru6, Horse2, and Type IV reported in other studies (Gong *et al.*, 2021; Gomes-Gonçalves *et al.*, 2024; Meng *et al.*, 2023). Notably, the zoonotic genotype D, common in many hosts, was also detected in African pygmy hedgehogs (Meng *et al.*, 2023), further supporting the cross-species transmission risk.

In China, populations of the Western European hedgehog have been on the decline for the past 20 years. The main factors are hunting, natural predators, environmental pollution, extensive use of chemicals and lack of insects as food, and harvesting of internal organs to be used in traditional Chinese medicine (Hua

et al., 2012; Zhang and Wang, 2006). Another likely threat to the wild hedgehog is parasitic disease. This statement is supported by previous studies (Sangster *et al.*, 2016; Krawczyk *et al.*, 2015; Lada and Jana, 2019). Our results indicated that *E. bieneusi* DNA can be found in hedgehog feces; this finding improves our understanding of *E. bieneusi* epidemiology in wild animals. More importantly, the presence of zoonotic *E. bieneusi* genotypes suggests a potential public health risk that warrants attention, although no direct link to human infection has been established in this study and the phylogenetic inference is limited by low bootstrap support for certain nodes. The identification of zoonotic *E. bieneusi* genotypes in wild, captive, and pet hedgehogs across different continents (Gomes-Gonçalves *et al.*, 2024; Meng *et al.*, 2023; Baz-González *et al.*, 2023) underscores the need for increased awareness and further surveillance of these animals as potential reservoirs for human microsporidiosis.

In conclusion, our results provide the first evidence for the presence of *E. bieneusi* in captive wild hedgehogs from Henan and Anhui Provinces, China. The previous identification of the zoonotic genotypes (IV, D, K, WildBoar3, and the novel AHCW-1) suggests that captive wild hedgehogs may serve as potential reservoirs, though further studies are needed to assess actual transmission risks to humans. These results improve our knowledge of the epidemiology and genotypes of *E. bieneusi* in wild animals in China.

Conflict of interest: The authors declare they have no conflict of interest.

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Declaration of artificial intelligence use: No artificial intelligence (AI) tools or large language models were used in the writing, editing, or data analysis of this manuscript. The authors take full responsibility for the content and originality of this work.

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